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Original Article

A human apolipoprotein E mimetic peptide reduces atherosclerosis in aged apolipoprotein E null mice

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Abstract: Apolipoprotein E (apoE) is well known as an antiatherogenic protein via regulating lipid metabolism and inflammation. We previously reported that a human apoE mimetic peptide, EpK, reduced atherosclerosis in apoE null (apoE$^{-/-}$) mice through reducing inflammation without affecting plasma lipid levels. Here, we construct another human apoE mimetic peptide, named hEp, and investigate whether expression of hEp can reduce atherosclerotic lesion development in aged female apoE$^{-/-}$ mice with pre-existing lesions. We found that chemically synthesized hEp significantly decreased cholesterol accumulation induced by oxidized low density lipoprotein and the expression of inflammatory cytokines TNFα and IL-6 induced by lipopolysaccharide in macrophages. In an *in vivo* study, Lv-hEp-GFP lentiviruses were intravenously injected into 9 month-old apoE$^{-/-}$ mice. Mice were then fed a chow diet for 18 weeks. Results showed that in comparison to the Lv-GFP lentivirus injection (Lv-GFP) group, Lv-hEp-GFP lentivirus injection achieved hepatic hEp expression and secretion in apoE$^{-/-}$ mice. It was observed that hEp expression significantly reduced plasma VLDL and LDL cholesterol levels and decreased aortic atherosclerotic lesions. This was accompanied by an increase of LDL receptor expression and a reduction of TNFα and IL-6 mRNA levels in the liver. Moreover, expression of hEp increased plasma paraoxonase-1 activity and decreased plasma myeloperoxidase activity and serum amyloid A levels. Our study provides evidence that hEp may be developed as a promising therapeutic apoE mimetic peptide for atherosclerosis-related cardiovascular diseases through its induction of plasma VLDL/LDL cholesterol clearance as well as its anti-oxidative and anti-inflammatory activities.

Keywords: Apolipoprotein E, mimetic peptide, atherosclerosis, LDL receptor, anti-oxidation, inflammation, apolipoprotein E null mice

Introduction

Apolipoprotein E (apoE), a secreted multifunctional protein, is recognized to suppress the progression of atherosclerosis. Beyond its pivotal role in cholesterol metabolism by mediating hepatic uptake of atherogenic lipoproteins and promoting macrophage cholesterol efflux, apoE also exhibits anti-oxidative and anti-inflammatory properties [1-3]. Human apoE is a polymorphic protein with three main isoforms, E2, E3, and E4, resulting from Cysteine-Arginine interchanges at residues 112 and 158 [1]. It has been reported that genetic variation of apoE is linked to plasma lipid levels and is an independent risk determinant of atherosclerosis and coronary heart disease (CHD). ApoE-null (apoE$^{-/-}$) mice develop hypercholesterolemia, chronic inflammation, and spontaneous atherosclerosis, while addition of exogenous apoE in various ways in animal models can substantially reduce hypercholesterolemia and atherosclerosis [4-7]. These findings have led to attempts at the design of apoE supplements for the treatment of dysfunctional apoE-related cardiovascular diseases. Considering that there are a number of limitations to the production and administration of this 299 amino acid protein, the development of apoE-derived mimetic peptides may be an effective strategy to ameliorate dyslipidemia and to halt the progression of atherosclerosis [8-10].

ApoE is mainly synthesized in the liver; it consists of two structural and functional domains separated by a hinge region. The four-helix-bun-

dle N-terminal domain contains the low density lipoprotein receptor (LDLR) binding region (residues 130-169) and heparin sulfate proteoglycans (HSPGs) binding region, and the amphipathic α-helical motif in the C-terminal domain (residues 244-272) is mainly responsible for lipid binding and triggers the conformational change of the N-terminal domain [11-13]. It is well documented that the apoE LDLR-binding domain can promote the endocytic clearance of plasma lipoproteins, especially VLDL and remnant lipoproteins and also exert anti-inflammatory actions, while lipid binding regions can promote cholesterol efflux from macrophages [14, 15]. In recent years, a series of studies conducted in Anantharamaiah's lab have shown that an apoE-derived peptide, Ac-hE18A-NH2, which links the LDLR-binding region of human apoE (residues 141-150) with an 18-residue amphipathic helical peptide, is capable of reducing plasma cholesterol and promoting cholesterol efflux from macrophages and possesses anti-inflammatory and anti-oxidative properties. Also, compared to the well-studied apolipoprotein AI (apo AI) mimetic peptide 4F, intravenous injection of the Ac-hE18A-NH2 peptide into apoE$^{-/-}$ mice is more effective in reducing atherosclerotic lesion formation [16-19].

