The LOX-1 receptor ectopically expressed in the liver alleviates atherosclerosis by clearing Ox-LDL in the circulation

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Abstract:

Objective: Ox-LDL is the core factor in the development of atherosclerosis. However, there are few therapy aimed at eliminating Ox-LDL. Here in this study, we investigate whether the expression of the lectin-like oxidized low density lipoprotein receptor (LOX-1) in the liver could lead to the phagocytosis and degradation of circulating Ox-LDL and prevent the deposition of oxidized lipids in the vascular wall, thereby alleviating the progression of atherosclerosis.

Methods: ApoE^{-/-} mice were randomly divided into three groups, the control group, the AAV8-TBG-eGFP group and AAV8-TBG-LOX-1 group. In the viral group, mice received an injection of AAV8-TBG-LOX-1 (1.16×10¹¹ virus genome (vg)/animal/100 µl). The mice in the control group and the AAV8-TBG-eGFP group received the same amount of sterile saline and AAV8-TBG-eGFP injections. The expression of LOX-1 in the liver was detected by immunofluorescent, Western blot and immunohistochemistry. The safety was assessed by H&E staining and blood biochemical analyses. The function of LOX-1 in the liver was detected by the co-localization of LOX-1 and Dil-Ox-LDL under laser scanning confocal microscope. The Ox-LDL in plasma was detected by ELISA. Changes in blood lipids were assessed through blood biochemical analysis. The progression of atherosclerotic lesions was detected by oil red O (ORO) staining. And the expression of VCAM-1 in endothelial cells and the extent of macrophages in plaques were detected by immunofluorescence staining. The protein expression in liver was assessed by qRT-PCR and Western blot.

Results: The expression of LOX-1 was stable in liver within 4 weeks. Ectopically expressed LOX-1 in the liver phagocytosed and degraded Ox-LDL and reduced Ox-LDL in circulating plasma but did not have a significant effect on blood lipid levels. After the expression of LOX-1 in liver, Ox-LDL can be cleared by the liver, thereby reducing VCAM-1 expression in vascular endothelium and migration of macrophages in plaques, and eventually alleviating the progression of atherosclerosis. Hepatic LOX-1 expression may facilitate the metabolic clearance of Ox-LDL by upregulating the expression of ABCG5/G8.

Conclusions: Ectopic liver-specific expression of LOX-1 alleviates the progression of atherosclerosis by clearing Ox-LDL in circulation.

Keywords: Ox-LDL, LOX-1, TBG promoter, AAV8, atherosclerosis

1. Introduction

Atherosclerosis is a systematic pathological change caused by the abnormal deposition of cholesterol-rich lipoproteins in the vascular wall[1]. Atherosclerotic cardiovascular diseases account for the leading cause of death and the majority of the world's disease burden, of which the incidence has continued to increase by 21.1% in the last decade[2,3]. Through hundreds of years of research and practice on AS, modern clinical practice can lower low-density lipoprotein (LDL) cholesterol to a very low state via the inhibition of two inputs and the enhancement of one output of cholesterol: statins inhibit key enzymes in hepatic cholesterol synthesis, ezetimibe inhibits the major acceptor of intestinal cholesterol absorption, and proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9) inhibitors (PCSK9i) increases hepatic cholesterol LDL receptor recycling and enhance the hepatic uptake of processed LDL, achieving great success in the inhibition of AS[4]. However, for the world's leading fatal disease, the complexity of its pathogenesis is still far from being completely understood, and simply reducing LDL is not sufficient. AS interventions still need to be explored from multiple perspectives to develop effective treatment methods.

Although lipid metabolism disorders are the leading cause of AS, the development of Ox-LDL and lipid-driven inflammatory responses in the vascular wall are the primary mechanism of AS formation[5-7]. Ox-LDL in AS patients not only exists in atherosclerotic plaques deposited in the vascular wall but also exists in circulating plasma. There are more than 20 clinical studies on the association between Ox-LDL and atherosclerotic cardiovascular disease, and several studies enrolled 1,000 to 2,000 AS patients. After correcting for the exclusion of LDL cholesterol risk factors, the risk of atherosclerotic cardiovascular disease in patients with elevated Ox-LDL increased 1.66-2.88 times. Several case-control studies were divided into four components based on Ox-LDL levels. Excluding the influencing factors of LDL cholesterol, the risk of cardiovascular disease in 14 groups with the highest Ox-LDL level and 14 groups with the lowest Ox-LDL level increased 1.67-5.03 times, with an average increase in cardiovascular events of approximately 2.93 times for every 1 U increase in circulating Ox-LDL[8]. Ox-LDL levels, including circulating Ox-LDL levels, are a strong risk factor independent of LDL cholesterol. For patients who used PCSK9i in the FOURIER and ODYSSEY-OUTCOMES studies, PCSK9i persistently reduced the LDL level to approximately 30 mg/dL (0.78 mmol/L), which is near the physiological limit of cholesterol in the human body. The FOURIER and Odyssey-OUTCOMES studies both reported significantly reduced rates of cardiovascular events, by 15%, at three and four years of follow-up, respectively; in these 2 studies, cardiovascular events were reduced from 14.6% in the control group to 12.6% in the treatment group and from 11.1% in the control group to 9.5% in the treated group, respectively, but the residual risk of cardiovascular disease was still obvious[9,10]. Although the residual risk for patients was more related to extralipid factors, the core component of AS was still cholesterol, indicating that lowering plasma LDL alone could significantly reduce Ox-LDL levels but could not completely inhibit Ox-LDL production and macrophage phagocytosis as well as vascular wall deposition. Fundamentally reducing the deposition of Ox-LDL in the vascular wall should be a more specific treatment strategy.

