Silencing ferritin alleviates atherosclerosis in mice via regulating the expression levels of matrix metalloproteinases and interleukins

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INTRODUCTION

Cardiovascular and cerebrovascular diseases caused by atherosclerosis are common and leading causes of disability and death (Barquera et al., 2015). Atherosclerosis is a chronic inflammatory disease affecting large and medium arteries (Pathkullina et al., 2016; Moss et al., 2018). A better understanding of cellular and molecular mechanisms underlying the pathogenesis of atherosclerosis is important for developing new prevention and treatment strategies as well as therapies for the disease. Studies have shown that inflammatory response plays an important role in the pathogenesis of atherosclerosis. For example, inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6 and lipid mediators could promote inflammatory reactions in atherosclerotic plaque (Auguet et al., 2016; Gostner & Fuchs, 2016). The abnormally elevated expression of matrix metalloproteinases (MMPs) can lead to atherosclerotic plaque rupture, acute myocardial infarction and other cardiovascular events (Newby, 2016). On the other hand, other cytokines such as IL-10, transforming growth factor β (TGF-β), and extracellular matrix (ECM) proteins can reduce atherosclerotic inflammation, leading to plaque regression (Hassan et al., 2018; Rahman et al., 2017).

Ferritin is an iron storage protein whose level reflects iron stores may be a risk factor for cardiovascular disease and have a causal role in the pathogenesis of atherosclerosis (Lauffer, 1991; MacDonald, 1993). Since pro-inflammatory and anti-inflammatory macrophages within arterial plaques have a different amount of intracellular iron (Kraml, 2017), it is still largely unclear how ferritin participates and regulates the atherosclerotic process, although iron level appeared to increase in the atherosclerotic plaques in deceased patients with coronary heart disease (Vlad et al., 1994). In addition, high ferritin was shown to predict poor prognosis in patients with coronary artery disease (Zhu et al., 2006).

To better understand the role of ferritin in pathogenesis of atherosclerosis, we examined the effect of ferritin...
(FTH1 gene) on atherosclerosis and related inflammatory reactions using the mouse model of atherosclerosis. The findings would provide new insights on the relationship between ferritin and atherosclerosis and clues for the treatment of atherosclerosis.

MATERIALS AND METHODS

Animals

Specific pathogen-free male apolipoprotein E-knockout (ApoE<sup>−/−</sup>) mice, aged 6 to 8 weeks, weighing 21.2 to 25.5 g, were purchased from Tsinghua Animals, Beijing, China. All experimental protocols for the use of animals were approved by the Animal Care and Use Committee of Hebei Medical University. All animal experiments complied with the ARRIVE guidelines. Mice were housed under pathogen-free conditions, had access to standard mice feed and water ad libitum and were maintained at a 12/12 hour day/night cycle in climate-controlled conditions (22±1°C, 40–70% humidity). Animals were sacrificed by CO<sub>2</sub> asphyxiation after completion of the experiments and tissues were collected. CO<sub>2</sub> was supplied at a flow rate of 20% of the cage volume per minute (5 L/min). The death after exposure to carbon dioxide was confirmed based on a careful assessment of the mice for cardiac arrest.

Reagents and instruments

Multi-shRNA vector pLKO.1-TRC was obtained from Addgene, USA; 293A cells were purchased from ThermoFisher Scientific, USA; automatic biochemical analyzer was purchased from Sigma-Aldrich, USA; blood lipid test reagents and ELISA kits were purchased from Wallysong Biotechnology, Wuhan, China; Trizol reagent (CW0580S) and Ultrapure RNA Extraction Kit (CW0581M) were products of CWbiotech, Beijing, China; cDNA Synthesis Kit (CW2141S) was obtained from CWbiotech, Beijing, China; 2x SYBR Green PCR Master Mix (A4004M) was obtained from ThermoFisher, Beijing, China; mouse anti-human monoclonal antibodies against phospho-Ser63 c-Jun (pc-Jun) (1,50, ab195924) and phospho-Thr183/185 JNK (p-JNK) (1,50, ab131499) were obtained from Abcam, Cambridge, UK; HiScript II SuperMix for qPCR (R223-01) was from Vazyme, USA; PVDF membrane (IPVH00010) was a product of Millipore, USA; cDNA Synthesis Kit (CW2141S) was obtained from ThermoFisher Scientific, USA.