In a previous study, we reported that a human apoE3-derived peptide, EpK, which contains an LDLR-binding region (residues 141-150) and a lipid-binding region (residues 234-254) linked by six Lys residues, could enhance the ability of high-density lipoproteins (HDL) to mediate cholesterol efflux and suppress inflammatory responses in cultured macrophages [20]. A subsequent *in vivo* study showed that lentivirus-mediated hepatic EpK expression and secretion could reduce atherosclerosis in apoE$^{-/-}$ mice through its significant anti-inflammatory properties. However, we also found that both peptide injection and hepatic EpK expression had little effect on plasma lipid levels in apoE$^{-/-}$ mice [21].

Considering the important role of the apoE receptor binding domain on lipid transport via binding to cell surface receptors, we further designed a novel human apoE3-derived peptide, named hEp, which directly links a longer N-terminal LDLR binding region (residues 131-162) and C-terminal lipid-binding region (residues 244-272). A previous study showed that synthesized hEp peptide injection could immediately lower plasma cholesterol level within 4 hours in mice by exhibiting both lipid-binding and LDLR-binding activity [22]. Here, we report that lentivirus-mediated hepatic expression of hEp significantly reduces the progression of atherosclerosis in aged female apoE$^{-/-}$ mice with existing aortic lesions. The mechanism may involve reducing macrophage lipid accumulation, inhibiting inflammation, and improving anti-oxidative status. Also simply ameliorating hypercholesterolemia *in vivo* may be important for the observed anti-atherogenic effect. These findings suggested that hEp expression may be more effective than EpK expression, and demonstrated that hEp may be a promising apoE mimetic peptide to prevent the progression of atherosclerosis.

Materials and methods

Reagents

Dulbecco's modified Eagle medium (DMEM) was purchased from Thermo Fisher, USA. Fetal bovine serum (FBS), lipofectamine 2000 and Trizol reagent were purchased from Invitrogen, USA. Human plasma low density lipoprotein (LDL) was purchased from Prospect Biosystems, USA and oxidized with 10 μM $CuSO_4$ for 24 h at 37°C. A 61-residue hEp mimetic peptide (MW: 7405.63 Da) with the amino acid sequence EELRVRLASHLRKLRKRLLRDADDLQKRLAVYEE QAQQIRLQAEAFQARLKSWFEPLVEDM was synthesized and purity (> 98.3%) was determined by HPLC (GL Biochem Ltd., China). Lipopolysaccharide (LPS), paraoxon and Oil Red O was purchased from Sigma-Aldrich, USA. Anti-His-tag antibody and anti-PON1 antibody were from Abcam, USA. Enhanced chemiluminescence kit (ECL) kit was purchased from GE healthcare, USA. PCR primers were purchased from Sangon Biotech., China.

Determination of macrophage lipid levels and inflammation

Mouse primary macrophages from apoE$^{-/-}$ mice were harvested by peritoneal lavage 3-4 days after intraperitoneal injection of 3 ml 3% thioglycollate. Attached cells were cultured in DMEM containing 10% FBS [23]. For testing the effect of exogenous hEp on macrophage inflammation, macrophages were plated at a density of 2.5×10^6/well in 6-well plates and treated with 100 ng/ml LPS and 20 μg/ml hEp for 6 h,

then medium was collected for ELISA, and the cells were harvested with Trizol reagent. For determining the effect of hEp on macrophage cholesterol efflux, the cells were cholesterol-enriched by incubation for 48 h in DMEM containing with 50 μg/ml oxidated LDL (ox-LDL). Cells were washed with 0.5 ml of 1% BSA in DMEM, then fresh DMEM containing hEp (20 μg/ml) was added; the cells were further cultured for 24 h. Cellular lipids were extracted by incubating overnight in isopropanol. The amount of total cholesterol (TC), free cholesterol (FC), and triglyceride (TG) were determined by enzymatic colorimetric assay kits (Mind Bioengineering, China). Cell proteins were solubilized by addition of 1N NaOH to the wells, and the protein content was measured using the Lowry method. Lipid content was normalized to macrophage protein content.

Generation of recombinant lentiviral vector

To generate a lentiviral vector for hEp expression, a 255 bp DNA fragment of recombinant hEp peptide (N-terminal linked 18 residues of apoE$_3$ signal peptide and 6×His tag) was synthesized and amplified by PCR, then subcloned into the *Pac* I site of a bicistronic lentiviral vector PWPI containing a green fluorescent protein (GFP) tag. The final hEp-lentiviral construct PWPI-hEp-GFP was verified by DNA sequencing. General procedures for lentivirus production were described in a previous study [24]. The lentivirus for expression of hEp was named Lv-hEp-GFP, and the control lentivirus was named Lv-GFP.