Existing drugs do not sufficiently inhibit the oxidation of circulating and tissue lipids because excess LDL is too easy to oxidize and modify. The ideal way to prevent the progression of AS is for the LDL level to be as low as possible, resulting in the lowest possible initiation of lipid oxidation and inflammation. The proposed approach is to convert the clearance mechanism for circulating pathogenic lipid particles from inflammatory clearance mechanisms by the existing innate immune system to normal metabolic mechanisms. In this field, the existing lipid-lowering drugs have achieved sufficient LDL-lowering effects, but the clearance of Ox-LDL remains a problem[11,12]. Theoretically, the adequate clearance of LDL to avoid oxidation and facilitate its removal can inhibit the deposition of atherosclerotic lipids and inflammation processes. Therefore, the clearance of Ox-LDL may be more meaningful. Among the possible strategies, the aim of this study was to start with the LOX-1 scavenger receptor and liver lipid metabolism to explore possible ways to achieve this goal.

LOX-1 is a transmembrane glycoprotein that, structurally, is in the C-type lectin family and a class ESR (a scavenger receptor with a haemagglutinin structural domain). It is mainly expressed in vascular endothelial cells but can also be expressed in macrophages, dendritic cells and fibroblasts. The expression level of the LOX-1 receptor in vascular endothelial cells is very low under physiological conditions, but it is significantly upregulated during oxidative stress and inflammatory responses[13]. Ox-LDL

is the major specific ligand of the LOX-1 receptor. Vascular endothelial cells mainly bind, phagocytose and degrade Ox-LDL through the LOX-1 receptor, thereby causing endothelial cell dysfunction and associated damage[14]. For the SRs mentioned above, as a transmembrane glycoprotein receptor that binds and internalizes Ox-LDL, LOX-1 differs from CD36 and SR-A receptors: the ligands recognized by the latter two are more extensive and can only conduct phagocytosis after the extensive oxidation of LDL particles, while LOX-1 is more specific for Ox-LDL and can also interact with mildly oxidated LDL. Therefore, LOX-1 may have better prospects as a potential intervention target for the clearance of Ox-LDL[15,16].

The liver is the hub of metabolic transformation of the entire body, has a highly sophisticated metabolic system and is also the core organ for lipid metabolism and transformation. The liver also has a large capacity for storage and regeneration. Unlike macrophages and endothelial cell pattern recognition receptors, which have no downregulation function after Ox-LDL phagocytosis, hepatocyte lipid metabolism receptors are involved in downregulation functions and multiple metabolic regulatory pathways. A large number of hepatocytes, lipid metabolism factories, cannot exert their important metabolic clearance function simply because they lack corresponding receptors. Based on existing highly specific liver transgenic expression technology[17-19], this study envisioned the use of adeno-associated virus type 8 (AAV8) containing a liver-specific thyroxine binding globulin (TBG) promoter as a vector to mediate the ectopic expression of LOX-1 in mouse hepatocytes to clear Ox-LDL from the circulation. We hope to achieve the physiological degradation of Ox-LDL so as to avoid lipid deposition in the vascular wall and atherosclerosis formation through multiple lipid metabolism pathways in hepatocytes, especially Ox-LDL lysosomal degradation and biliary cholesterol excretion, which are two major hepatic pathways for handling excess lipid molecules.

2. Materials and Methods

2.1. Study Design and Animals

AAV expressing LOX-1 fused with enhanced green fluorescent protein (eGFP) under the control of hTBG promoter, the vector backbone (plasmid) is WY3081. The high titers of engineered AAV (AAV2/8-hTBG-LOX-1-P2A-eGFP-3xFlag-WPRE-SV40pA, 1.16×10^{13} v.g/ml) and the negative control virus (AAV2/8-hTBG-eGFP-WPRE-pA, 1.76×10^{13} v.g/ml) were produced by Shanghai Genechem Co., Ltd. In order to test the specific transfection effect of the AAV in vivo, we use the empty virus (1.76×10^{11} virus genome (vg)/animal/100 μ l) for pre-experiment.

