Analysis of interleukin-17 and interleukin-18 levels in animal models of atherosclerosis

XIUMING TANG

Department of Cardiology, The Affiliated Hospital to Changchun University
of Chinese Medicine, Changchun, Jilin 130021, P.R. China

Abstract. This study investigated the correlation between the levels of interleukin (IL)-17 and IL-18 and atherosclerotic plaques. A total of 60 Apo E gene (Apo E-/-) mice were fed with high-fat diet in the model group and 20 wild male C57BL/6 mice were fed with the basic diet in the control group. The serum levels of IL-17 and IL-18 were determined by enzyme-linked immunosorbent assay. Carotid artery ultrasonography was performed and divided into stable plaque, unstable plaque and non-plaque groups. The severity of plaque was estimated by semi-quantitative method and divided into grades I, II and III. The expression levels of low-density lipoprotein cholesterol, plasma total cholesterol and blood glucose level in the model group induced by high-fat diet were significantly higher than those in the control group (P<0.05). The level in the model group was significantly higher than in the control group at the 16th week (P<0.05). The expression of IL-17 and IL-18 in the model group was significantly higher than that in the control group (t=6.903, 11.02, P<0.05). The concentration of IL-17 and IL-18 in the non-plaque group was significantly lower than that in the stable plaque and unstable plaque groups (P<0.05). The concentration of IL-17 and IL-18 in the stable plaque group was significantly lower than that in the unstable plaque group (P<0.05). Based on the correlation of IL-17 and IL-18 expressions in the model group, the expression of IL-18 increased with the expression of IL-17, indicating that the expression of IL-17 was positively correlated with that of IL-18 (r=0.7195, P<0.001). In conclusion, serum IL-17 and IL-18 played an important role in the formation and development of atherosclerotic plaque, and were related to the stability and severity of plaque. The expression of IL-17 and IL-18 was positively correlated.

Correspondence to: Dr Xiuming Tang, Department of Cardiology, The Affiliated Hospital to Changchun University of Chinese Medicine, 1478 Gongnong Avenue, Changchun, Jilin 130021, P.R. China
E-mail: tnnzs6@163.com

Introduction

Atherosclerosis (AS) is the main cause of myocardial infarction, cerebrovascular accident or peripheral vascular disease, leading to death from cardiovascular disease (1). AS is considered to be an inflammatory disease caused by high plasma cholesterol levels and hypertension (2). Many studies have revealed that the formation and development of atherosclerotic plaques are closely related to neovascularization (3,4). Inflammatory cell aggregation results from the formation of neovascularization, which may cause plaque hemorrhage and plaque rupture. Therefore, the reduction of neovascularization in plaque can be regarded as an important target for the treatment of AS plaque (5). The formation and development of atherosclerotic plaque are closely related to the expression and activity of inflammatory markers. Therefore, the study of related inflammatory markers has become a hot topic to study the occurrence and development of atheromatous plaques (6).

The pathological process of AS is closely related to the process of inflammation, which is a slow inflammatory process caused many inflammatory factors in the body, such as interleukin (IL)-23 and IL-17 (7). IL-17, one of the ILs, was secreted by Th17 (8). Th17 is mainly produced in the thymus, some of which are transformed from other cells (9). Previous studies suggested that IL-17 could accelerate the progress of intracranial AS plaques, affecting the formation of thrombus, or even the stability of plaques (10). IL-18 is a multipotent proinflammatory cytokine and inflammation marker, which is related to the occurrence and development of AS (11). Animal studies (12,13) suggested that IL-18 is a strong AS-causing factor that promotes the increase of atherosclerotic plaque areas and enhances interferon and T lymphocyte in the plaque, suggesting that IL-18 can induce AS and increase the instability of plaques.

To the best of our knowledge, there is currently no study on the expression and correlation of IL-17 and IL-18 in AS mice. This study investigated the relationship between the expression of IL-17 and IL-18 and the stability of plaque, and the correlation of IL-17 and IL-18 in AS.