Expression of hEp in HepG2 cells

HepG2 hepatocellular cell line was obtained from Classic Specimen Culture and Storage Center at Wuhan University (Wuhan, China). The cells were cultured and transfected with the PWPI-hEp-GFP vector or the control PWPI-GFP vector using lipofectamine 2000 as described in a previous study [24]. GFP expression in lentivirus-transfected cells was directly visualized as GFP fluorescence using an Olympus microscope, and hEp protein level in the medium was detected by western blot using anti-His-tag antibody (1:1000).

Mice

Female apoE$^{-/-}$ mice (on a C57BL/6 background) were purchased from Vital River Laboratory Animal Technology Company, China and housed in microisolator cages in the Wuhan University Animal Center. Mice were fed a chow diet in a temperature-controlled facility maintained at 22 ± 1°C and 60% humidity with a 12 h light: 12 h dark cycle. All animal studies were performed under the regulations of the Institutional Animal Care and the Ethics Committee for Animal Experiments of Wuhan University, conforming to the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee. Twenty female apoE$^{-/-}$ mice at 9 months of age were randomly divided into two groups for lentiviral injection.

Lentivirus-mediated hepatic expression of hEp in apoE$^{-/-}$ mice

In order to achieve hepatic expression of hEp, all apoE$^{-/-}$ mice were injected with 200 μl of lentivirus containing 1.5~1.6×10^8 particles of Lv-hEp-GFP (Lv-GFP as control) via the retoorbital venous plexus under anesthesia with 3% isoflurane as previously described [24]. The mice were maintained on a chow diet for 18 weeks and then euthanized; blood and tissues were harvested. Expression levels of hEp in the liver and plasma hEp levels were detected by immunohistochemistry or Western blot analysis.

Plasma lipid, lipoprotein, and biochemical analyses

Mouse blood samples were collected after overnight fasting by retroorbital venous plexus puncture at 2- to 8-week intervals. Plasma was immediately separated by centrifugation at 10,000 g for 10 min at 4°C, and then aliquoted and stored at -80°C. Plasma TC and TG levels were determined as described above. Plasma lipoprotein cholesterol profiles were analyzed by fast protein liquid chromatography (FPLC) using a Superose 6 10/300 GL column on an AKTA purifier (GE Healthcare) as described previously [7, 24].

Plasma paraoxonase-1 (PON1) activity was measured using paraoxon as a substrate as described previously [25]. Plasma myeloperoxidase (MPO) activity was determined using MPO Detection Kit (Nanjing Jiancheng Bioengineering Institute, China). Plasma IgG concentrations were determined by enzyme-linked immunosor-