6-week-old male apolipoprotein E (ApoE) knockout mice in a C57 background were selected and housed in a single-cage single-chamber system at the specific-pathogen-free (SPF) grade Animal Experimental Center, Tongji Medical College, Huazhong University of Science and Technology. The animals were fed a normal diet for 1 week until they adapted to the environment. Body weight was measured weekly and recorded. The condition of the mice was observed. Mice were randomly divided into three groups: the control group, the AAV8-TBG-eGFP group and the AAV8-TBG-LOX-1 group. The first intervention was conducted in the first week (7 weeks old), and the second intervention was performed in the 5th week. Mice were sacrificed the 1st week, 2nd week, 3rd week, 4th week and 8th week after virus injection, and samples were collected for the following analyses.

Intervention programme: AAV8-TBG-LOX-1 vector (1.16×10^{11} virus genome (vg)/animal/100 μ l) and AAV8-TBG-eGFP vector (1.76×10^{11} virus genome (vg)/animal/100 μ l) was freshly prepared by diluting the vector to a viral titre of 1.16×10^{13} vg/ml (the titre of AAV8-TBG-eGFP is 1.76×10^{13} vg/ml) with sterile phosphate-buffered saline (PBS) and then stored in an ice box to avoid the influence of repeated freeze-thaw cycles on the virus titre. For the LOX-1 group and the eGFP group, 1.16×10^{11} vg/100 μ l virus dilution was injected through tail vein, and for the control group, 100 μ l of sterile saline was injected through the tail vein.

2.2. Blood Collection

To avoid the influence of Dil-labelled Ox-LDL (Dil-Ox-LDL) (Yiyuan Biotech, YB-0010) on the Ox-LDL content in the circulation, we have to collect blood from the orbital intracanthal vein before the probe injection. Mice were fasted for 8h before blood collection, and the glass capillary tube was tilted at 30° and slowly rotated to puncture the angular vein for blood collection. After the blood sample is collected, use the corresponding ELISA (CUSABIO, CSB-E07933m) kit to detect Ox-LDL in serum. The specific operation steps are as described in the previous study[20]. Blood collection from the orbital sinus by quickly removing the eyeball from the socket (the mice were sacrificed) was used for the detection of other blood parameters, such as ALT, AST, etc. The blood sample obtained from the above method was placed at room temperature for three hours and then centrifuged at room temperature for 15 minutes at 3500 r/min. The supernatant was collected in an Eppendorf (EP) tube and stored in a -80°C freezer.

2.3. Blood Lipid Panel and Biochemical Indices

A fully automated biochemical analyser was used to detect the levels of blood lipids, namely, total cholesterol (TC), triglyceride (TG), HDL, and LDL. Liver and kidney function indicators, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), serum albumin (ALB), creatinine (Cr) and blood urea nitrogen (BUN) were detected using a fully automated biochemical analyser (Servicebio, Wuhan, China).

2.4. Detection of Related Protein Expression Using Laser Scanning Confocal Microscopy

The gene sequence of eGFP (enhanced green fluorescent protein) was carried in an AAV8 vector. After the injection of Dil-Ox-LDL via the tail vein, cryosectioning of liver tissue was performed. After immunofluorescence staining, laser scanning confocal microscopy was used to detect the expression of LOX-1 and the phagocytosis of Ox-LDL by hepatocytes. The procedures were as follows. 1. The mice of the viral and control groups were fasted for 12 h to obtain blood samples, followed by the injection of 30 µg/100 µl Dil-Ox-LDL (freshly prepared and protected from light during injection) into the tail vein. Thirty minutes later, mice were intraperitoneally anaesthetised and sacrificed. 2. For each mouse, the heart was exposed, and pre-cooled saline was used for cardiac perfusion. Aorta, heart, liver, spleen, lung and kidney specimens were collected. 3. For the cryosections, cryosectioning equipment was prechilled to -20 °C, the thickness of each liver section was 10 µm, and 6 to 8 specimens were collected from a liver sample. 4. After the sections were prepared, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and the sections were observed under a laser confocal microscope. The same parameters were used for each view. 5. Cryosections of aortic root tissue were slightly different, with a slice thickness of 6 µm, and cryosections of spleen, kidney, and lung tissue were generated using the same method as that for the liver sections.

2.5. Western Blot

The protein expression levels of LOX-1 (Abcam, ab203246), ABCG5 (Boster, PB0381), ABCG8 (Boster, A01482) and SR-BI (GeneTex, GTX113645) in liver tissues were detected using Western blot. Liver tissues were homogenized in RIPA buffer (AntGene, ANT060) supplemented with protease and phosphatase inhibitors at 4 °C for 30 min. After centrifugation at 12,000 g for 15 minutes at 4°C, the supernatant was collected and measured using the Pierce™ BCA Protein Assay Kit according to the instructions. After the system was prepared, it was electrophoresed on a 10% polyacrylamide SDS gel, and then transferred to the membrane. The membrane was blocked with 5% skimmed milk powder at room temperature for 2 hours, and then it was incubated in the indicated antibody at 4°C overnight. The next day, incubate with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature, and then observe using ECL Kit. Chemiluminescence was detected by exposure to film and quantified using Image Lab.