Materials and methods

Establishment of experimental animal models. A total of 60 8-week-old Apo E (Apo E-/-) male mice (10-15 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) [License no. SCXK (Beijing) 2012-0001] and used as the model group. A total of 20 wild male C57BL/6 mice (10-15 g) were used as the control group. All animals were raised in Specific Pathogen-Free (SPF) system of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) [License no. SYXK (E) 2010-0028]. Mice were allowed to acclimatize for 1 week. Mice in the control group were fed with the basic diet and mice in the model group were fed with high fat diet [10% lard, 5% white sugar, 4% cholesterol, 0.5% sodium cholate, 0.2% propylthiouracil (PTU), 80.3% basic diet]. Vitamin D3 (150,000 U/kg) was injected into abdominal cavity each month. Feeding conditions were as follows: room temperature at 21±2˚C, humidity at 63±5%, free drinking water from tap water, controlled indoor lighting from day (8:00-20:00) to night (20:00-8:00) for 20 weeks. The experimental animals were nursed according to protocol synopsis instructions - institutional animal care and use from the Institute of Basic Theory for Chinese Medicine, Chinese Academy of Chinese Medicine Science. Animal studies in the experiment were approved by the Medical Ethics Committee of The Affiliated Hospital to Changchun University of Chinese Medicine (Changchun, China).

Laboratory reagents and instruments. Automatic biochemical analyzer (PUZS-300; Shanghai Huanxi Medical Instrument Co., Ltd., Shanghai, China), color Doppler ultrasonic diagnostic apparatus (DC-N2S; Beijing Mindray, Beijing, China), mouse IL-18 and IL-17 ELISA kits (Shanghai Yanjin Biological Science and Technology Co., Ltd., Shanghai, China), automatic quantitative enzyme marker (352; LabSystems Multiskan MS Helsinki, Finland), centrifuge [KDC-40; Anhui University of Science and Technology of China (USTC) Innovation Co., Ltd., Huainan, China], and Olympus microscope; Olympus Co., (Tokyo, Japan) were used in the present study.

Serum extraction. The weight of mice was recorded and observed each week. Mice were fasted before death and weighed. Blood was collected from the orbit and kept at room temperature for 1 h, and centrifuged at 2,500 x g for 15 min at 4˚C to obtain the supernatant and then put into EP tube to get the serum sample, which was stored in a refrigerator at -80˚C to detect serum IL-17, IL-18 and blood lipids.

Determination of blood lipids. Blood lipids in the separated serum was tested by PUZS-300 automatic biochemical analyzer, including total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and blood glucose levels (Glu).

Ultrasonic testing. The transducer frequency of color Doppler ultrasonic diagnostic apparatus was 7.5 MHz after the animals were sacrificed. The plaque was scanned along the carotid artery. Intima-media thickness (IMT) was the vertical distance between lumen intima to media and adventitia. IMT >1.5 mm indicates the formation of AS plaque. Plaques were divided into hard, flat, ulcer and mixed plaques based on the size, shape and echo, among which stable plaques include hard and flat plaques, unstable plaques include soft, ulcer and mixed plaques. The severity of the plaque was estimated by semi-quantitative method as follows: unilateral plaque ≤2.0 mm indicates grade I. Unilateral plaque >2.0 mm or bilateral plaque with one side ≤2.0 mm indicates grade II. Bilateral plaque >2.0 mm indicates grade III.

Detection of IL-18 and IL-17. The concentration of IL-17 and IL-18 in serum of mice in the model and the control groups was determined by ELISA according to the instructions. A total of 10 standard wells were placed on the enzyme label plate, 100 μl of standard and 50 μl of standard diluent were blended in wells 1 and 2, then100 μl mixed liquor was taken from wells 1 and 2 and added to wells 3 and 4 and 50 μl of standard diluent was added. Then 50 μl of the mixed liquor in well 3 and 4 was discarded and 50 μl was added to wells 5 and 6, and 50 μl of standard diluent was added. Each of the 10 wells after the dilution was 50 μl. The blank wells and the tested samples were set (the blank control wells without the enzyme-labeled reagents and samples were the same as the above steps). The tested wells were added with 40 μl of the sample diluent and 10 μl diluent (5 times sample dilution). The well wall was not touched but shaken gently when operating, and was incubated in a water bath or incubator at 37˚C for 30 min. The sealed film was uncovered and the liquid was discarded using blotting paper. Each well was filled with scrubbing solution. After 30 sec, this step was repeated 5 times and the well was patted dry. Then 50 μl of the enzyme labeling reagent was added to each well and the mixture was incubated at 37˚C for 30 min except for the blank wells and 50 μl of substrate A and B was added to each well. The color was developed at 37˚C for 15 min and 50 μl of the stop solution was added to each well and zeroed with a blank well. The OD value (optical density value) of each well was measured at a wavelength of 450 nm in 25 min. The levels of IL-18 and IL-17 in the serum were calculated.