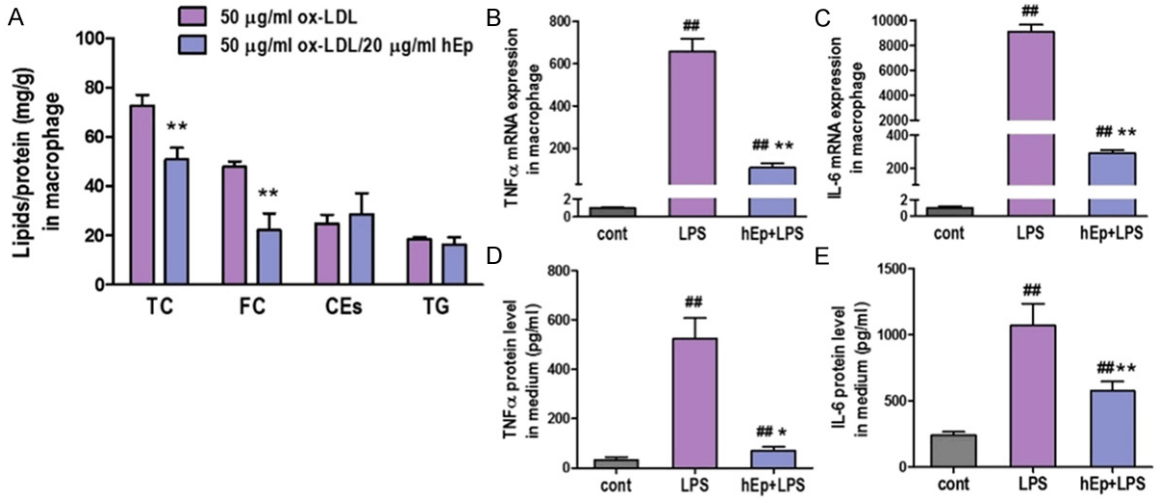

Figure 1. The secondary structure of hEp peptide and its effects on primary peritoneal macrophages from apoE$^{-/-}$ mice. A: Primary peritoneal macrophages were loaded with 50 μg/ml ox-LDL for 48 h, and cultured with 20 μg/ml hEp in fresh DMEM for 24 h, before the total lipids in cells were extracted. The amounts of TC, FC, CEs, and TG were determined. n=3. *P < 0.05 and **P < 0.01 vs control group. B-E: Primary macrophages were treated with 100 ng/ml LPS, and 20 μg/ml hEp for 6 h before culture medium was collected and cellular RNA was extracted. TNFα and IL-6 mRNA levels were determined by qPCR with the expression level of each gene normalized to the level of 18 s RNA. TNFα and IL-6 levels in medium were determined by ELISA. n=3. **P < 0.01 vs LPS group. ##P < 0.01 vs control group.

bent assay (ELISA) kits (eBioscience, USA) according to the manufacturer's instructions. Plasma alanine aminotransferase (ALT) activity was determined using Mohum's method.

Quantification of atherosclerotic lesions

Eighteen weeks after lentiviral injection, the extent of atherosclerosis was examined using Oil Red O-stained cross sections of the aortic root (8 μm serial sections) and by *en face* analysis of the aorta. Quantification with Image-Pro Plus 6.0 software was performed as described previously [21, 23, 24].

Quantitative real-time PCR (QPCR)

Total RNA of macrophages or mouse liver tissue was isolated with Trizol reagent and reverse transcribed into cDNA using a PrimerScript® RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Target mRNA levels were measured by qPCR with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Mouse genes were normalized with 18 s rRNA as internal control. PCR primers for amplification of mouse genes, including LDLR, tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), were described previously [24].

Western blot analysis

Western blot was used to measure the target protein levels in cell lysate, plasma, or liver homogenate. The protein concentrations of cell lysate and liver homogenate were determined by the Lowry method using a DC protein assay kit (Bio-Rad), and appropriate amounts of proteins were loaded and separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Target proteins were detected using specific primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. The signal was detected using an ECL kit and analyzed with Image J software (NIH, USA). The detailed information about loading amount of proteins and primary antibodies against mouse LDLR, PON1, apolipoprotein AI (apoAI), serum amyloid A (SAA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [24].

Statistical analysis

Data are represented as the mean ± SD. Statistical analyses were performed using Student's t test and one-way ANOVA between the groups. Differences were considered to be significant at P < 0.05.

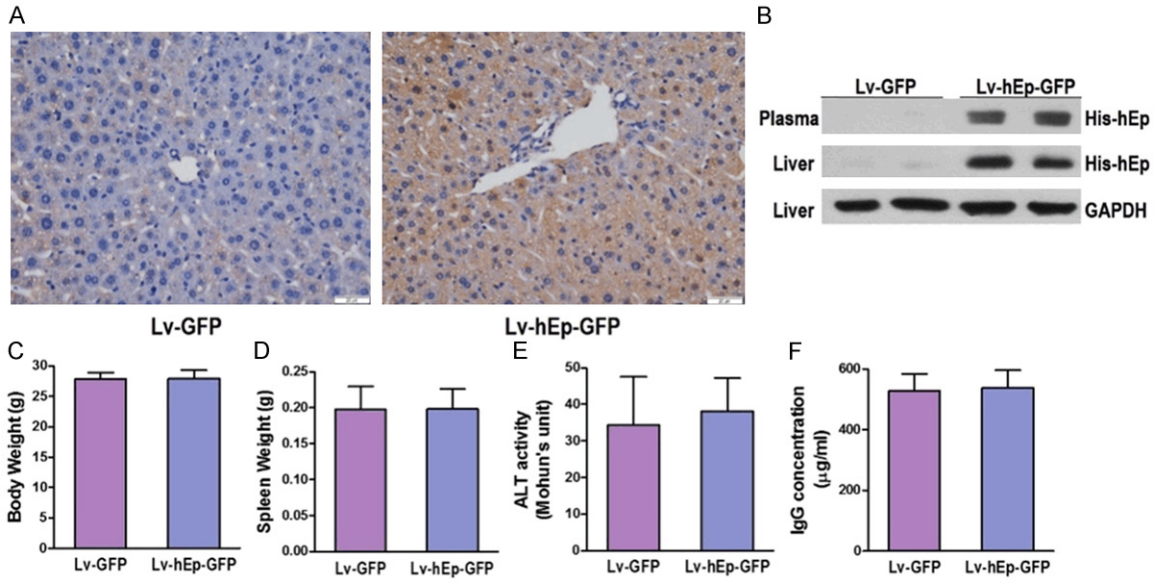

Figure 2. Lentivirus-mediated hEp expression in apoE$^{-/-}$ mice and the effects on body weight and spleen weight. Nine-month-old apoE$^{-/-}$ mice were intravenously injected with lentiviruses Lv-hEp-GFP or Lv-GFP and euthanized 18 weeks later. A: Expression of hEp in the liver was determined by immunohistochemistry. B: hEp in liver and plasma was detected by western blot using anti-His-tag antibody. C, D: Mouse body weights and spleen weights were measured at the end point, n=8. E, F: Plasma IgG concentrations were determined by ELISA, and ALT was detected using Mohum' method. n=7-8.