2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from livers using Trizol reagent (Invitrogen, 15596026) according to the producer's protocol. To obtain cDNA, we used a SYBR Premix Ex Taq™ Kit (Takara, RR420A) on a thermocycler (Bio-Rad, Hercules, CA, USA). The primer sequences were: GAPDH (forward) 5'- ATGGGTGTGAACACGAGA-3' and (reverse) 5'- CAGGGATGATGTTCTGGGCA-3'; SR-BI (forward) 5'-AGTTGGTGAGATCCTGTGGG-3' and (reverse) 5'- TCTTGCTGAG-TCCGTTCCAT-3'; ABCG8 (forward) 5'-CTGGCACCCCTATCTACCTG-3' and (reverse) 5'- TTCTAGGAACAGGGCTGCAA-3'; ABCG5 (forward) 5'- AGAACAACACGCTAAAGGGC -3' and (reverse) 5'- GAAAATGACCGTGGCGATGA -3'. The mRNA expression levels of SR-BI, ABCG8 and ABCG5 were determined by comparing that of the GAPDH, which was chosen as internal controls.

2.7. H&E Staining

As previously described[21], H&E staining was conducted to assess cellular morphology in the tissue. Images were captured by a Nikon Eclipse microscope with the NIS Elements software.

2.8. Oil Red O Staining

The aorta perivascular adipose tissue and the surrounding connective tissue were carefully dissected under a stereomicroscope, and the portion from the aortic arch to the iliac artery bifurcation was completely preserved. After fixation with 4% paraformaldehyde, the aorta was stained with Oil Red O (ORO). After staining, digital images were taken with a microscope and stored. Plaque area was observed, calculated and analyzed using Image-Pro Plus 6.0 (The results are expressed as the percentage of the lesion area with lipid accumulation to the total gross area of the aorta). To further detect the lesion of atherosclerosis, ORO staining were also used on serial sections (6 µm thick) of aortic root. Then digital images were acquired under low-magnification and high-magnification fields of an optical microscope (the view in which all of the tricuspid valve was visible was retained). Plaque area was observed under a microscope and calculated (results were expressed as the percentage of the lesion area with lipid accumulation to the total lumen area), and the results were statistically analyzed using Image-Pro Plus 6.0 software.

2.9. Immunohistochemistry and Immunofluorescence

To determine whether the liver had expressed LOX-1 (Proteintech, BC022295) successfully, we used Immunohistochemistry and Immunofluorescence staining to assessed it. The specific steps are as previously described[22]. For immunohistochemistry staining, serial sections of the liver were immunostained with antibody against LOX-1 (Proteintech, BC022295). And for immunofluorescence staining, serial sections were incubated with antibody LOX-1 (Proteintech, BC022295). To verify that hepatocytes can phagocytose Ox-LDL after expressing LOX-1, we performed immunofluorescence co-localization of the two. The specific steps are

as described above. Serial sections were stained by using DAPI to observe nuclei. And the localization of LOX-1 in hepatocytes by using antibody against LOX-1(Proteintech, BC022295). Since eGFP (green) and Dil-Ox-LDL (red) are self-fluorescent, the slices can be directly placed under a laser confocal microscope for the fluorescence analyses after staining LOX-1(purple).

To determine the extent of macrophage and VCAM-1(Servicebio, GB11336) in the plaque, we performed the corresponding immunofluorescence staining. The specific steps are as described above. We chosed CD68 (Servicebio, GB14043) as the marker of macrophage.

2.10. Statistical Analysis

Quantitative data were collected from at least three independent replicates, and the measurement data are expressed as the mean $\pm$ standard deviation (SD). All data were statistically analyzed using SPSS 22.0 statistical software. As for the methods of statistical, the Student's t-test or one-way analysis of variance (ANOVA) was used when the data were normally distributed with homogeneity of variance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Adeno-associated virus with specific promoter TBG has good tissue specificity

Figure 1A shows the construction design for the AAV8-TBG-LOX-1 vector and a flow chart for the whole experiment. The AAV8 vector carries the eGFP gene sequence, and the empty AAV-TBG-eGFP vector was injected through the tail vein. Mouse heart, liver, spleen, lung, kidney were collected, cryosectioned, and viewed under a laser scanning confocal microscope (Figure 1B). To show the tissue and cell structure of the corresponding organs more clearly, we use HE staining and fluorescent staining to correspond to each other (Figure 1B). Under the same imaging parameters, abundant eGFP expression was observed in the liver of ApoE^{-/-} mice, almost no eGFP was expressed in the heart, spleen and kidney, and a small amount of eGFP expression was observed in the lungs. These results indicated that the AAV8 vector carrying the liver-specific TBG promoter mediated the specific transfection of target genes in the liver of ApoE^{-/-} mice, with very little impact on other organs and tissues.

