CXCL16 may be a predisposing factor to atherosclerosis: An animal study

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Abstract. Atherosclerosis (AS) is a chronic inflammatory process initiated when lipoprotein is retained in the arterial wall. Leukocyte recruitment accelerates this process. CXC chemokine ligand 16 (CXCL16) acts as a chemokine to attract immune cells and also facilitates the phagocytosis process of modified low-density lipoprotein. Whether CXCL16 promotes or inhibits the pathological process of AS remains to be elucidated. To clarify this, CXCL16 gene was introduced into C57BL/6J wild-type mice to establish a stable CXCL16 overexpression mouse model. The initial changes of AS in mice were induced by high-fat diet (HFD). To study how the interaction of HFD and CXCL16 affected fatty acid metabolism and deposition, body weight and plasma lipid profile were assessed. Soluble CXCL16, matrix metalloproteinase-9, monocyte chemoattractant protein-1 and intercellular adhesion molecule-1 were detected by immunohistochemistry and ELISA to identify how CXCL16 affects AS lesion formation. The present study suggested that overexpression of CXCL16 combined with HFD lead to atherogenesis by upregulating the aforementioned inflammatory related genes at a protein level. The present study was the first, to the best of the authors' knowledge, to build a CXCL16 homozygous transgenic mice model to study how overexpressed CXCL16 is associated with AS for intervening in the occurrence and development of AS.

Introduction

Atherosclerosis (AS) is a chronic inflammatory disease as well as a cholesterol storage disease, which is often asymptomatic at the early stages but can lead to serious ischemic vascular disease and thrombosis such as coronary heart disease and carotid artery disease at an advanced stage (1-3).

Risk factors of AS, such as obesity, smoking, stress and hypertension, are all intimately linked with a higher level of local or global inflammation (4-8). CXC chemokine ligand 16 (CXCL16), a member of the CXC chemokine family, serves an important role in the initiation and maintenance of inflammation (9,10). Evidence links it with the formation and progression of AS (11). CXCL16 expresses in aortic smooth muscle cells exclusively within atherosclerotic plaques (12). Its two distinct existing isoforms *in vivo*, transmembrane and soluble form, give it great influence over inflammation (13). The transmembrane isoform functions as a selective cell adhesion molecule for CXC receptor 6^+ cells, such as monocytes and T lymphocytes, to facilitate cell-cell interaction (14), while the soluble isoform secreted into extracellular matrix or plasma exerts a relatively long distance recruitment of immune cells and regulates a global inflammation level (15,16). Another role CXCL16 performs is a scavenger receptor which facilitates internalization of fatty acids in several central cell types involved in AS, such as macrophages and T lymphocytes (17). Clinical researches investigating the role of CXCL16 in AS or carotid artery disease are controversial, for instance, as to whether CXCL16 promotes or inhibits lesions (18). Decreased and increased plasma levels of CXCL16 are identified to be related to cardiovascular or cerebrovascular events, such as coronary artery disease and atherosclerotic ischemic stroke (11,19). It is probably due to the methodological limitation of clinical research that cross-sectional studies or a relatively short period of prospective study cannot well determine causal relationship. The present study attempted to address this problem by introducing CXCL16 gene into C57BL/6J wild-type mice, a canonical strain for metabolism disorder, to create a predisposed high expression level of CXCL16 during a relatively long period of time. The present study also focused on how this high level of CXCL16 expression affected atherogenicity in an inflammatory aspect by testing several well-established atherosclerotic inflammation markers.

Materials and methods

Laboratory animals. Animal experiments were approved by the Laboratory Animal Welfare and Ethics Review Committee

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of University of South China (approval no. SYSK2013-0010). The present study was performed according to the requirements in the Guide for the Care and Use of Laboratory Animals (20). Specific pathogen-free (SPF) C57BL/6J wild-type mice were purchased from Shanghai Model Organisms Center, Inc. (SMOC) and raised under SPF conditions. A total of 45 mice (30 females and 15 males; age, 6-8 weeks; weight, 17-20 g) were housed in standard plastic cages at 20-26˚C, 40-70% humidity with a 12 h light/dark cycle and had free access to water and food. The mice were given 2 weeks to acclimate for breeding before any treatment.

Vector construction. The goal of the present study was to subclone mouse CXCL16 cDNA into the pGL3-basic mammalian expression vector. PCR amplification was used to multiply Murine CXCL16 cDNA and simultaneously as part of the primer a sequence (5' to 3') of flag tag (GATTACAAGGATGAC GACGATAAG) was attached to the N terminus. Later scavenger receptor (SR)-enhancer, SR-promoter and CXCL16-flag were all respectively inserted into pGL3-basic between restriction sites *Kpn*I and *Nhe*I, *Xho*I and *Hin*dIII, *Hin*dIII and *Xba*I. A genomic DNA isolation kit (Bio Basic Inc.) was used to extract and purify DNA (the yield of 5-10 μg) from miniprep according to the manufacturer's protocols. Verification was performed through Sanger sequencing (21) using an ABI 3730XL automatic sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). A triple cut site *Cla*I was set to cut the vector in to three parts that were respectively 1.8, 2.7 and 0.1 kbp. The 1.8 kbp fragments including CXCL16 cDNA were collected and purified from agarose gel electrophoresed in TBE running buffer using the gel recovery kit (Bio Basic Inc.), according to the manufacturer's instructions, for further use.

