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ORIGINAL ARTICLE

YKL-40 promotes the progress of atherosclerosis independent of lipid metabolism in apolipoprotein $E^{-/-}$ mice fed a high-fat diet

Lei Chen[1] · Jianlei Zheng[2] · Qi Xue[2] · Yan Zhao[2]

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Abstract

YKL-40 is recently regarded as a pro-inflammatory cytokine involved in the pathological process of atherosclerosis and lipid metabolism. However, whether YKL-40 can directly influence the development of atherosclerosis and levels of lipid parameters is unknown. The aim of this study is to explore the effects of YKL-40 on atherosclerotic features, the levels of serum lipids, and biomarkers in apolipoprotein (E)-deficient ($ApoE^{-/-}$) mice fed a high-fat diet. $ApoE^{-/-}$ mice were injected with a recombinant adenovirus expressing mouse YKL-40 or control adenovirus through the caudal vein. The levels of serum YKL-40, interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-alpha), matrix metalloproteinase-9 (MMP-9), and soluble vascular cell-adhesion molecule 1 (sVCAM-1) were measured by ELISA. Lipid metabolism parameters were measured using immunoturbidimetric assay. The size of plaque area in aorta was evaluated by Oil Red O and hematoxylin/eosin (HE) staining. The content of collagen fibers was stained with Masson, and the content of macrophages and smooth muscle cells (SMCs) in atherosclerotic lesions was investigated by immunohistochemistry. The serum levels of total cholesterol and triglycerides were similar between these two groups. Compared with the control, the levels of serum YKL-40, IL-6, TNF-alpha, MMP-9, plaque size, and macrophages in plaques were significantly increased in mice with adenovirus overexpressing YKL-40. However, the content of collagen fibers and SMCs was remarkably decreased in mice with adenovirus overexpressing YKL-40 than that in control. YKL-40 prompts the progress of atherosclerosis maybe involved with its role of pro-inflammation, but does not affect lipid metabolism in $ApoE^{-/-}$ mice fed a high-fat diet.

Keywords Atherosclerosis · Inflammation · Lipid metabolism · YKL-40

Introduction

Atherosclerosis is considered as a chronic disease which displays numerous pathological characteristics such as endothelial dysfunction, vascular inflammation, accumulation of cholesterol, and calcium in the medium- and large-sized arteries [1, 2]. The initiation and development of atherosclerosis are a complicated process involved with fatty streak and plaque formation, coronary lumen stenosis, and plaque rupture [3]. Not only the size of the plaques, but also the plaque features determine the prognosis of the cardiovascular disease. It has been evidently demonstrated that the plaque stability is subject to the proportion of macrophages, lipid core, SMCs, extracellular matrix, and collagen fibers in atherosclerotic lesions [4, 5].

YKL-40 (also known as human cartilage glycoprotein-39 or 38-kDa heparin-binding protein) is recently found as an inflammatory cytokine in atherosclerotic lesions, which is released by macrophages, SMCs, and other cells [6]. In clinical studies, several studies demonstrated that elevated serum YKL-40 levels were independently associated with the presence and extent of coronary artery disease and even higher YKL-40 levels were observed in patients with myocardial infarction [7, 8]. Moreover, the increase of serum YKL-40 levels has also been found to be associated with all-cause as well as cardiovascular mortality in an elderly population [9]. In our previous study, we investigated that angiographical coronary progression was related to elevated

Lei Chen and Jianlei Zheng contributed equally to this work.

✉ Jianlei Zheng
zhengjianlei@zjheart.com

[1] Department of Pathology, Zhejiang Provincial People's Hospital, Hangzhou 310014, Zhejiang, China