Figure 1 Construction and in vivo specific expression of AAV8-TBG-eGFP. (A) Schematic showing the experimental strategy for high-fat diet feeding and analysis. (B) AAV8 carrying the TBG promoter can mediate the specific expression of target genes in the liver without affecting other organs in ApoE^{-/-} mice (laser scanning confocal microscopy, 400×).

3.2. The Stable Expression of LOX-1 in the Liver and LOX-1 Phagocytosis of Circulating Ox-LDL

The effects of transduction on the liver were assessed using Western blot. The results (Figures 2A, B) showed that compared with that in the control group, hepatic LOX-1 expression in the AAV8-TBG-LOX-1 viral group was low at the 1st week and increased at the 2nd week, with stable expression observed at the 4th week. In order to see the localization of LOX-1 in hepatocytes more clearly after transfection, we used immunohistochemistry and immunofluorescence staining for detection (Figure 2C-D). The

results showed that LOX-1 was mainly expressed in the cell membrane and cytoplasm of hepatocytes after transfection, and most of the hepatocytes were successfully transfected. Under the laser confocal microscope (Figure 2E), we observed that compared with the control group, the expression of eGFP (green fluorescence) in the liver of the virus group was obvious, indicating that the virus has been successfully transfected. Meanwhile, LOX-1 (purple fluorescence) was successfully expressed in the cytoplasm and cell membrane of hepatocytes in the virus group, and the results of co-localization with Dil-Ox-LDL (red fluorescence) showed that LOX-1 successfully engulfed Ox-LDL into the liver cytoplasm. From these results, we reasonably speculate that LOX-1 expressed in hepatocytes after transfection can phagocytose circulating Ox-LDL.

Figure 2 Functional expression of LOX-1 in the liver of ApoE^{-/-} mice. **(A)** Western blot detection of LOX-1 expression in liver tissue at different time points after transduction. **(B)** Statistical analysis of the results from Figure 2A. ** P < 0.01 compared with the control group. **(C)** Detection of LOX-1 expression in liver tissue from mice in each group (immunohistochemistry, n = 7, 400×). **(D)** Detection of LOX-1 expression in liver tissue from mice in each group (immunofluorescence, n = 7, 400×). **(E)** Detection of LOX-1 expression in liver tissue and LOX-1-mediated Ox-LDL phagocytosis in hepatocytes (laser scanning confocal microscopy, n = 7, 200×).

3.3. Expression of LOX-1 in the Liver Can Reduce Circulating Ox-LDL Without Affecting Blood Lipid Levels

Plasma Ox-LDL was detected at the 2nd, 4th, 6th, and 8th weeks after virus transduction. As shown in Figure 3A, after 8 weeks of a high-fat diet, plasma Ox-LDL in the control group and AAV8-TBG-eGFP group rapidly increased within 4 weeks and slowly increased after 4 weeks and plasma Ox-LDL in the AAV8-TBG-LOX-1 group was slightly higher than the initial level at the 2nd week. The results in Figures 3B-E show that compared with those in the control and AAV8-TBG-eGFP group, the plasma levels of TC, TG, and LDL-C in the AAV8-TBG-LOX-1 group were lower, while the plasma level of HDL-C was higher, but none of the differences were statistically significant. These results suggest that hepatic LOX-1 expression can reduce Ox-LDL in circulating plasma by recognizing, binding, and phagocytosing Ox-LDL without affecting TC, TG, LDL and HDL levels in the plasma.

Figure 3 Ox-LDL and lipid levels in circulating plasma. **(A, B)** Detection of Ox-LDL in circulating plasma (ELISA, n = 7). **(C)** plasma LDL-C levels in mice 8 weeks after virus transduction (n = 7). **(D)** plasma TC levels in mice 8 weeks after virus transduction (n = 7). **(E)** plasma HDL-C levels in mice 8 weeks after virus transduction (n = 7). **(F)** plasma TG levels in mice 8 weeks after virus transduction (n = 7). Values are expressed as mean ± SD (n = 7). Student's t-test was applied for determining the significance of data. *P < 0.05 and **P < 0.01 compared with the control group. #P < 0.05 and ###P < 0.01 compared with the AAV8-TBG-eGFP group.