Founder mouse generation. A total of 30 C57BL/6J x CBA F1 hybrid female mice (age, 2-4 weeks; weight, 7-10 g) supplied by SMOC were housed in standard plastic cages at 20-26˚C and 40-70% humidity with a 12 h light/dark cycle. These mice were used as their oocytes exhibit high survival and maturation rates after microinjection (22). At the age of ~6-8 weeks old, 12 h before dark cycle, they were injected with 10 IU PMSG and ~48 h afterwards with 10 IU human chorionic gonadotropin i.p. In the same afternoon they were mated 1:1 with fertile male C57BL/6J mice. For all mouse experiments, the mice were anesthetized with 50-60 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA) and sacrificed by cervical dislocation. The next day, if a vaginal plug was present, they were set as donors and fertilized eggs were excised from oviducts into M2 culture medium (Sigma-Aldrich; Merck KGaA). After washing, screening and removal of cumulus cells, the zygotes were transferred into M16 medium (Sigma-Aldrich; Merck KGaA) prior to microinjection. Linear DNA constructs (1.8 kbp) prepared as aforementioned were injected into the pronuclei of zygotes using micromanipulator (Narishige Group). After ~20-30 min rest in M16, zygotes (20-30 each) were transferred into the oviducts of pseudopregnant C57BL/6J mice prepared beforehand. Integration of the transgene was determined by a double check of transgene-specific PCRs with genomic DNA isolated from tail biopsies of the progenies. To distinguish genomic CXCL16 from transgenic CXCL16, special upstream and downstream primers were located in SR-promoter and CXCL16-flag respectively. The primer sequences (purchased from Sangon Biotech Co., Ltd.) were given in Table I.

Table I. Primer sequences used.

Name	Sequence
CXCL16-FP	5'-TAAAGCGGCCTAAATGGGGTG-3'
CXCL16-RP	5'-CAAGAAAAGGAAGAACGCAAGAGA-3'
β-Actin-FP	5'-CATCCTGCGTCTGGACCTGG-3'
β-Actin-RP	5'-TAATGTCACGCACGATTTCC-3'

CXCL16, CXC chemokine ligand 16; FP, forward primer; RP, reverse primer.

Transgenic mice strain generation. PCR positive offspring were mated with wild-type C57BL/6J mice according to female/male ratio (1:1 or 1:2). To generate homozygous transgenic strain, sib-mating was applied most of the time and backcrossing when necessary. Genomic DNA was extracted from the descendants to sequence for the verification of transgene presence. PCR was used as a screen method. When all progenies (n>5) of two consecutive generations were all homozygous, the mouse was considered homozygous. After 10 generations of selective breeding, a $CXCL16^{+/+}$ transgenic line was successfully established. In total, 16 $CXCL16^{+/+}$ C57BL/6J mice from F11 combined with 16 wild-type C57BL/6J mice were chosen as subjects.

Treatment. $CXCL16^{+/+}$ and wild-type mice were randomly selected and divided into two treatment groups. One group was fed with high-fat diet (HFD, 67.5% standard chow, 15% lard, 10% yolk powder, 3% cholesterol, 4% whole milk powder and 0.5% Sodium cholate; Amresco, LLC) and the other standard chow, termed low-fat diet (LFD) for 18 weeks (23-25). Body weight, plasma total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL) were measured at week 18. Mice in each group were intraperitoneally injected with sodium pentobarbital (50 mg/kg) and were sacrificed by cervical dislocation followed by decapitation.

ELISA. ELISA kits for Mouse CXCL16 (cat. no. 10030301; Cusabio Technology LLC), Mouse matrix metalloproteinase-9 (MMP-9; cat. no. EK1462), monocyte chemoattractant protein-1 (MCP-1; cat. no. EK1351) and intercellular adhesion molecule-1 (ICAM-1; cat. no. EK1726; Wuhan Boster Biological Technology, Ltd.) were used according to the manufacturer's protocols. Absorbance was measured at 450 nm using an 800 TS Absorbance Reader (BioTek Instruments, Inc.). ELISA calc software (BioTNT; V0.1) was used to draw standard curve and calculate concentration.

Hematoxylin-eosin (HE) staining and immunohistochemistry. Biopsies were obtained from the aortic root and artery sinus and were fixed overnight in 4% formaldehyde solution at room temperature. Aortic root HE staining was performed