Results

HEp peptide diminished macrophage lipid accumulation and inhibited macrophage inflammation

A 61-residue hEp mimetic peptide was designed; it contains an N-terminal LDLR binding region (residues 131-162) and a C-terminal lipid binding region (residues 244-272) of apoE. We performed an *in vitro* functional assessment with mouse peritoneal macrophages using chemically synthesized hEp peptide (98% purity). First, we tested the effect of hEp on cholesterol-loaded macrophages. Macrophages from apoE$^{-/-}$ mice were loaded with cholesterol by incubation for 48 h with 50 μg/ml ox-LDL, and then incubated with DMEM containing 20 μg/ml hEp. As shown in **Figure 1A**, TC and FC accumulation induced by ox-LDL was significantly attenuated in hEp treated macrophages ($P < 0.01$). Next, we examined whether hEp inhibits macrophage inflammatory responses to LPS. Peritoneal macrophages were cultured in DMEM containing 100 ng/ml LPS with or without 20 μg/ml hEp for 6 h. qPCR results showed that hEp significantly decreased LPS-induced mRNA expression of inflammatory cytokines TNFα and IL-6 in macrophages (**Figure 1B**, **1C**). The levels of TNFα and IL6 in the medium were also significantly decreased (TNFα: 70.3 ± 16.2 pg/ml vs. 523.7 ± 84.0 pg/ml; IL6: 577.9 ± 68.7 pg/ml vs. 1072.5 ± 162.7 pg/ml; $P < 0.01$; **Figure 1D**, **1E**). These data demonstrated that the hEp peptide diminished macrophage cholesterol accumulation and inhibited macrophage inflammation.

Generation of hEp lentiviral vector and induction of hEp expression in transfected HepG2 cells

In order to achieve secretion of the hEp peptide in mice for *in vivo* studies, the PWPI-GFP lentiviral expression system was used to construct a recombinant hEp lentivirus. The inserted hEp sequence was designed to link an apoE3 signal peptide containing 18 amino acids, a 6 His-tag and the 61-residue hEp as depicted in additional file 1: Figure S1A. We constructed a bicistronic lentiviral vector PWPI-hEp-GFP, to express hEp and GFP simultaneously. The construct was verified by DNA sequencing.

Subsequently, PWPI-hEp-GFP lentiviral vector was transfected into HepG2 cells to confirm the expression and secretion of hEp. We found that GFP, as a reporting protein for the lentiviral vec-