3.4. Expression of LOX-1 in the Liver does not cause significant side effects in vivo

Liver sections from mice in the control, AAV8-TBG-eGFP and AAV8-TBG-LOX-1 groups were stained with H&E to observe the effect of viral transduction on liver structure. As shown in Figure 4A, 8 weeks after viral transduction, the morphology and structure of hepatocytes in all three groups did not change significantly. Changes in serum levels of key indicators of liver function (ALT, AST, TBIL and ALB) and renal function (Cr, BUN) in all three groups after 8 weeks of a high-fat diet were assessed using a fully automated biochemical analyser to evaluate the safety of the AAV vector (Figures 4B-G). The results indicated that compared with those in the control and AAV8-TBG-eGFP groups, liver function and renal function indicators in the AAV8-TBG-LOX-1

group changed slightly, but no significant difference was identified. Thus, the expression of LOX-1 in the liver does not cause significant side effects in vivo. In other words, AAV8-TBG was safe as a gene therapy vector for atherosclerosis.

Figure 4 The injection of AAV8 did not cause significant side effects in vivo. (A) H&E staining of liver sections 8 weeks after virus transduction (n = 7). (B) plasma ALT levels (n = 7). (C) plasma AST levels (n = 7). (D) plasma TBIL levels (n = 7). (E) plasma ALB levels (n = 7). (F) plasma Cr levels (n = 7). (G) plasma BUN levels (n = 7). Values are expressed as mean ± SD (n = 7). Student's t-test was applied for determining the significance of data.

3.5. Clearance of Circulating Ox-LDL by Ectopic Expression of LOX-1 in the Liver Significantly Inhibited atherosclerosis

To determine the effect on atherosclerosis of clearance of circulating Ox-LDL, we observe the plaques in the aorta and aortic root sections using ORO. The percentages of aortic plaque area in mice in the control group AAV8-TBG-eGFP group and AAV8-TBG-LOX-1 group were $26.9 \pm 4.9\%$, $23.8 \pm 4.5\%$ and $10.5 \pm 2.1\%$, respectively (Figures 5A, C); the percentage of aortic plaque area in the AAV8-TBG-LOX-1 group was much lower than that in the control and AAV8-TBG-eGFP groups. For the aortic root (Figures 5B, D), at the same magnification, the plaques in the control group were obvious, with cholesterol crystals present, and the plaques in the AAV8-TBG-LOX-1 group were significantly smaller; the percentages of aortic root plaques in the mice in the control, AAV8-TBG-eGFP and AAV8-TBG-LOX-1 groups were $66.1 \pm 3.8\%$, $65.0 \pm 3.8\%$ and $33.6 \pm 3.9\%$, respectively. These results suggested that the ectopic expression of LOX-1 in the liver to clear circulating Ox-LDL indeed inhibited the formation of atherosclerosis.

Figure 5 Effects of hepatic LOX-1 expression on atherosclerosis in ApoE^{-/-} mice. **(A-B)** Representative images of aorta obtained from the study groups stained with Oil red O **(A)**. The percentage of the plaque area in the aorta is presented among the indicated groups (n = 7) **(B)**. **(C-D)** Representative images of aortic root sections obtained from the study groups stained with Oil red O **(C)**. The percentage of the plaque area in the aortic root is presented among the indicated groups (n = 7) **(D)** Values are expressed as mean ± SD. Student's t-test was applied for determining the significance of data. *** P < 0.001 compared with the control group. ### P < 0.001 compared with the AAV8-TBG-eGFP group.

3.6. Clearance of Circulating Ox-LDL by Ectopic Expression of LOX-1 in the Liver Significantly Reduce VCAM-1 Expression in Vascular Endothelium and Migration of Macrophages in Plaques

To further determine the effect of clearing of circulating Ox-LDL, we also detect the extent of macrophage and VCAM-1 in the plaque. The results suggested that compared with the control group (43.07%±5.96%), the content of macrophages in the plaque of the virus group (7.77%±5.36%) was significantly reduced (Figures 6A, B). Consistent with this, the content of VCAM-1 in the virus group (0.34%±0.06%) was also greatly reduced (Control group set as 1) (Figures 6C, D). From these data, we reasonably speculate that elimination of Ox-LDL by hepatocytes can reduce the activation of vascular endothelial inflammation and subsequent subendothelial migration of macrophages, which could help to reduce intravascular inflammation and foam cell formation.

Figure 6 Clearance of circulating Ox-LDL by ectopic expression of LOX-1 in the liver significantly reduce VCAM-1 expression in vascular endothelium and migration of macrophages in plaques. **(A-B)** Representative images of aortic root sections obtained from the control and AAV-TBG-LOX-1 groups stained with CD68. **(A)** DAPI were used to stain nuclei (blue). The percentage of the plaque area that was occupied by CD68 is presented among the indicated groups **(B)**. **(C-D)** Representative images of aortic root sections obtained from the control and AAV-TBG-LOX-1 groups stained with VCAM-1 **(C)**. DAPI were used to stain nuclei (blue). The relative fluorescence density is presented among the indicated groups (Control group set as 1) **(D)**. Values are expressed as mean $\pm$ SD (n = 7). Student's t-test was applied for determining the significance of data. ** P < 0.01 compared with the control group.