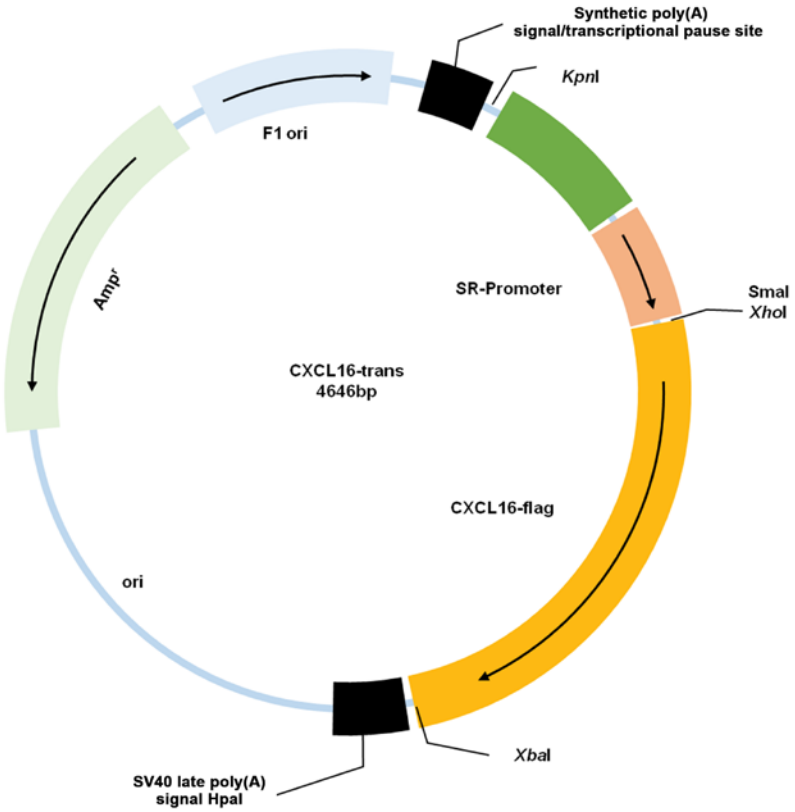

Figure 1. Diagram of transgenic mice strain generation. SR, scavenger receptor; CXCL16, CXC chemokine ligand 16; ori, origin; Ampr, ampicillin resistance gene; SV40, simian virus 40.

to examine the degree of fatty acid infiltration among the groups. The sections were stained in hematoxylin solution for 10 min and eosin solution for 1 min at room temperature. Immunohistochemical staining of the lesions was performed at room temperature for 1 h using rabbit polyclonal MMP-9 antibody (1:1,000; cat. no. ab38898), rabbit polyclonal MCP-1 antibody (1:1,000; cat. no. ab73680) (both Abcam) and rabbit polyclonal ICAM-1 antibody (1:200; cat. no. PB9081; Wuhan Boster Biological Technology, Ltd.).

Statistical analysis. Each experiment was repeated in triplicate. Data analysis was performed with SPSS v17.0 (SPSS, Inc.) and presented as the mean ± standard deviation. Results were determined by unpaired Student's t test and one-way ANOVA (post hoc test: Tukey's multiple comparison test) or two-way ANOVA (post hoc test: Bonferroni). $P<0.05$ was considered to indicate a statistically significant difference. All diagrams were generated by GraphPad Prism7 (GraphPad Software, Inc.).

Results

Transgene construct. Following several rounds of insertion, a plasmid with the backbone of pGL3-basic, SR-enhancer, SR-promoter, CXCL16 CDS and flag was successfully constructed (Fig. 1) and confirmed by sequencing.

CXCL16$^{+/+}$ transgenic mice. A total of 426 microinjected eggs were obtained and 20 eggs as one unit were transferred to oviducts of 20 pseudo mothers. Among which 12 became pregnant and 171 young were born. Following PCR screening, 26 were proved to be carriers of the target gene. After 4 weeks, another double-blind PCR was performed on 26 transgenic and four wild-type mice, and confirmed the stability of transgene expression. Founder mice were mated with wild-type C57BL/6J mice. The PCR-positive offspring were then mated with positive siblings in order to obtained homozygous transgenic mice. When all progenies (n>5) of two consecutive generations were all homozygous, the parent mice were considered homozygous. F8-1-2 (female) and F9-2-3 (male) were thus proved homozygous and, when mated with wild type mice, their offspring were all positive. Backcross F8-1-2 with F9-2-3, 5 descendants were obtained and termed C10-1-1 to C10-1-5. Sib-mate C10-1 and 19 descendants were obtained and eight were randomly chosen as CXCL16$^{+/+}$ transgenic subject mice. In addition, eight from the 11th generation of wild-type mice were randomly selected as subjects.

Transgene expression in CXCL16$^{+/+}$ transgenic mice. CXCL16 mRNA expression on artery AS lesion and plasma protein expression was measured at week 18. In the two HFD groups or the two LFD groups, CXCL16$^{+/+}$ transgenic mice had a higher CXCL16 mRNA expression (Fig. 2A). CXCL16 mRNA expression of CXCL16$^{+/+}$ transgenic mice or wild-type mice fed with HFD was significantly higher than those fed with LFD (Fig. 2A). CXCL16 plasma protein expression was result similar to CXCL16 mRNA expression on artery AS lesion (Fig. 2B).

Plasma lipid profile and visceral fat accumulation. Body weight and plasma lipid profile were measured at the end of the week 18. No significant difference was observed

Table II. Counts of mice with liver disease in each experimental group (n=8).

Group	n	Cirrhosis	Fatty liver	Healthy liver	Nos. of livers with pathological changes	Percentage
HDF CXCL16$^{+/+}$	8	4	4	0	8	100
HDF wild-type	8	0	4	4	4	50
LDF CXCL16$^{+/+}$	8	0	0	8	8	0
LDF wild-type	8	0	0	8	0	0

CXCL16, CXC chemokine ligand 16; HDF, high-fat diet; LDF, low-fat diet.