Statistical analysis. The experimental data were analyzed by SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). The measurement data were expressed by mean ± standard deviation and compared by paired t-test between the two groups. One-way ANOVA test was used for comparison among groups. LSD method was used for comparison between two groups. Pearson's was used for the correlation between IL-17 and IL-18. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of blood lipid levels between AS and normal mice. As shown in Fig. 1, the expression levels of LDL-cholesterol (LDL-C), TC, Glu in the model group induced by high-fat diet were significantly higher than those in the control group (P<0.05). There was no significant difference in TG and HDL-cholesterol (HDL-C) between the model and the control groups (P>0.05).
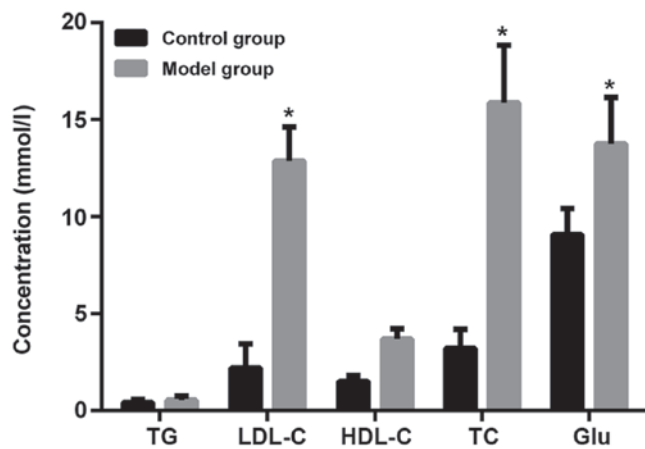
Figure 1. Comparison of blood lipid levels between AS and normal mice. The expression levels of LDL-C, TC and Glu in the model group induced by high-fat diet were significantly higher than those in the normal control group (*P<0.05). There was no significant difference in TG and HDL-C between the model and the control groups (P<0.05). AS, atherosclerosis; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; Glu, blood glucose levels; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.
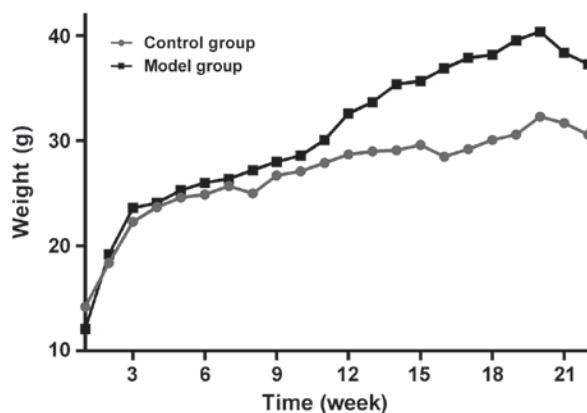

Figure 2. Changes of BMI in mice. There was no significant difference in initial weight between the model and the control groups (P>0.05). The body weight of the two groups increased with feeding time. The body weight of the two groups decreased at the 21st week. Weight of mice in the model group were significantly higher than those in the control group at the 16th week (P<0.05). BMI, body mass index.

Changes of body mass index (BMI) in mice. As shown in Fig. 2, there was no significant difference in initial weight between the model and the control groups (P>0.05). The body weight of the two groups increased with feeding time. The body weight of the two groups decreased at the 21st week. Weight in the model group was significantly higher than that in the control group at the 16th week (P<0.05) until the end of the experiment.

Comparison of IL-17 and IL-18 expression in serum of AS and normal mice. The expression of IL-17 and IL-18 in the model group was significantly higher than that in the control group (t=6.903, 11.02, P<0.05; Table I).

Comparison of IL-17 and IL-18 concentrations of non-plaque mice in the model group. The concentration of IL-17 and IL-18

Table I. Comparison of IL-17 and IL-18 expression in serum of AS mice and normal mice (mean ± standard deviation).

Group	Cases	IL-17 (μg/l)	IL-18 (pg/ml)
Control	20	23.13±4.32	37.76±5.39
Model	60	37.23±8.76	61.23±8.98
t		6.903	11.02
P-value		<0.001	<0.001

IL, interleukin; AS, atherosclerosis.