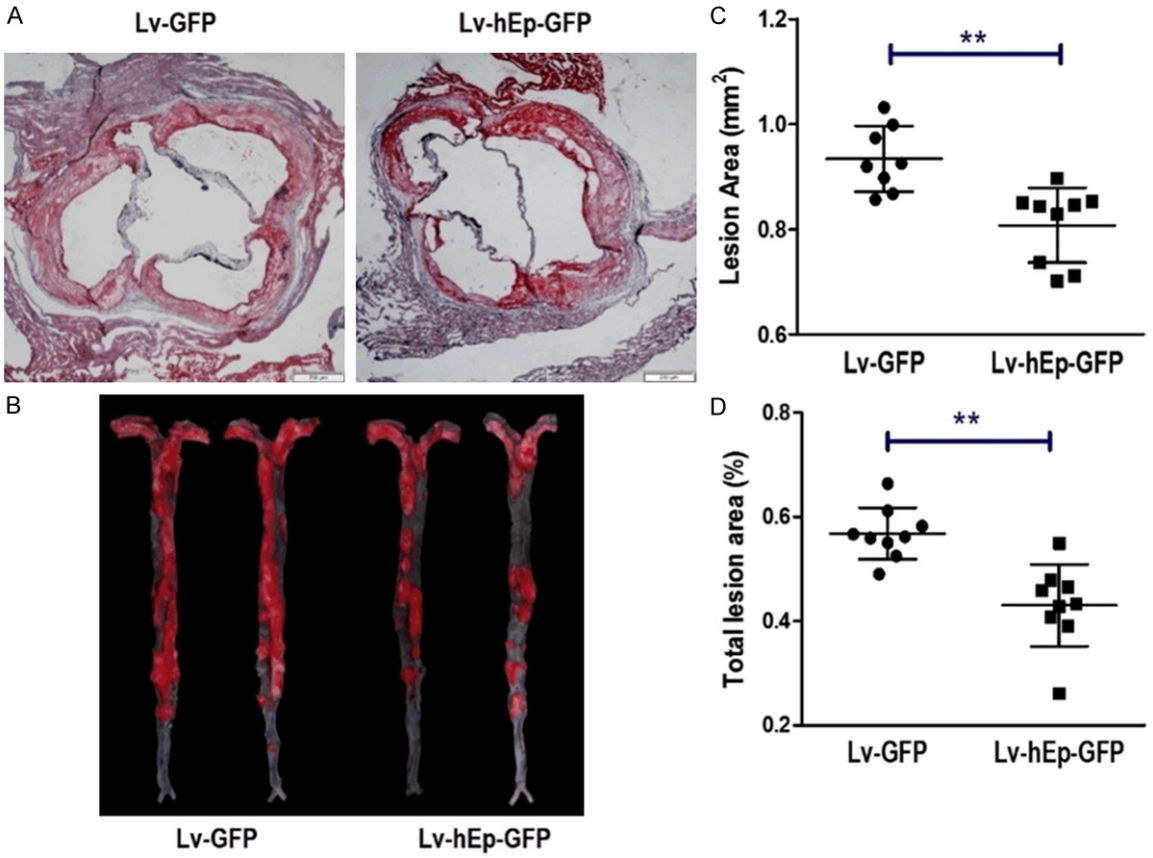

Figure 3. Expression of hEp reduced atherosclerotic lesions in apoE$^{-/-}$ mice. Lv-GFP mice and Lv-hEp-GFP mice were fed a chow diet for 18 weeks after lentivirus injection. The atherosclerotic lesions were examined using Oil Red O-stained cross sections of the aortic root (8 μm serial sections) and by *en face* analysis of the aorta and were quantified using Image-Pro Plus 6.0 software as described previously. A, B: Representative images and quantification of lesions in cross-sections of the aortic roots. C, D: Representative images and quantification of *en face* aortas. n=9. *$P < 0.05$.

tor, was directly visualized with fluorescent microscopy. Also, high expression levels of the His-tag portion of the hEp peptide were also detected in the cell culture medium using western blot with anti-His antibody, indicating hEp was expressed in transfected hepatocytes and secreted into the medium (Additional file 1: Figure S1B, S1C).

Secretion of hepatic hEp in Lv-hEp-GFP mice

Lv-hEp-GFP or Lv-GFP lentiviruses were intravenously injected into 9-month-old female apoE$^{-/-}$ mice, and the mice were euthanized at 18 weeks after the lentiviral injection. hEp in liver and plasma was detected by immunohistochemistry or western blot using an anti-His-tag antibody. The results showed that intravenous injection of lentivirus resulted in high levels of hepatic expression of hEp, and that hEp was secreted into the circulation of Lv-hEp-GFP mice (**Figure 2A**, **2B**). It is important to note that high levels of lentivirus-mediated expression of hEp in the liver and its excretion into plasma did not cause *in vivo* toxicity, and no alterations were observed in body weight, spleen weight, plasma ALT activity, or plasma IgG concentrations (**Figure 2C**, **2D**).

Expression of hEp reduced atherosclerosis

The impact of hepatic hEp expression on atherosclerotic lesion development in apoE$^{-/-}$ mice was examined 18 weeks after lentiviral injection. Representative atherosclerotic lesions in cross sections of aortic roots and *en face* aorta images stained with Oil Red O are shown in **Figure 3A**, **3B**. Quantitative analysis of the aortic root cross sections demonstrated a significant decrease in mean lesion area in Lv-hEp-