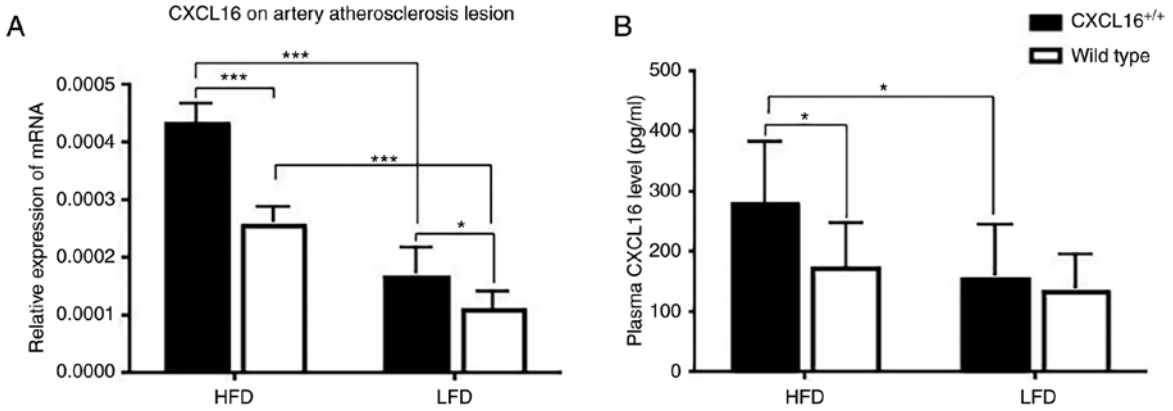

Figure 2. CXCL16 expression in CXCL16$^{+/+}$ transgenic mice. (A) Relative mRNA expression of CXCL16 on artery atherosclerosis lesion in the HFD and LFD groups. (B) Plasma CXCL16 level at week 18 in the HFD and LFD groups. All data are expressed as the mean ± standard deviation and analyzed by one-way or two-way analysis of variance (n=8). *P<0.05, ***P<0.001. CXCL16, CXC chemokine ligand 16; HFD, high-fat diet; LFD, low-fat diet.

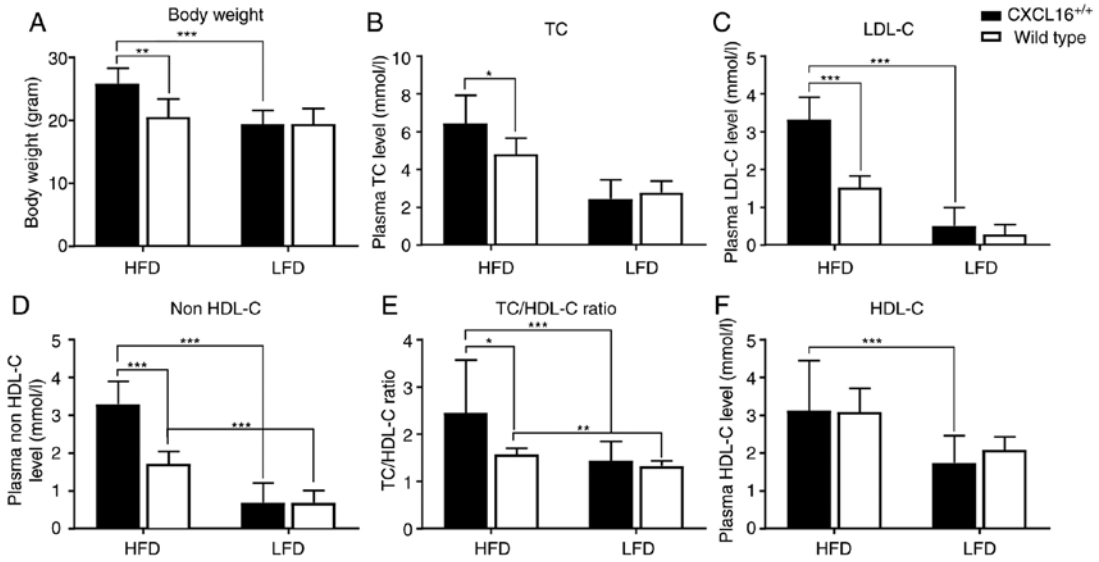

Figure 3. Body weights and plasma lipid profiles in different groups. (A) Body weights and plasma level of (B) TC, (C) LDL-C, (D) non HDL-C, (E) TC/HDL-C ratio and (F) HDL-C. All data are expressed as the mean ± standard deviation and analyzed by one-way or two-way analysis of variance (n=8). *P<0.05, **P<0.01, ***P<0.001. TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; HFD, high-fat diet; LFD, low-fat diet.

between the two LFD groups, but the average body weight of CXCL16$^{+/+}$ transgenic mice fed with HFD was significantly highly (Fig. 3A) compared with that of wild-type mice fed with the same regimen. Similar results were observed in plasma TC level, LDL-cholesterol (LDL-C) level, non HDL-C level and TC/HDL-cholesterol (HDL-C) ratio (Fig. 3B-E). Another significant change was identified between CXCL16$^{+/+}$ transgenic mice fed with HFD and LFD. When fed with HFD, CXCL16$^{+/+}$ transgenic mice tend to maintain much higher plasma lipoprotein levels such as LDL-C and HDL-C