Table II. Comparison of IL-17 and IL-18 concentrations of non-plaque mice in the model group (mean ± standard deviation).

Group	Cases	IL-17 (μg/l)	IL-18 (pg/ml)
Stable plaque	21	32.87±6.76	56.09±7.34
Unstable plaque	28	38.36±9.76[a]	67.32±8.67[a]
Non-plaque	11	26.28±6.09[a,b]	40.98±6.23[a,b]
F		8.954	46.29
P-value		0.0004	<0.001

[a]Compared with stable plaque group (P<0.05); [b]Compared with unstable plaque group (P<0.05). IL, interleukin.

Table III. Expression of IL-17 and IL-18 in different degrees of inflammation in AS mice (mean ± standard deviation).

Severity	Cases	IL-17 (μg/l)	IL-18 (pg/ml)
I	18	27.12±5.98	41.76±6.32
II	30	33.76±7.99[a]	56.13±7.22[a]
III	12	40.01±10.34[a,b]	69.34±9.13[a,b]
F		9.638	51.76
P-value		0.0002	<0.001

[a]Compared with stable plaque group (P<0.05); [b]Compared with unstable plaque group (P<0.05). IL, interleukin; AS, atherosclerosis.

in the non-plaque group was significantly lower than that in the stable plaque and unstable plaque groups (P<0.05). The concentration of IL-17 and IL-18 in the stable plaque group was significantly lower than that in the unstable plaque group (P<0.05; Table II).

Expression of IL-17 and IL-18 in AS mice with different degrees of inflammation. According to grades of AS, the levels of IL-17 and IL-18 at grade I were significantly lower than those at grades II and III (P<0.05). The levels of IL-17 and IL-18 at grade II were significantly lower than those at grade III (P<0.05; Table III).
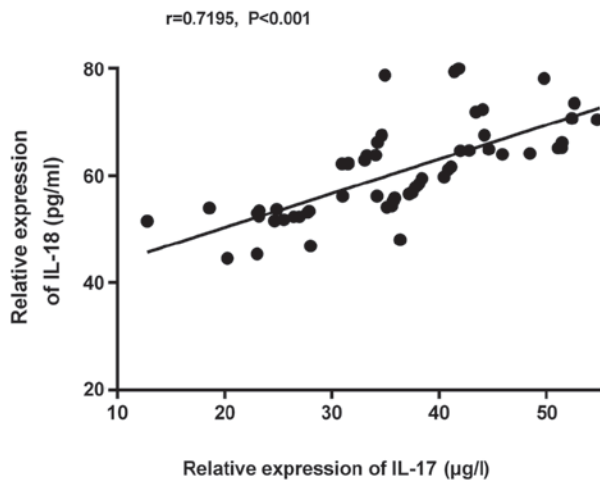
Figure 3. Correlation of IL-17 and IL-18 expression in serum. Based on the correlation of IL-17 and IL-18 expression in the model group, the expression of IL-18 increased with the expression of IL-17, indicating that the expression of IL-17 was positively correlated with that of IL-18 (r=0.7195, P<0.001). IL, interleukin.

Correlation of IL-17 and IL-18 expression in serum. Based on the correlation of IL-17 and IL-18 expression in the model group, the expression of IL-18 increased with the expression of IL-17, indicating that the expression of IL-17 was positively correlated with that of IL-18 (r=0.7195, P<0.001; Fig. 3).

Discussion

The occurrence and development of AS is a chronic inflammatory process, among which inflammatory cells and mediators are involved (14). Many studies suggested that the rupture and shedding of vulnerable atherosclerotic plaques were independent risk factors for acute cerebral infarction (15,16). Therefore, the prevention and treatment of cardiovascular disease is to identify plaque at earlier stages (17). Studies indicated that unstable plaques were soft plaques composed of lipids, with ulcers or bleeding plaque on the surface, which were prone to acute ischemia (18).

As a proinflammatory cytokine, IL-17 is secreted by Th17 cells, which activates macrophages, vascular smooth and endothelial cells, resulting in inflammatory cytokines and the formation of AS (19). Previous findings have shown that the downregulation of IL-17 expression was associated with the intraperitoneal injection of anti-IL-17 antibodies. IL-17 antibody inhibits the formation of plaques in Apo E-/- model mice, playing an important role in the formation of AS plaques (20). IL-18, not only promoted the proliferation of T cells and increased the activity of T cells and NK cells, but also promoted the production of cytokines such as tumour necrosis factor (TNF) TNF-α, IL-1, and IL-8 and the reduction of IL-10. The excessive secretion of IL-18 caused invasive inflammatory cells, or even damaged cells (21).