[2] Department of Cardiology, Zhejiang Provincial People's Hospital, 158 Shangtang Road, Hangzhou 310014, Zhejiang, China

serum YKL-40 level [10]. It was reported that fasting serum levels of YKL-40 were positively associated with plasma triglyceride levels in individuals at high risk of type 2 diabetes, and another study showed the similar result between plasma YKL-40 and triglycerides in general population [11, 12]. Except the triglycerides, the correlation between serum cholesterol levels and YKL-40 was also reported in different populations [13, 14]. In addition, YKL-40 as a proliferation cytokine prompts the cell migration and growth, including SMCs and various tumor cells [15, 16]. YKL-40 also takes part in angiogenesis, and anti-YKL-40 antibody or YKL-40 short hairpin RNA (shRNA) can inhibit the tube formation of microvascular endothelial cells [17, 18]. Angiogenesis is not only the prognosis factor for tumor growth and metastasis, but also for unstable plaques in atherosclerosis [19]. All of the above studies showed that YKL-40 played important roles in the pathophysiological process including inflammation, lipid metabolism, angiogenesis, and proliferation.

However, the effects of YKL40 on the development and features of atherosclerosis are still seldom reported. The present study is to demonstrate whether the serum levels of IL-6, TNF-alpha, sVCAM-1, MMP-9, and lipid parameters are influenced by the overexpression of YKL-40. On the other hand, we further investigate whether YKL-40 affect the stability of atherosclerosis by regulating the expression of macrophages, collagen fibers, and SMCs in atherosclerotic plaques.

Methods

Mouse atherosclerosis model

The present study was approved by the Ethics Committee of the Animal Research Institute of Zhejiang Provincial People's Hospital (Zhejiang, China). All the procedures were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twenty male ApoE$^{-/-}$ mice aged 8 weeks on the C57BL/6J background were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Mice were maintained on 12 h dark/12 h light cycles in air-conditioned rooms (23.5 ± 0.5 °C, 50 ± 5% humidity) and access to chow diet and water ad libitum for 2 weeks. At age of 10 weeks, mice were randomly assigned into two groups ($n = 10$, each group), and all the ApoE$^{-/-}$ mice were provided with high-fat diet (HD012; purchased from BiotechHD Co. Ltd., Beijing, China) for 10 weeks. The high-fat diet mainly contains 21.1% proteins, 38.6% carbohydrate, and 22.5% fat (all by weight). High-fat diet intake was closely monitored and did not differ between two groups. At age of 14 and 16 weeks, one group was injected into 200 µl recombinant adenovirus-YKL-40 per time (virus titre was 1.0×10^{10}/ml) provided by Shandong ViGene Biosciences (Jinan, Shandong, China.) and another was injected into 200 µl control adenovirus without YKL-40 per time through tail veins. Body weight and food intake were monitored throughout the study. All the mice were killed after the infusion of sodium pentobarbital overdose at age of 20 weeks to collect the aorta which was fixed in paraformaldehyde and embedded in paraffin for atherosclerosis analysis. Serum was collected to analyze lipid parameters and serum levels of IL-6, TNF-alpha, YKL-40, sVCAM-1, and MMP-9.

Serum concentrations of lipids, fasting blood glucose and biomarkers

Whole blood samples were collected from the orbital sinus after full anaesthesia with sodium pentobarbital and stored in sterilized Eppendorf tubes. Then, the blood was allowed to clot at room temperature before the serum was separated by centrifugation at 1000*g* for 10 min. Serum was stored at − 80 °C for following use. Fasting blood glucose, lipid levels including triglycerides and total cholesterol (TC) were measured with standard laboratory techniques on a Hitachi 912 Analyzer (Roche Diagnostics, Germany). The commercially available ELISA kits for sVCAM-1 and MMP-9 were provided by Meimian Biotechnology (Jiangsu, China), and IL-6, TNF-alpha, and YKL-40 were provided by Jianglai Biotechnology (Shanghai, China). Serum samples were assayed according to the manufacturer's instruction.

Analysis of atherosclerotic lesions

The adventitial fatty tissues were removed using pincettes. Aortas were opened longitudinally from the ascending aorta to the abdominal aorta, and fixed in 4% paraformaldehyde for 36 h. Then, the fixed aortas were stained with Oil Red O. To quantify areas of the atherosclerotic lesions, the stained aortas were photographed using a digital camera; then, the proportion of atherosclerotic plaques to the total aortic areas were measured according to the Oil Red O staining (Sigma-Aldrich, USA). Transverse sections of aorta paraffin were stained with hematoxylin/eosin (HE) (Maiwei, Xiamen, China) to evaluate the size of atherosclerotic lesions in the aortic lumen. Masson staining (Maiwei, Xiamen, China) was used to evaluate the content of collagen fibers in atherosclerotic plaques. Quantitative analysis of lesions was performed with Image-Pro Plus 6.0.