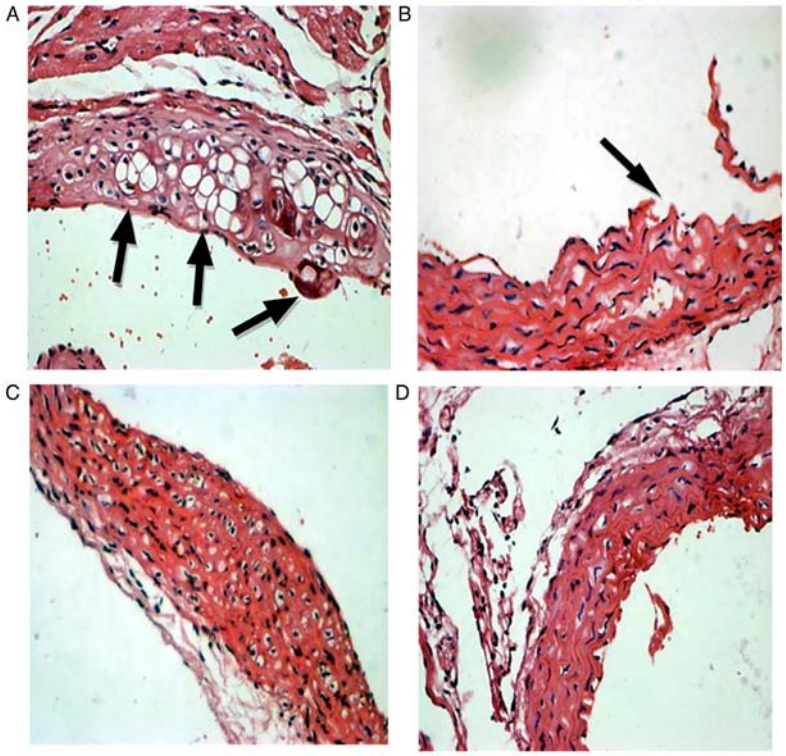

Figure 4. HE staining of aortic root sections in different groups. Representative images of aortic root sections stained with HE to exam total fatty acid infiltration. Black arrows indicate fatty acid infiltration. (A) HFD CXCL16$^{+/+}$, (B) HFD wild type, (C) LFD CXCL16$^{+/+}$, (D) LFD wild type. Magnification, x400. HE, hematoxylin-eosin; CXCL16, CXC chemokine ligand 16; HFD, high-fat diet; LFD, low-fat diet.

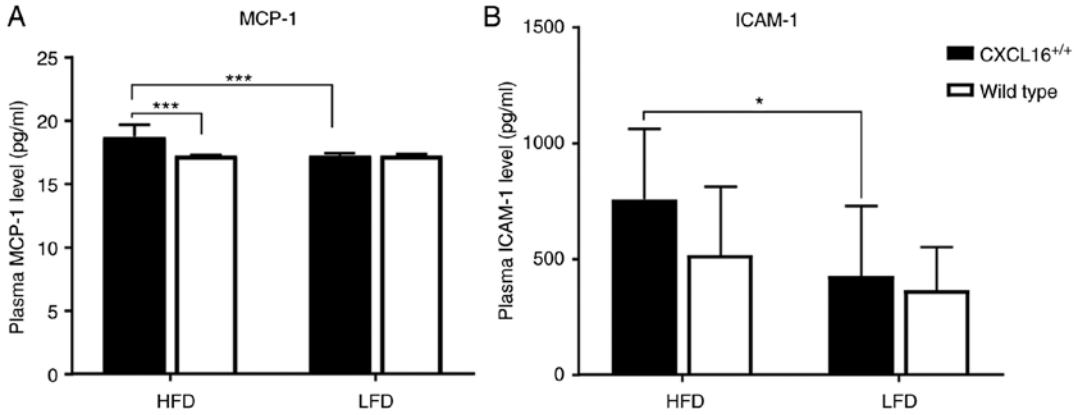

Figure 5. Plasma MCP-1 and ICAM-1 levels in different groups. Quantitative analysis of plasma MCP-1 and ICAM-1 levels in different groups was conducted using ELISA. (A) MCP-1, (B) ICAM-1. All data are expressed as the mean ± standard deviation (n=8) and analyzed by one-way or two-way analysis of variance. *P<0.05, ***P<0.001. MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule 1; HFD, high-fat diet; LFD, low-fat diet.

compared with the LFD groups (Fig. 3C and F), even when no significant differences were observed between the expression levels of the wild-type mice groups. This suggested that CXCL16 exerted an effect on fatty acid metabolism, but it was only significant when a higher intake of fatty acid was present.

Visceral fat accumulation was recorded in the form of images (data not shown). Fibrosis status was determined according to the Ishak staging system (26). Table II shows the percentage of mice with different degree of liver disease; after 18 weeks of HFD, 100% of mice with CXCL16$^{+/+}$ developed liver diseases, while only 50% of wild-type mice in HFD did. The degree of fibrosis status was also relatively less. None of mice in the LFD groups developed fatty liver disease.

Aortic root HE staining. For aortic root HE staining, CXCL16 double positive mice in the HFD groups demonstrated the most marked contrast compared with healthy aorta (Fig. 4).