Construction of silencing and overexpression vectors

Using ThermoFisher’s online RNAi Designer (https://www.thermofisher.com/us/en/home/life-sciences/rmi/vector-based-rmi.html), short hairpin RNA sequences for ferritin (shRNA, 5′-ATTTTGGCAACTGC-CTCTG) were designed and inserted into pLKO.1-TRC. The lentivirus vector was amplified in 293A cells, tittered according to the manufacturer’s protocol using primers for ferritin (forward 5′-GCTGCAGATGCTTCTCACAAGCCCTCCGC- CGCTTCG, reverse 5′-GGTACCCATGCGCTGTA CGCATTTCCTTCGAAGCGA). The amplified DNA fragment was recovered from the gel and cloned into pENTR™ Directional TOPO to generate pENTR-ferritin according to the manufacturer’s instructions.

Atherosclerosis model and treatments

ApoE<sup>−/−</sup> mice were fed a high-fat diet (containing 21.0% fat and 1.5% cholesterol, Research Diets, New Brunswick, NJ, USA) to generate an atherosclerosis model or regular diet (10% fat and 0% cholesterol, Research Diets, USA) for use as a control. After 24 weeks, three mice were randomly selected from each group to examine blood lipid profiles to determine whether atherosclerosis modeling was successful. The model animals were then randomly divided into three groups (n=10) to receive an intravenous injection of silencing construct (10 µl at 10<sup>6</sup> UT/µL), overexpression construct (5 µg at 1 µg/µL), or empty vector (5 µg at 1 µg/µL) via tail vein as previously reported (Gorgens et al., 2019). After injection, the animals were reared at the same conditions as described above for 20 days and sacrificed for analysis.

Blood lipid measurements

Venous blood was collected from three randomly selected mice per group and analyzed for total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) using COD-PAP and GPO-PAP methods according to the supplier’s instructions.

Hematoxylin and eosin (HE) staining

HE staining was carried out to examine the plaque as described previously (Fischer et al., 2008). Briefly, the thoracic aortas were isolated and fixed in 4% paraformaldehyde, dehydrated in 70%, 80%, 90% and 100% alcohol, dehydrated in xylene. Dehydrated tissue was embedded in paraffin, sectioned, dewaxed, and hydrated. The sections were stained with aqueous hematoxylin solution for 3 min, differentiated with hydrochloric acid for 15 s, briefly washed, and counter-stained with eosin for 3 min. After being washed in distilled water, dehydrated and cleared, the sections were sealed and examined under a microscope for aortic morphological changes.

Real-time fluorescent quantitative PCR (qRT-PCR)

Total RNA was isolated from blood 5 days after injection of constructs using the Trizol reagent according to the manufacturer’s instructions and was reversely transcribed to cDNA for mRNA expression analysis using cDNA Synthesis kit according to the manufacturer’s protocols. HiScript II qRT SuperMix for qPCR was used for quantification of cDNA according to the manufacturer’s protocol using primers for ferritin (forward, GCCGAGAAGCTGAGTGAACGCTG, reverse, GCACGACTCCATGATTA CGCC). Normalization was performed with GAPDH. The PCR was carried out in a total volume of 10 µl containing 1.5 µl of diluted and pre-amplified cDNA, 10 µl of 2x SYBR Green PCR Master Mix and 1 µl of each fluorescence probe. The cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 45 cycles, each one consisting of 10 s at 95°C and 30 s at 58°C. Samples were run in triplicate and the mean value was calculated for each treatment. The data were managed according to the previously described protocol (Livak & Schmittgen, 2001).
Table 1. Levels of serum lipids and atherosclerotic lesions parameters in mice after feeding with the high-fat diet (model) and the normal diet (ND) for 24 weeks.