Immunohistochemistry analysis

The aortic arch was sliced into 5 µm thick for morphometric analyses. Histological sections from the aorta were treated with 3% hydrogen peroxide to block endogenous peroxidase activity, and immunohistochemical staining was performed

with anti-alpha-SMA and anti-MAC-2 antibodies (both are 1:100 dilution, Abcam, UK). Then incubated with a biotinylated secondary antibody; and finally counterstained with HE. All cross sections were analyzed under an upright microscope (Nikon, Tokyo, Japan). The positive areas were measured in five non-overlapping fields and analyzed with Image-Pro Plus 6.0.

Statistical analysis

Data are presented as means ± SD. A two-tailed Student's *t* test was used to determine significance. Differences were considered significant for $*p<0.05$ and $**p<0.01$. Analyses were done using the statistical software SPSS 11.0 (SPSS, Inc., Chicago, IL, USA).

Results

The serum levels of lipid parameters, fasting blood glucose, and biomarkers

Table 1 shows the data of serum YKL-40, IL-6, TNF-alpha, sVCAM-1, MMP-9, fasting blood glucose, and lipid levels of the mice. As a result, the levels of serum YKL-40, IL-6, TNF-alpha, and MMP-9 were significantly higher in YKL-40 overexpression group than those in control group (all $p<0.05$). However, the levels of serum fasting blood glucose, TC, triglycerides, and sVCAM-1 were no significant difference between these two groups. The correlation between serum YKL-40 and lipid parameters was not statistically significant as well. Specifically, TC and triglycerides did not correlate with serum YKL-40 (Pearson's $r=0.105$, $p=0.773$ and Pearson's $r=0.214$, $p=0.552$, respectively).

The analysis of atherosclerostic constituent

The Oil Red O staining results displayed the atherosclerotic plaques in entire aorta, and atherosclerotic lesions in group of YKL-40 overexpression are remarkably increased than those in controls (14.1 ± 0.9% vs 11.3 ± 0.8%, $p<0.05$) (Fig. 1a, c). Compared with control mice, the area of atherosclerotic plaques significantly increased in the aorta of mice injected with adenovirus encoding YKL-40 ($p<0.01$) (Fig. 1b, d). Aorta cross-sectional Masson staining results showed that there were significantly less collagen fibers in aortic plaques after intervention with overexpression of YKL-40 in $ApoE^{-/-}$ mice (58.0 ± 2.8% vs 49.4 ± 3.2%; $p<0.05$) (Fig. 2c, f). Our data indicated that increased YKL-40 level prompted the formation of atherosclerotic areas and decreased the content of collagen fibers in the aorta of $ApoE^{-/-}$ mice fed with a high-fat diet.

The content of macrophage cells and SMCs in atherosclerotic lesions

Aorta cross-sectional immunochemistry analysis displayed that the number of macrophages in YKL-40 overexpression group was significantly increased compared with that in control group (6.3 ± 0.5% vs 4.3 ± 0.8%, $p<0.05$) (Fig. 2a, d). Meanwhile, the content of smooth muscle cells in YKL-40 overexpression group was remarkably decreased than that in control group (15.5 ± 1.5% vs 23.3 ± 1.6%, $p<0.01$) (Fig. 2b, e). Alterations in the composition of the atherosclerotic plaques showed that the overexpression of YKL-40 prompted the instability of the atherosclerotic lesions and aggregated the progress of atherosclerosis, which was probably associated with the pro-inflammation effect of YKL-40.