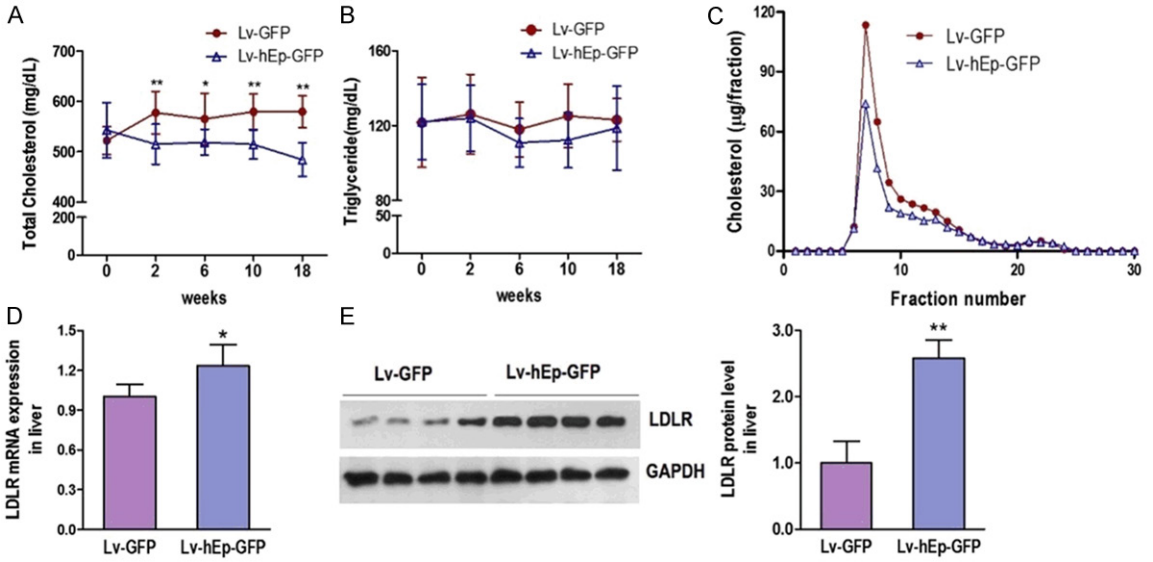

Figure 4. Effects of hEp on plasma lipid levels and hepatic LDLR expression in apoE$^{-/-}$ mice. A, B: TC and TG levels of fasting plasma collected at the indicated time points were measured enzymatically. n=8. *P < 0.05, **P < 0.01. C: Plasma lipoprotein profiles in Lv-hEp-GFP mice or Lv-GFP mice at 18 weeks after lentiviral injection were analyzed. D: The mRNA levels of LDLR in livers of lentivirus treated mice were determined by qPCR. n=3-4. *P < 0.05. E: LDLR protein level was determined by Western blot. n=3-4. **P < 0.01 vs Lv-GFP group.

GFP mice compared to Lv-GFP mice (0.94 ± 0.06 mm^2 vs. 0.81 ± 0.07 mm^2; P < 0.01; **Figure 3C**). *En face* analysis of pinned-out aortas revealed that the percentage of lesion area in Lv-hEp-GFP mice was reduced by 24.2%, compared to Lv-GFP injected mice (P < 0.01, **Figure 3D**).

HEp ameliorated plasma lipid levels in apoE$^{-/-}$ mice

Plasma lipid levels were assessed on the day before lentiviral injection and several times thereafter. Fasting plasma TC and TG levels and FPLC profile were determined. Our data showed that secretory expression of hEp reduced plasma TC levels in apoE$^{-/-}$ mice as early as 2 weeks after lentiviral injection, and the reduction was maintained during the whole experimental period, while plasma TG levels were not significantly altered (**Figure 4A**, **4B**). The FPLC profiling indicated that the reduction in plasma TC was due to a decrease in VLDL and LDL fractions, but not in HDL fraction (**Figure 4C**). We further found that hepatic LDLR mRNA and protein levels were up-regulated by hEp expression in Lv-hEp-GFP mice (**Figure 4D**, **4E**), suggesting that enhanced LDL uptake mediated by LDLR in the liver was involved in plasma VLDL/LDL cholesterol clearance in apoE$^{-/-}$ mice.

HEp ameliorated oxidative stress and inflammation in apoE$^{-/-}$ mice

Considering the anti-oxidative and anti-inflammatory properties of apoE, these potential effects of hepatic hEp expression were assessed. To examine the plasma redox and inflammatory status in mice, we measured the activities of oxidation related enzymes PON1 and MPO, and the protein levels of PON1, apoAI, and acute phase reactant SAA. The data showed that plasma PON1 activity was significantly increased, accompanied by enhanced PON1 protein level, whereas plasma MPO activity and SAA levels in Lv-hEp-GFP mice were significantly decreased, and the protein levels of apoAI did not change (**Figure 5A-C**). In addition, we have also determined the gene expression of TNFα and IL-6 in liver, and found that the mRNA levels of TNFα and IL-6 were significantly reduced in Lv-hEp-GFP mice compared to Lv-GFP control mice (**Figure 5D**, **5E**).