Plasma level of MCP-1 and ICAM-1. Plasma MCP-1 and ICAM-1 expression levels were determined by ELISA at the end of the week 18. Fig. 5A demonstrates the overall expression level of MCP-1 in the four groups. MCP-1 expression pattern among the LFD groups showed no observable discrepancy (Fig. 5A) while among the HFD groups, a significant difference was observed; with a higher expression of CXCL16, MCP-1 expression levels were also higher (Fig. 5A).

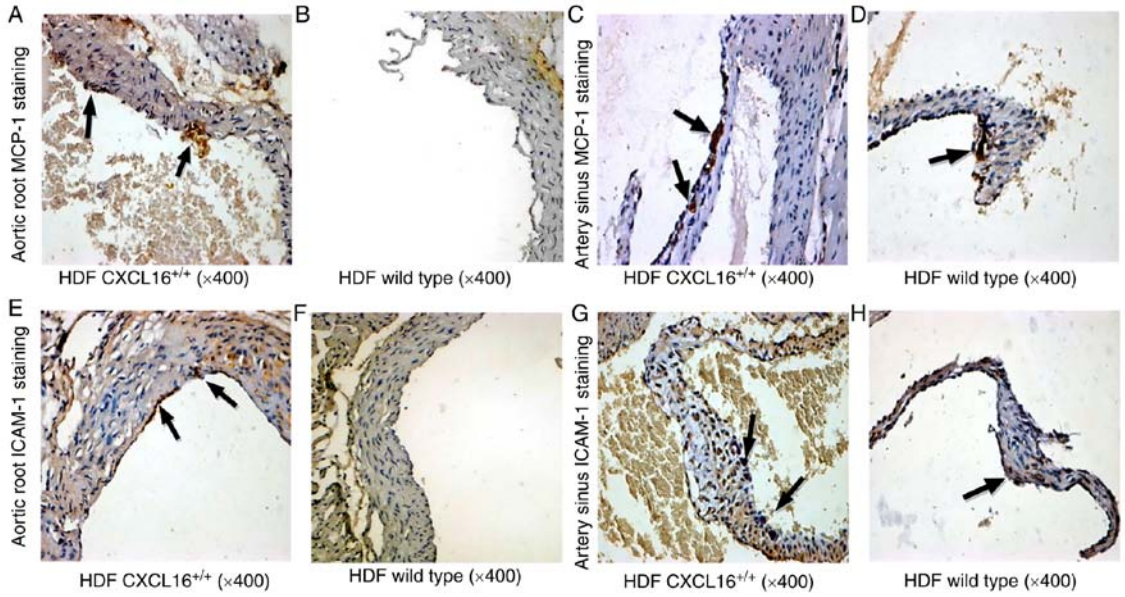

Figure 6. Reprehensive images of HFD group aortic root and artery sinus sections showing MCP-1 and ICAM-1 local expression. Black arrows indicate positively stained areas. (A and B) MCP-1 aortic root Immunohistochemistry. (C and D) MCP-1 artery sinus immunohistochemistry. (E and F) ICAM-1 immunohistochemistry of aortic root. (G and H) ICAM-1 immunohistochemistry of artery sinus. Magnification, x400. HFD, high-fat diet; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1.

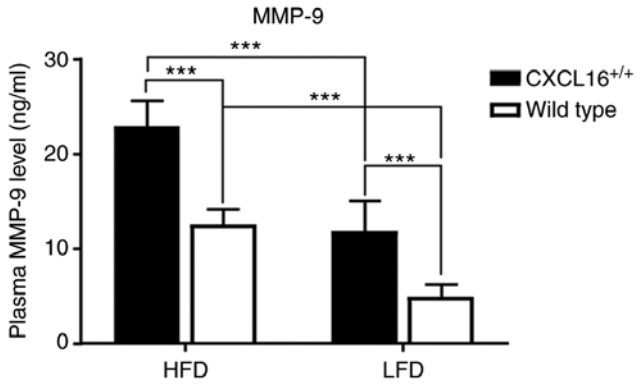

Figure 7. Plasma MMP-9 level in different groups. Quantitative analysis of plasma MMP-9 level in different groups assayed by ELISA. All data are expressed as the mean ± standard deviation and analyzed by one-way or two-way analysis of variance (n=8). ***P<0.001. ELISA, enzyme linked immunosorbent assay; MMP-9, matrix metalloproteinase-9; HFD, high-fat diet; LFD, low-fat diet.

ICAM-1 expression levels showed an incremental gradient from LDF wild-type, LDF CXCL16$^{+/+}$, HDF wild-type to HDF CXCL16$^{+/+}$. This had no statistical significance except for the difference between CXCL16$^{+/+}$ transgenic mice fed with HFD and LFD (Fig. 5B).

Artery sinus expression of MCP-1 and ICAM-1. Immunohistochemistry staining also indicated a higher local expression of MCP-1 and ICAM-1 in transgenic mice at the aortic root and artery sinus (Fig. 6). Positive MCP-1 signals were seen within the AS lesions, while accumulation of ICAM-1 was mainly located on the aortic intima surface (Fig. 6E-H). An overall higher expression of MCP-1 and ICAM-1 was observed at the artery sinus compared with the aortic root (Fig. 6).