Table 1 Data on fasting blood glucose, lipid profile, and serum biomarkers in both groups

	YKL-40 group ($n=6$)	Control ($n=6$)	*p* value
Fasting blood glucose (mmol/l)	6.4 ± 0.5	6.5 ± 0.5	0.759
TC (mmol/l)	26.5 ± 3.3	25.3 ± 3.6	0.589
Triglycerides (mmol/l)	3.5 ± 0.3	3.3 ± 0.4	0.364
IL-6 (pg/ml)	43.3 ± 7.5	32.3 ± 8.4	0.038
TNF-alpha (pg/ml)	78.5 ± 7.6	63.3 ± 8.8	0.010
MMP-9 (μg/l)	65.5 ± 8.7	51.5 ± 6.1	0.018
sVCAM-1 (μg/l)	279.8 ± 39.6	261.5 ± 13.9	0.356
YKL-40 (ng/ml)	11.83 ± 1.7	8.6 ± 0.9	0.005

Values are expressed as mean ± SD

TC total cholesterol, *IL-6* interleukin-6, *TNF-alpha* tumour necrosis factor-alpha, *MMP-9* matrix metalloproteinase-9, *sVCAM-1* soluble vascular cell-adhesion molecule 1

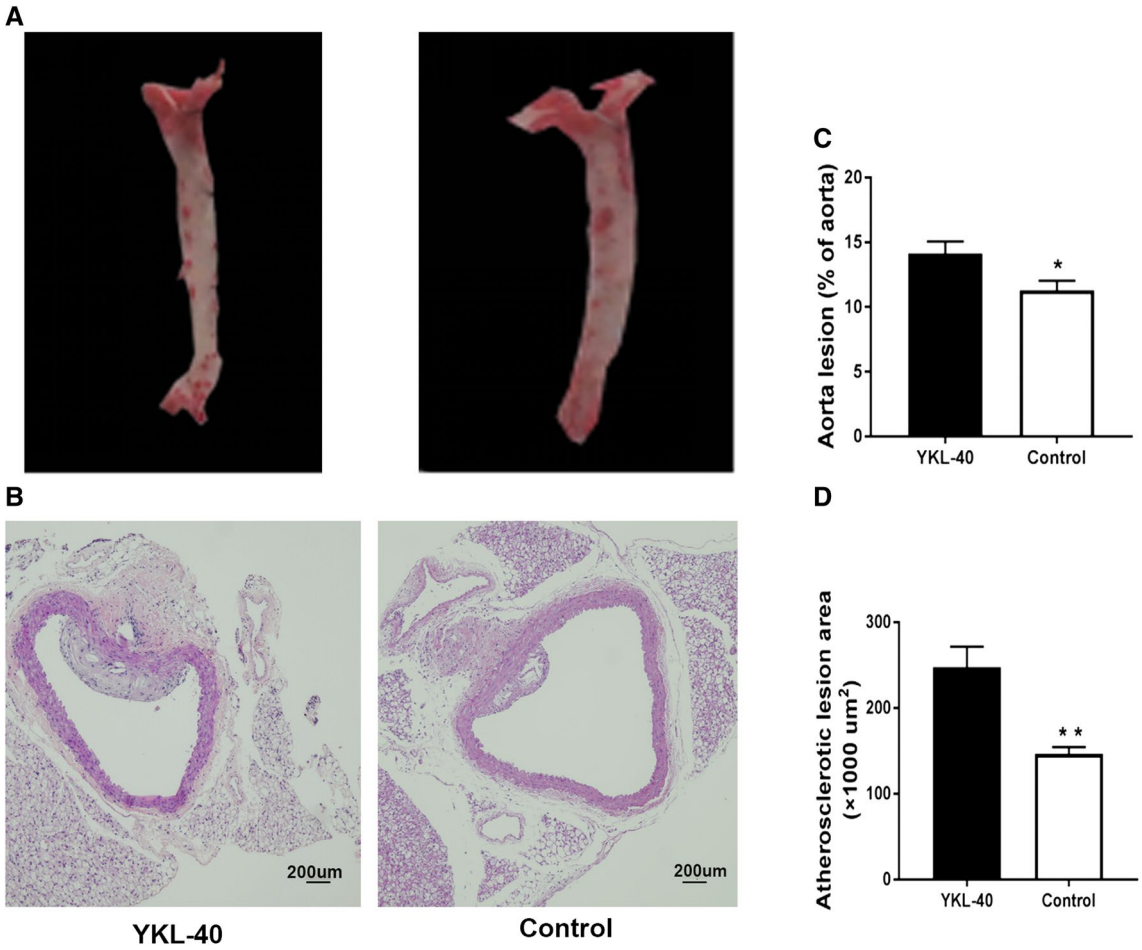

Fig. 1 Atherosclerotic lesion formation and plaque area in aortic sections from ApoE$^{-/-}$ mice. **a** Aortic en face flat from the ascending aorta to the abdominal aorta stained with Oil Red O. **b** Transverse section of atherosclerotic lesions strained with HE. **c** Relative area percent of the atherosclerotic lesion to the whole aorta in two groups. **d** Quantification of total atherosclerotic lesion area in serial cross sections of the aorta in two groups. Data are mean ± SD, $n = 3$. *$p < 0.05$ and **$p < 0.01$

Discussion

The abnormal lipid metabolism and inflammatory response are considered as two of the most important factors involved in the development and instability of atherosclerotic plaques [20]. YKL-40 is recently found as an inflammatory cytokine and related to lipid metabolism [6, 11, 13]. In our current study, we demonstrated that