<table>
<thead>
<tr>
<th>Lipids and atherosclerotic lesions</th>
<th>ND, mmol/L (n=6)</th>
<th>Model, mmol/L (n=6)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>0.88±0.23</td>
<td>4.89±0.63</td>
<td>2.811</td>
<td>0.022</td>
</tr>
<tr>
<td>TC</td>
<td>10.06±1.23</td>
<td>25.06±2.23</td>
<td>4.221</td>
<td>0.011</td>
</tr>
<tr>
<td>LDL-C</td>
<td>8.06±1.11</td>
<td>15.76±2.23</td>
<td>2.183</td>
<td>0.031</td>
</tr>
<tr>
<td>HDL-C</td>
<td>2.09±0.23</td>
<td>2.09±0.33</td>
<td>2.875</td>
<td>0.082</td>
</tr>
<tr>
<td>Minimal lumen diameter (mm)</td>
<td>0.82±0.08</td>
<td>0.55±0.03</td>
<td>7.815</td>
<td>0.012</td>
</tr>
<tr>
<td>Lumen area (mm²)</td>
<td>0.44±0.02</td>
<td>0.35±0.03</td>
<td>6.215</td>
<td>0.017</td>
</tr>
<tr>
<td>Atherosclerotic plaque size (mm²)</td>
<td>0.04±0.01</td>
<td>0.13±0.02</td>
<td>5.236</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from mice 20 days after injection. Serum concentrations of IL-1β, TNF-α, IL-10, MMP8, MMP12, and MMP13 were measured using ELISA kits according to the manufacturer’s protocols. The absorbance (OD value) was read with a plate reader at a wavelength of 450 nm within 15 min after adding the stop solution.

Western blot

Thoracic aorta tissues were lysed with RIPA buffer containing protease inhibitors to extract proteins. Total protein was quantitated using BCA kit according to the manufacturer’s instructions. After denaturation, 50 µg of protein was separated using polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Non-specific binding was blocked by incubating in 5% non-fat milk in 1X Tris-buffered saline containing 0.1% Tween 40 for 4 hours at room temperature and then the membranes were incubated with primary antibodies (mouse anti-human phospho-Thr183/185 JNK (p-JNK) (1:50) and mouse anti-human phospho-Ser63 c-Jun (p-c-Jun) (1:50)) overnight. The blots were then incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:20000) and the immunoreactive bands were visualized with a chemiluminescence kit. The gray values of bands were analyzed using Quantity One software.

Statistical analysis

All data were expressed as means ± standard error of the mean (S.E.M.) obtained from at least three independent experiments. Statistical comparisons between groups were performed using one-way ANOVA followed by the Tukey post-hoc test. A value of P<0.05 was considered statistically significant.

RESULTS

High fat-diet generated pro-atherogenic lipid profiles and atherosclerotic lesions in ApoE-/- mice

After being fed with a high-fat diet for 24 weeks, the animals were tested for serum lipid levels. The results showed that compared to the mice fed with normal diet (ND), high-fat diet feeding resulted in significantly elevated levels of pro-atherogenic lipids in ApoE-/- mice. The levels of TG, TC, LDL-C and HDL-C increased from 0.88, 10.06, 8.06 and 2.06 to 4.89, 25.06, 15.76 and 2.09 mmol/L, respectively (P<0.05, Table 1), while HDL-C remained unchanged (P>0.05, Table 1). The size of atherosclerotic plaques was significantly greater in mice fed with the high-fat diet than fed with the normal diet. In addition, the vessel lumen was significantly narrowed (P<0.05, Table 1).