Plasma level of MMP-9. Plasma MMP-9 level was tested at the end of the week 18. It showed high specificity among the four groups. HDF CXCL16$^{+/+}$ mice had the highest plasma level of all, and it was statistically significant when compared with HDF wild-type or LDF CXCL16$^{+/+}$ mice (Fig. 7). Additionally, LDF CXCL16$^{+/+}$ mice also expressed a higher level of plasma MMP-9 than LDF wild-type mice (Fig. 7).

Artery sinus MMP-9 expression. Local expression of MMP-9 in artery sinus was also tested among the HFD groups (Fig. 8A and B). Atherosclerotic plaques of CXCL16$^{+/+}$ mice possessed a higher percentage of cells that were MMP-9 positive (Fig. 8C).

Discussion

Growing attention has been paid to chemokines involved in the pathogenesis of AS (27). CXCL16 is one of those chemokines, although a large-scale review of overexpression of CXCL16 in transgenic mice is still required. CXCL16 is specifically expressed in arteries with AS and is mainly undetectable in normal arteries (14). The present study was the first, to the best of the authors' knowledge, to examine how overexpression of CXCL16 directs the progress of AS under LFD and HFD. Although apolipoprotein E (ApoE) knockout mice are used by numerous research groups for AS changes, they possess some limitations (28,29). ApoE is a multifunctional protein that has an effect on inflammation, oxidation, lipid transport and AS pathogenesis (30). These functions might affect atherosclerotic plaque development in ApoE$^{-/-}$ mice, independent of plasma lipid levels (29). It has been reported that HFD for 10-20 weeks can cause typical AS lesions in the aortic sinus valve of mice, which are mainly filled with foam cells and lipid streaks (23,31). The present study identified that a high expression level of CXCL16 combined with an HFD presented a strong pro-atherogenesis

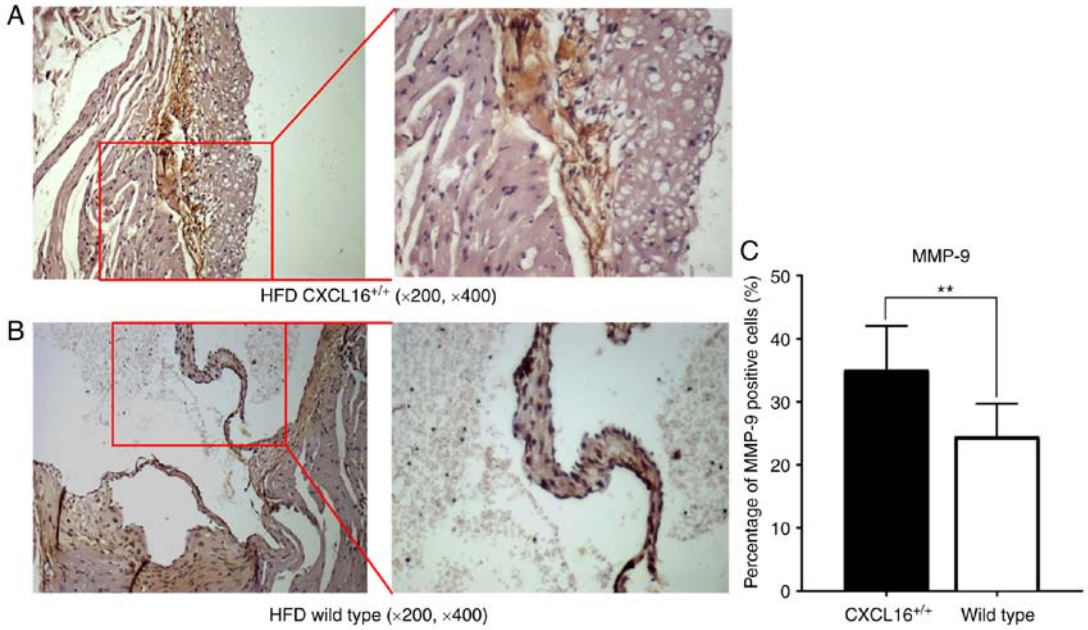

Figure 8. MMP-9 local expression. Reprehensive images of the HFD groups artery sinus sections showing MMP-9 local expression. (A) CXCL16$^{+/+}$ transgenic mice, (B) wild type mice. (C) Percentage of MMP-1 positive cells at artery sinus. All data are expressed as the mean ± standard deviation and analyzed by Student's t test (n=8). **P<0.01. Magnification, x200 and x400. MMP-9, matrix metalloproteinase-9; HFD, high-fat diet; CXCL16, CXC chemokine ligand 16.

ability. Blood lipid levels have a regulatory effect on the expression of CXCL16. A higher level of lipid profile was identified in CXCL16$^{+/+}$ transgenic mice fed with HFD and greater fatty acid accumulation was observed in the liver and the artery wall. Higher plasma levels of AS markers and denser foci expression of risk indicators were detected, while the levels in transgenic mice fed with LFD showed no significant difference with its corresponding control. It was a limitation to the present study that oil Red O staining was not performed to check the formation of lipid plaques and to observe the changes in foam cells. The observation of changes in plaques and fibrous caps will be added in future studies, which may further illustrate this part of the results.