the overexpression of YKL-40 had no effects on the levels of serum lipid parameters. However, YKL-40 overexpression significantly increased the level of serum IL-6, TNF-alpha, MMP-9, the size of atherosclerotic plaques, and the content of macrophages in atherosclerotic lesions. The content of collagen fibers and the number of SMCs in atherosclerotic lesions were remarkably decreased in the aorta from the mice injected with adenovirus encoding YKL-40. In addition, YKL-40 overexpression tends to slightly increase serum sVCAM-1 levels, although without statistical significance. These results indicated that the effects of YKL-40 on atherosclerosis are quite complicated.

Atherosclerosis is a complicated pathological process, and the lipid metabolism disorder takes part in the initiation and development of atherosclerosis. Foam cells accumulation and lipid streak formation in the arterial wall are leading characteristic for early atherosclerosis [3]. The high serum cholesterol levels are associated with high prevalence of cardiovascular diseases, which were well established in the previous studies [21]. Diet with high-fat is an ideal model to construct hypercholesterolemia in ApoE$^{-/-}$ mice. It is now well accepted that early atherosclerosis is mainly displayed with a local immune inflammatory response to lipid deposition within the arterial subendothelial compartment [22]. Up to now, the association between inflammatory factor of YKL-40 and lipid metabolism is uncertain. Recently published studies showed that there were correlations between serum YKL-40 and the levels of serum triglycerides and