Ferritin silencing reduced atherosclerotic lesions

The atherosclerotic model mice were then intervened with ferritin silencing and over-expression constructs and assessed for atherosclerosis-related changes. PCR assay showed that five days after injection of silencing and overexpression vectors, the serum ferritin mRNA levels were significantly down and up-regulated (Fig. 1), indicating that the vectors were effective in reducing and increasing ferritin expression, respectively. 20 days after the intervention, compared to mice treated with an empty vector, the area of atherosclerosis plaques was significantly increased in the mice treated with ferritin over-expression vector and significantly decreased in the mice treated with the silencing vector (P<0.05, Table 2). The diameter and area of arterial lumen were significantly decreased in the mice treated with ferritin over-expression vector and significantly increased in the mice treated with ferritin silencing vector (P<0.05, Table 2).

Ferritin silencing changed IL-1β, IL-10, and TNF-α levels

We then measured IL-1β, IL-10, and TNF-α levels in the mice 20 days after injection. Results showed that compared to mice treated with an empty vector the levels of IL-1β and IL-10 levels were significantly increased or decreased in the mice treated with ferritin over-expression or silencing vector (P<0.05, Table 3), respectively. On the other hand, the levels of TNF-α levels were significantly decreased when the mice were treated with ferritin over-expression vector and significantly increased when the mice were treated with ferritin silencing vector (P<0.05, Table 3).

![Figure 1. Serum ferritin mRNA levels following delivery of overexpression and silencing vectors in mice. **denotes P<0.01 vs control.](image)
Ferritin silencing changed the expression of MMP8, MMP12, and MMP13

In addition, MMP8, MMP12, and MMP13 levels were measured and results showed that they were increased significantly following treatment with ferritin over-expression vector and significantly decreased after treatment with ferritin silencing vector ($P<0.05$, Table 4).

Ferritin silencing activated p-JNK/c-Jun signaling pathways

The expression of proteins in the p-JNK / c-Jun signaling pathways was assessed and results are shown in Figure 2. Compared to the control ferritin over-expression resulted in increased expression of p-JNK, while ferritin silencing decreased the expression of the protein ($P<0.05$, Fig. 2A). On the other hand, the levels of pc-Jun remained unchanged ($P>0.05$, Fig. 2B).

**DISCUSSION**

Ferritin is an important storage protein for iron supply and its role in atherosclerosis has not been fully elucidated at the molecular level, although clinical studies showed that patients with carotid atherosclerosis displayed elevated serum ferritin levels (Ma et al., 2015; Xu et al., 2017) and a population-based study in northeast Germany showed that there is a relationship between serum ferritin level and carotid atherosclerosis that was potentiated by LDL cholesterol (Wolff et al., 2004). The present study found that silencing FTH1 gene expression could alleviate the development of atherosclerotic lesions in atherosclerotic mice. FTH1 gene codes ferritin which is a major intracellular iron storage protein in prokaryotes and eukaryotes (Muhoberac & Vidal, 2019). Silencing this gene also reduced the levels of inflammatory cytokines and MMPs and down-regulated p-JNK signaling pathways. On the other hand, overexpressing the gene promoted the development of atherosclerotic lesions, increased the levels of inflammatory cytokines and MMPs, and up-regulated p-JNK signaling pathways. Since overexpression of ferritin would result in increased body iron
load, this may lead to enhanced progression of atherosclerosis as previously reported (Araujo et al., 1995; Lee et al., 1999). Furthermore, previous studies showed that the expression of the ferritin gene is up-regulated in atherosclerotic vessels (Pang et al., 1996), which is consistent with our observations that over-expression of ferritin has atherogenic activity, leading to increased plaque size and reduced aorta lumen diameter.