3.7. Hepatic Expression of LOX-1 Upregulates the Expression of ABCG5 and ABCG8 in the Cholesterol Biliary Excretion Pathway

To explore the possible mechanisms of Ox-LDL binding, phagocytosis, and degradation by hepatocytes, we investigated two pathways, biliary cholesterol excretion and reverse cholesterol transport. Therefore, the expression levels of representative molecules in these two pathways, i.e., ABCG5, ABCG8 and SR-BI, were examined. From the results of qRT-PCR we can see that the mRNA level of SR-BI almost remained unchanged, while the mRNA levels of ABCG8 and ABCG5 were obviously higher (Control group set as 1) (Figure 7A). The results of Western blot (Figure 7B-E) indicated that compared with those in the control group, the expression level of SR-BI, a protein in the reverse cholesterol transport pathway, was not significantly different and the expression levels of ABCG5 and ABCG8, markers of the biliary cholesterol excretion pathway, were significantly increased in the viral group. Based on this result, we speculated that after the uptake of Ox-LDL by hepatocytes via LOX-1, Ox-LDL was degraded into amino acids and free cholesterol in lysosomes, and cholesterol was secreted into the intestinal lumen in the form of bile acids after the expression of the cholesterol transporters ABCG5 and ABCG8 was upregulated, thus achieving Ox-LDL degradation; furthermore, the reverse cholesterol transport pathway plays a minor role in this process.

Figure 7 qRT-PCR and Western blot analysis of SR-BI, ABCG5, and ABCG8. **(A)** Set the protein expression level of the control group to 1, and the relative mRNA expression of SR-BI, ABCG8 and ABCG5 detected by qRT-PCR. The expression of mRNA were normalized to that of GAPDH. **(B)** Western blot analysis of the hepatic lipid metabolism core pathway-related proteins SR-BI, ABCG5, and ABCG8 4 weeks after virus transduction. **(C)** Set the protein expression level of the control group to 1, and the relative protein expression level of SR-BI in the virus group. **(D)** Set the protein expression level of the control group to 1, and the relative protein expression level of ABCG8 in the virus group. **(E)** Set the protein expression level of the control group to 1, and the relative protein expression level of ABCG5 in the virus group. Values are expressed as mean $\pm$ SD. Student's t-test was applied for determining the significance of data. ** $P < 0.01$ compared with the control group.

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Figure 8 Schematic diagram of hepatic LOX-1 Ox-LDL uptake and lipid metabolism pathways. AAV8-TBG-LOX-1 induces hepatocyte-specific expression of LOX-1, and hepatocytes endocytose LDL and Ox-LDL via LDLR and LOX-1; LDL and Ox-LDL are then degraded into amino acids and free cholesterol by lysosomes. Free cholesterol can induce an increase in biliary cholesterol excretion in hepatocytes; furthermore, it can reduce cholesterol synthesis through feedback inhibition of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase. Hepatocytes can maintain stable lipid metabolism through other cholesterol metabolism pathways, such as the reverse cholesterol transport pathway.

Note: The entire square region is a hepatocyte, and the nucleus (green sphere) is the centre of metabolic regulation. The nuclear transcription factor LXR/RXR located in the nucleus regulates the expression of the cholesterol transporters ABCG5 and ABCG8, which secrete cholesterol into the lumen of the small intestine (the lower right area in the figure) as bile acids. SREBP-2 (RNA helix-like icon in the upper right of the figure) transcribed into the cytoplasm through the nucleus regulates the dynamic equilibrium between LDLR and PCSK9. LDLR (green arc-shaped transmembrane receptor in the figure), located on the cell membrane surface, is responsible for the uptake and metabolism of circulating LDL (near the cell membrane and cytoplasmic molecules and organelles in the upper left of the figure), and the ability of LDLR to recycle to the cell membrane after binding LDL and internalizing into hepatocytes is closely related to the regulation of PCSK9 (the red icon in the upper right of the figure). HDL-C is taken up through SR-BI (light green receptor with corresponding green spherical HDL icon in the lower right of the figure) on the surface of hepatocytes. In the endoplasmic reticulum (green reticular membrane icon in the cytoplasm in the middle-upper portion of the figure), HMG-CoA reductase synthesizes cholesterol, of which some is packaged into VLDL and secreted into the blood and some is stored after ACAT esterification.

4. Discussion

Authors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted. The development and progression of atherosclerosis is closely related to the subendothelial deposition of lipids and chronic inflammation in the vascular wall[23]. Despite tremendous achievements in the inhibition of atherosclerosis through modern medical drug treatment and lifestyle interventions, atherosclerosis still has the highest morbidity among diseases and is the leading cause of mortality in the world[24]. A more in-depth study of its pathophysiological mechanisms and the search for crucial intervention pathways and targets are necessary and urgent.