Lipid metabolism disorder is considered to be the cause and pathological basis in the formation of AS. Excessive lipid in the plasma will enter the intima of the artery after injury and was accumulated in the artery wall. Deposit in the subintimal space leads to lipid deposition and early atherosclerotic lesions (22). The expression levels of LDL-C, TC and Glu in the model group induced by high-fat diet were significantly higher than those in the normal control group. There was no significant difference in TG and HDL-C between the model and the control groups. Aubin *et al* (23) indicated that no changes of TC, LDL-C, HDL-C and TG were found in plasma of SD rats after 8 weeks of high fat feeding, which was consistent with the results of HDL-C and TG in this study. However, the levels of LDL-C, TC and Glu in the model group were higher than those in normal control group, which might be related to longer feeding time. There was no significant difference in initial weight between the model and the control groups. The body weight of the two groups increased with feeding time. The body weight decreased in the two groups at the 21st week. Weight of the mice in the model group were significantly higher than those in the control group at the 16th week. Sun *et al* (24) studied on the changes of BMI in mice screened for atherosclerotic animal models. Mallat *et al* (25) suggested that the expression of IL-18 mRNA was higher in atherosclerotic plaques compared with normal arteries. The study indicated that the expression of IL-17 and IL-18 in serum of the model group were significantly higher than that in the normal control group (P<0.05). Underhill *et al* (26) showed that the levels of serum IL-23, IL-17 and CRP in the acute cerebral infarction group were higher than those in the control group (P<0.05). Some studies have suggested that the reduction of IL-17 expression was related to intraperitoneal injection of anti-IL-17 antibodies. IL-17 antibody inhibited the formation of plaques in Apo E-/- model mice, playing an important role in the formation of AS plaques (20). Mallat *et al* (27) indicated that IL-18 mRNA and related proteins were expressed in human carotid atherosclerotic plaques. The expression of IL-18 in unstable plaques was stronger than that in stable plaques, indicating that IL-18 was related to the stability of the plaque. The concentration of IL-17 and IL-18 in the non-plaque group was significantly lower than that in the stable plaque and unstable plaque groups. The concentration of IL-17 and IL-18 in the stable plaque group was significantly lower than that in the unstable plaque group. IL-17 and IL-18 are involved in the early inflammatory response of atherosclerotic plaque, which have an impact on the development and instability of plaque. Animal models (27) indicated that the inhibition of the process of AS plaque and the stable development of plaques can be achieved by inhibiting IL-18 antibodies. According to different grades of AS, the expression of IL-17 and IL-18 in the model group at grade I was significantly lower than that at grades II and III (P<0.05), while the expression of IL-17 and IL-18 at grade II was significantly lower than that at grade III (P<0.05). IL-17 and IL-18 antibodies play an important role in the development of AS. The higher the level of IL-17 and IL-18 antibodies, the more severe the AS of patients. The expression of IL-18 increased with the expression of IL-17, indicating that the expression of IL-17 was positively correlated with that of IL-18 (r=0.7195, P<0.001). IL-17 and IL-18 may be interrelated inflammatory factors in the formation and development of atherosclerotic plaque. There is a correlation between IL-18 and P-selectin in coronary atherosclerotic

plaques (28). There is no report on research on the correlation between IL-17 and IL-18 expression in AS.

This study investigated the expression of IL-17 and IL-18 in animal models through detecting animal models, which could be used as indicators of the severity of disease. However, the specific mechanism of IL-17 and IL-18 in the formation of atherosclerotic plaques is not clarified. The reduction of pro-inflammatory factors and increase of anti-inflammatory factors will need further study.

In conclusion, serum IL-17 and IL-18 played an important role in the formation and development of atherosclerotic plaques and were closely related to the stability and severity of plaques. The expression of IL-17 and IL-18 was positively correlated. Future studies are to focus on ?

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XT conceived and designed the study, collected, analyzed and interpreted the experiment data, drafted this study, and revised the manuscript critically for important intellectual content. XT read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Affiliated Hospital to Changchun University of Chinese Medicine (Changchun, China).

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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