Inflammatory response plays an important role in the development of atherosclerosis which is regarded as a form of chronic vascular inflammation lesions (Soeki & Sata, 2016; Taleb, 2016). Measurements showed that the levels of inflammatory cytokines such as IL-1β and IL-10 were significantly increased following treatment with ferritin overexpression construct, and significantly reduced once ferritin silencing construct was used, suggesting that ferritin may regulate atherosclerosis progression via inflammatory pathways. These findings are consistent with the previous observation that the secretion of serum ferritin is regulated by inflammatory hormones (Tran et al., 1997). For instance, it was found that IL-1β may increase the expression of both the heavy (H) and light (L) ferritin subunit (Rogers et al., 1994) and ferritin could stimulate secretion of IL-10 and TNF-α in mice (Wang et al., 2017). Since ferritin level is statistically correlated with the levels of inflammatory biomarkers such as TNF-α, IL-10, and high-sensitivity C reactive protein (hs-CRP) and mortality of patients with the peripheral arterial disease (PAD), including atherosclerosis (Depalma et al., 2010), it is likely that ferritin has pro-atherogenic activity and further study is needed to investigate the mechanisms underlying ferritin-induced elevation of inflammatory hormones.

In atherosclerotic lesions, monocyte chemotaxis is induced to transform monocytes into macrophages. An important component of the inflammatory response is the secretion of MMPs to promote plaque rupture and to produce inflammatory cytokines such as TNF-α, IL-1β as well as lipid mediators to promote the inflammation in the plaque (Moore et al., 2013; Ruytinx et al., 2018). On the other hand, some reparative macrophages, such as alternatively activated M2 macrophages, could phagocytize dead cells or damaged tissue and release IL-10, TGF-β and ECM proteins to resolve inflammation and stabilize or even reverse atherosclerosis (Barrett, 2020). Our study showed that ferritin increases the levels of IL-1β and IL-10 as well as the levels of MMPs, suggesting that ferritin may influence the polarization of macrophages, leading to an increased inflammatory response. Previously, it was found that iron overload could result in M2-like polarization of macrophages (Kao et al., 2020) and expression of the heavy subunit of ferritin is a key factor determining the macrophage polarization in isolated bone marrow-derived mouse monocytes (Bolisetty et al., 2015).

Our study showed that silencing of ferritin down-regulates the expression of MMP8, MMP12, and MMP13. MMPs and their endogenous tissue inhibitors (TIMPs) play complex dual role during late-stage progression and rupture of atherosclerotic plaques. MMPs can degrade and the fibrous cap of the lesion, resulting in the rupture of the lesion, subsequent thrombus formation (Zhang et al., 2017), and acute myocardial infarction (Hong-Brown et al., 2015). Therefore, selective MMP inhibition would help limit cardiovascular morbidity and mortality.

p-JNK/c-Jun signaling pathway is a major signaling cassette of the mitogen-activated protein kinase (MAPK) signaling pathway. It is involved in a number of cellular processes, including proliferation, embryonic development and apoptosis. Our data showed that JNK was down-regulated when the expression of ferritin was suppressed, suggesting that ferritin may regulate the progression of atherosclerosis via the p-JNK/c-Jun signaling pathway. Early works also showed that when berberine was used to suppress atherosclerosis (Wan et al., 2018) or the chemerin gene was knocked down to alleviate atherosclerotic lesion (Liu et al., 2019), p-JNK expression was down-regulated, suggesting that p-JNK expression is associated with atherosclerosis.

CONCLUSION

By overexpressing and silencing the ferritin gene, our work demonstrated that ferritin regulates atherogenesis in ApoE−/− mice. Silencing ferritin alleviated atherosclerotic lesions, reduced the levels of inflammatory cytokines and MMPs and deactivated the p-JNK/c-Jun signaling pathway, while ferritin overexpression resulted in opposite outcomes. Further study is needed to investigate the mechanism underlying the ferritin-mediated regulation of atherosclerosis.

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the Animal Care and Use Committee of Hebei Medical University, Hebei, China.

Consent for publication

Not applicable.

Availability of data and material

The datasets used during the current study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

MZ, LL and XQ designed the study. MZ, LL, YL and YL conducted the experiments, collected the data and performed analysis. YL, YL and XQ drafted the manuscript. All authors read and approved the final version of the manuscript.

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