Ox-LDL is an important risk factor for atherosclerosis and is present in both atherosclerotic plaques and circulating plasma[25]. Ox-LDL phagocytosis and pathological processing through pattern recognition receptors on the vascular wall is the core mechanism of lipid-driven inflammation in the vascular wall. Ox-LDL activates mitogen-activated protein kinases (MAPKs) after endocytosis via LOX-1 in vascular endothelial cells, thereby increasing the expression of chemokine monocyte chemoattractant protein 1 (MCP-1) and endothelial adhesion molecules. In addition, Ox-LDL promotes the subendothelial migration and differentiation of monocytes into macrophages[14,26]. Ox-LDL downregulates the expression of the antiapoptotic protein B-cell lymphoma 2 (Bcl-2) through LOX-1 and activates cysteine-aspartic acid proteases (caspase-3 and caspase-9) in the apoptotic signalling pathway to promote endothelial cell apoptosis[27]. Ox-LDL binds to LOX-1 and induces an increase in ROS expression and a reduction in carbon monoxide (NO) synthesis via the reductive coenzymes (NADPH) on the cell membrane, resulting in endothelial dysfunction[28]. Therefore, the targeted clearance of Ox-LDL is a possible strategy to reduce inflammatory responses driven by lipid deposition in the vascular wall and inhibit atherosclerosis.

The liver is the core organ of lipid metabolism in the body and has well-developed lipid metabolism and regulatory pathways[29,30]. In particular, the liver has cholesterol degradation capacity and a mechanism to inhibit cholesterol synthesis. Furthermore, the liver also excretes biliary cholesterol, accounting for half of the synthetic cholesterol intake by the liver. Therefore, the specific ectopic expression of LOX-1 on the membrane of hepatocytes through AAV8-TBG is a unique way to achieve Ox-LDL degradation in the liver. In this study, based on the current research frontier and preliminary studies in our laboratory, we used an AAV8 vector carrying the liver-specific promoter TBG to mediate LOX-1 gene transduction in the liver of ApoE^{-/-} mice to ectopically express LOX-1, an Ox-LDL-specific receptor, in the liver. Circulating Ox-LDL can be persistently cleared via the robust lipid processing capacity of the liver, and rigorous experimental studies were conducted to investigate the effect of circulating Ox-LDL clearance on atherosclerosis. We found that persistent liver-specific ectopic expression of LOX-1 could not only recognize, bind to, and phagocytose circulating Ox-LDL but also inhibit atherosclerosis progression by upregulating the expression of the cholesterol transporters ABCG5 and ABCG8 to excrete cholesterol into the intestinal lumen as bile acids[31], thereby enabling Ox-LDL degradation and avoiding Ox-LDL deposition in the vascular wall and lipid-driven inflammatory responses. The aim of this study was to explore a safe and reliable pathway to eliminate Ox-LDL, via the physiological clearance of circulating Ox-LDL, for anti-atherosclerosis studies and to elucidate the possible mechanism of action. Figure 7 better shows our experimental assumptions. However, our study has some limitations. In subsequent experiments, we could use cyclization recombinase (Cre) mice to specifically knock down LOX-1 in the vascular endothelium for a more sophisticated exploration. In addition, the inflammatory responses within the body after the degradation of circulating Ox-LDL by the liver could be studied in more detail.

In summary, our research basis is the clearance of Ox-LDL, the pathogenic core of atherosclerosis. The ectopic expression of LOX-1 in the liver was achieved through gene therapy, resulting in plasma Ox-LDL clearance. The traditional Ox-LDL phagocytosis by pattern recognition receptors on endothelial cells in the vascular wall, leading to a lipid-driven inflammatory response, was modified to Ox-LDL metabolic degradation by liver-specific receptors, leading to biliary cholesterol excretion to the intestinal lumen. This will provide new targets and new breakthroughs for the investigation of atherosclerosis and provide new ideas for the clinical treatment of atherosclerosis.

Conclusion:

All in all, we propose a novel therapeutic direction for atherosclerosis, the physiological removal of Ox-LDL. Here in this study, we used the principle of gene editing to make the LOX-1 ectopically express in hepatocytes, and with the strong lipid metabolism ability of hepatocytes to clear Ox-LDL in the circulation, ultimately alleviating the progression of atherosclerosis.

Data Availability Statement: The data presented in this study are available on request from the corresponding author on reasonable request.

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Acknowledgments: The authors thank AJE company for language editing of the manuscript.

Funding: Please add: This research received financial support by the National Natural Science Foundation of China (Grant No. 82070376, 81873491).

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Ethics declarations	518
Ethics approval and consent to participate: The study was conducted according to the guidelines of the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology (IACUC Number:2445, Date: 20210402).	519 520
Consent for publication: No applicable.	521 522
Conflicts of Interest: The authors declare no conflict of interest.	523 524