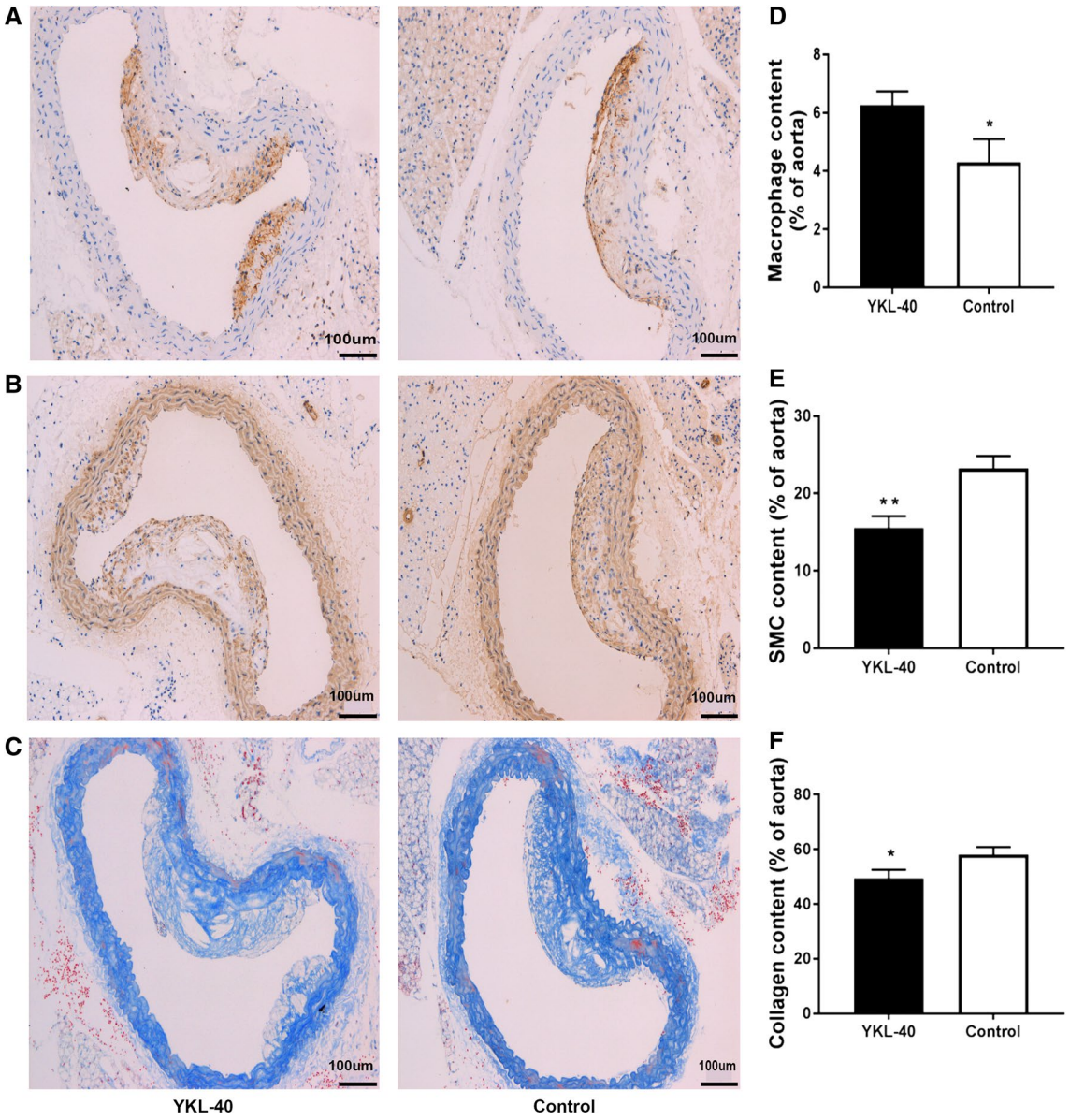

Fig. 2 Expression of macrophages, smooth muscle cells and collagen fibers in atherosclerotic lesions. Cross sections of the aorta were cut into slices and stained with anti-macrophage antibodies (**a**), anti-smooth muscle cell (**b**), and Masson (**c**). The percentages of macrophages (**d**), smooth muscle cells (**e**), and collagen fibers (**f**) in the whole aorta were calculated. Results are shown as mean ± SD; $n = 3$ per group; $^{*}p < 0.05$ and $^{**}p < 0.01$

cholesterol in different populations [11–14]. However, another study demonstrated that YKL-40 was not related to serum triglycerides in insulin-resistant hypertensive patients [23]. In our study, we also did not find that the overexpression of YKL-40 increased or decreased the concentration of serum lipid parameters. Although there is no relation between YKL-40 level and lipid metabolism, YKL-40 overexpression remarkably increase the size of atherosclerotic lesions in our study. In fact, overexpression of YK-40 in human colon cancer cells enhanced macrophage infiltration, migration of THP-1 cells, and tube formation of HUVECs [24]. Another study showed that YKL-40 overexpression promoted macrophage polarization to be M1 phenotype [25]. In addition, YKL-40 increased proliferation and migration of primary normal human bronchial smooth muscle cells by inducing IL-8 expression via MAPK- and NF-κB-signaling pathways [15]. Hence, we concluded that YKL-40 at a large scale prompted the development of atherosclerosis independent of lipid metabolism.