HDF CXCL16$^{+/+}$ mice expressed a significant high level of pro-inflammatory or pro-AS markers while the other three groups retained a relatively similar low level of such markers, suggesting that both HFD and high CXCL16 level were prerequisite for a severer degree of AS to occur. Overexpression of the CXCL16 gene in transgenic mice did have a pro-inflammatory effect, but not as great as the HFD. When an HFD was combined with high CXCL16 expression, it showed a predisposing effect greater than either of these factors (HFD or CXCL 16 overexpression) alone. As adipose tissue is a potential origin for inflammation, this may suggest that CXCL16 alone is not enough to initiate inflammation. However, CXCL16 when initiated is a potent booster of inflammation and, furthermore, AS.

MCP-1 and ICAM-1 are involved mainly in the initiation of atherosclerotic plaques (32). MCP-1 has a definite pro-atherogenesis effect via its potent chemotactic ability for monocytes and also helps them through endothelial cells by diapedesis (33). MCP-1 is predominantly expressed in macrophages, smooth muscle cells and endothelial cells (34). It induces transendothelial migration of monocytes so as to facilitate atherosclerotic lesion formation (35). Absence of MCP-1 results in a milder extent of AS. Modified LDL, local fluid stress or short-term of high glucose exposure may all be stimulants to upregulate MCP-1 expression (36-38). The present study proposed MCP-1 as an early stage in indicator of AS.

ICAM-1 by contrast, is an adhesion molecule. Adhesion molecules represent another group of factors closely linked with inflammation and AS (39). They are upregulated in the regions predisposed to atherosclerotic lesion. Deficiency of certain adhesion molecules exert a protective effect from atheroma formation (40,41). Among adhesion molecules, ICAM-1 and vascular cell adhesion protein 1 (VCAM-1) are upregulated in atherosclerotic plaques (32,43), while ICAM-1 is a more responsive marker to diet cholesterol compared with VCAM-1, which remains relatively stable during or after a high cholesterol diet (44). Thus, the present study proposed ICAM-1 as a marker representing adhesion molecules in AS.

Under the combined action of elevated blood lipids and CXCL16 overexpression, the expression levels of MCP-1 and ICAM-1 in the aortic root and artery sinus of transgenic mice with HFD were significantly higher compared with those of wild-type mice with HFD, suggesting that they may have a common regulatory pathway under the influence of CXCL16 overexpression. The distribution of inflammatory factors on the tissue surface is consistent with the location of the initial stage of AS. Overexpression of CXCL16 in ApoE knockout mice can also promote the expression of MCP-1 mRNA in arterial plaques, but its mechanism or signal pathway remain to be elucidated (45). These results of the present study can also indirectly support the hypothesis that CXCL16 has the effect of promoting AS lesions, which supports the views of Wuttge *et al* (46). In a future study, additional markers in CXCL16 inflammation-related pathways need to be investigated to further explain the process of promoting AS lesions.

MMP-9 is one of the zinc-dependent endopeptidases and has a broad spectrum of substrates including a full

length interstitial collagens, certain cytokine and adhesion molecules (47-49). MMP-9 is closely involved in AS in that it can regulate inflammatory processes by proteolyzing chemokines (50,51) or releasing chemokines from their functional location (50). MMP-9 may also serve a role in macrophage migration (50). Another function, which is also the main role it serves in AS, is that MMP-9 affects plaque stability (52). Clinical and cell experimental studies have shown that a high level of MMP-9 expression, active form foci or total plasma MMP-9 are correlated with plaque vulnerability to rupture (53-56). The present study proposed MMP-9 level as a marker for advanced AS and its corresponding myocardial infarction. Following an HFD, all mice had elevated peripheral blood MMP-9. Immunohistochemistry showed that the expression of MMP-9 in the aortic sinus AS lesions of transgenic mice was significantly increased. Unfortunately, there was the lack of protein expression analysis of MCP-1, ICAM-1 and MMP-9 using western blot analysis in the present study.

According to a pooled analysis conducted by NCD Risk Factor Collaboration, the global body mass index has been constantly increasing since 1975 (57). This adds an extra burden to the task of artery disease control, although the pathological conditions in the arterial wall are reversible before certain point of the disease (58). More studies similar to the present study could lead the way to a significant elucidation of the mechanism of AS and may show the way to prevention or cure.

In conclusion, the present study was the first, to the best of the authors' knowledge, to establish a CXCL16 homozygous transgenic mice model to study how overexpressed CXCL16 is associated with AS. It is hypothesized that overexpression of CXCL16 combined with HFD may promote the AS lesions by upregulating the aforementioned inflammatory-related genes at a protein level. Overexpression of CXCL16 may lead to high blood lipids and strong inflammatory response. Whether blocking the expression of CXCL16 could reduce the local inflammatory response in AS, thereby intervening in the occurrence and development of AS, remains to be elucidated.

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Availability of data and materials

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

Authors' contributions

JZ and JW conceived and designed the experiments. JZ, MY and JW performed the experiments. JZ and JW confirm the authenticity of all the raw data. JZ and JW analyzed the data. JW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed according to the guidelines of the Chinese Experimental Animals Administration Legislation and were approved by the Ethics Committee for Animal Care and Use of Shandong Academy of Medical Sciences.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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