Atherosclerosis is an inflammatory cardiovascular disease, IL-6 and TNF-alpha as systemic inflammatory factors substantially affected cardiovascular function and

morphology and promoted the atherosclerotic progression in human beings and animal experiments [26, 27]. In addition, Endothelium injury and increased matrix metalloproteinases (MMPs) in the aorta are important pathological features of atherosclerosis [28–30]. Endothelium injury commonly means the initial of atherosclerosis, and sVCAM is an important indicator for endothelial dysfunction. Increased circulating levels of MMP-9 have been shown in patients with acute coronary syndrome [31]. It has been reported that increased serum YKL-40 level prompted plaque instability of carotid atherosclerosis with CagA-positive helicobacter pylori infection and YKL-40 increased MMP-9 expression and activity in THP-1 monocytes [32, 33]. In our current study, we found that YKL-40 overexpression tended to increase serum IL-6, TNF- alpha, and MMP-9 levels in ApoE$^{-/-}$ mice fed a high-fat diet. Compared with control, the expression of sVCAM-1 was slightly increased in mice with YKL-40 overexpression, but there was no statistical difference between these two groups. Although there were conflicting associations between atherosclerotic cardiovascular disease and sVCAM-1 [34], we supposed that relatively small samples as another reason led to no significant difference in our study.

The composition of the plaques determines the stability of atherosclerosis, and ever-increasing evidence shows that inflammation plays an important role in instability and rupture of atherosclerotic lesions [35]. YKL-40 was regarded as a pro-inflammatory glycoprotein involved in cardiovascular diseases and other diseases [6]. YKL-40 levels were elevated in patients with acute myocardial infarction and increased YKL-40 concentration was associated with all-cause as well as cardiovascular mortality [7, 10]. All the above studies display that YKL-40 may prompt the instability of atherosclerotic plaques. In the present study, we demonstrated that YKL-40 notably increased the number of macrophages in atherosclerotic lesions. The content of SMCs in atherosclerotic lesions was significantly decreased in YKL-40-overexpressed mice, compared with the control. Increased content of SMCs expressing contractile protein markers such as smooth muscle α-actin was regarded as a phenotype for stable plaques [36]. However, it is well known that MMPs may facilitate the migration of SMCs and infiltration of leucocytes, which may lead to structural changes and progression of the atherosclerotic plaque [37]. In fact, inflammation circumstances tend to prompt SMCs from contractile phenotype to synthetic phenotype, and this "phenotypic switching" of SMCs has been considered to be an important contributor to the vulnerable plaques [38, 39]. In addition, the content of collagen fibers in ApoE$^{-/-}$ mice with YKL-40 overexpression was notably decreased than that in control. All the above investigations showed that YKL-40 overexpression significantly led to the instability of atherosclerosis.

In conclusion, YKL-40 had no effects on lipid metabolism in our present study, but it prompted the progress and instability of atherosclerosis was probably associated with the role of YKL-40 in promoting the accumulation of macrophages and decreasing the content of collagen fibers and SMCs in atherosclerotic plaques.

Limitation

The current study is only an observational results without referring to physiopathological mechanism. The majority of adenoviruses encoding YKL-40 survive in vivo about 2 weeks after injecting through tail veins. Considering the effect of YKL-40 overexpression is transient and unstable, therefore, the use of YKL-40 knockdown mice can more overwhelmingly answer the role of YKL-40 in the initiation and development of atherosclerosis. ApoE gene was not only associated closely with cognitive function, but related to the physiological effects in various nervous cells such as astrocytes involved in neuronal survival and synaptogenesis [40, 41]. Hence, it was believed that knockout for low density lipoprotein receptor (LDLR$^{-/-}$) mice would be a better model for atherosclerosis. In addition, due to the complicated pathological process of atherosclerosis, it is necessary to further explore whether YKL-40 has the effects on the polarization of macrophages, the angiogenesis of endothelial cells and the phenotypic switching of SMCs in the following studies, which will be more significant to answer the molecular mechanism of YKL-40 in adjusting the instability of atherosclerotic plaques.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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