Discussion

ApoE mimetic peptides represent a promising new therapeutic strategy for atherosclerosis-related cardiovascular diseases [9]. We have previously shown that an apoE mimetic peptide, EpK, binds to HDL and enhances HDL

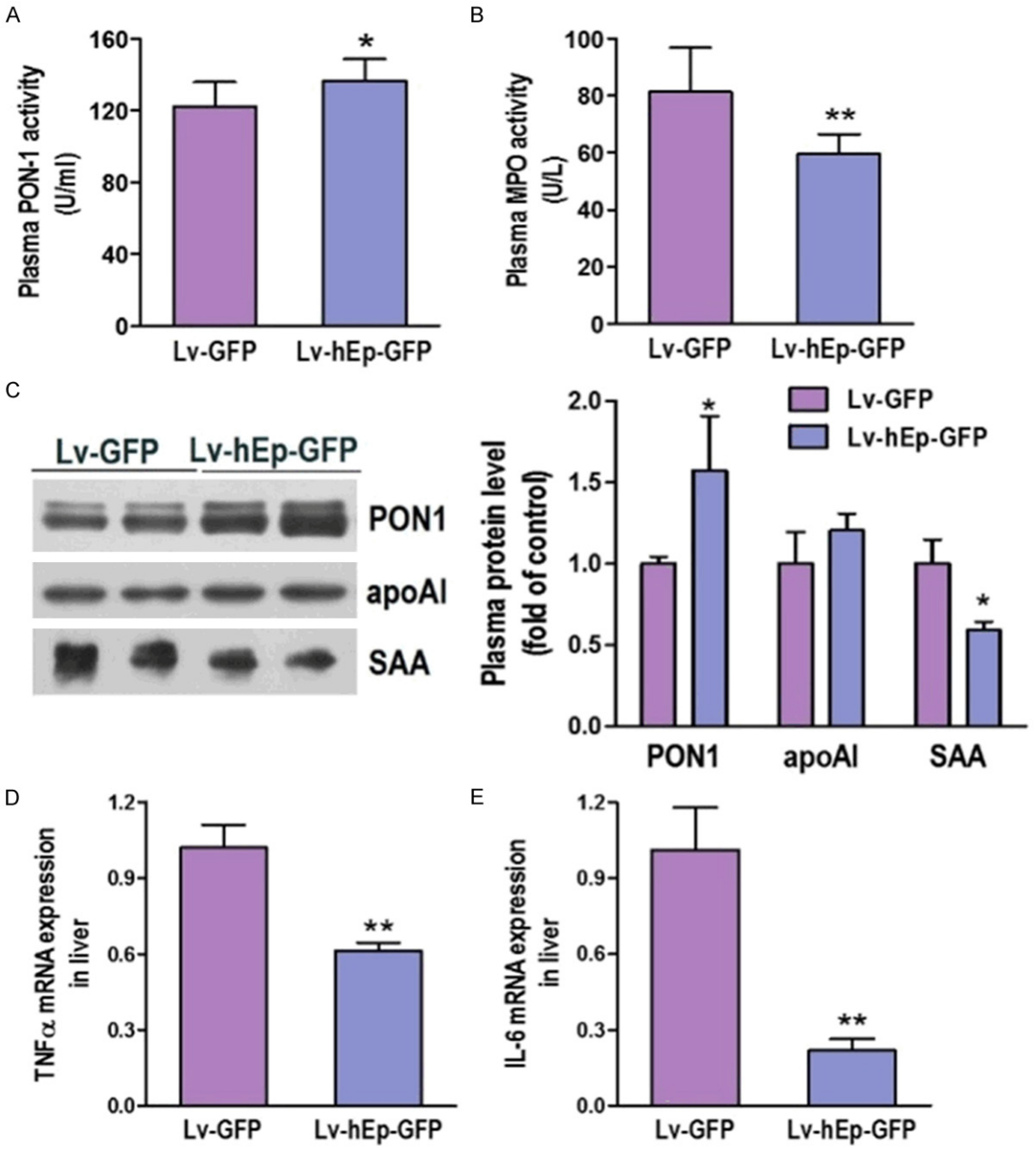

Figure 5. Expression of hEp ameliorated oxidative stress and inflammation in apoE$^{-/-}$ mice. A, B: The activities of MPO and PON1 in plasma were determined by enzymatic colorimetric assay. n=6-8. *P < 0.05, **P < 0.01. C: Plasma protein levels of PON1, apoAI, and SAA were determined by western blot analysis. Relative fold increase of each protein is shown. n=4-5. *P < 0.05. D, E: TNFα and IL-6 mRNA levels in liver were determined by qPCR, and the relative expression levels of genes were normalized to the level of 18 s RNA. n=4-5. **P < 0.01.

function in promoting macrophage cholesterol efflux and attenuating macrophage inflammatory responses [20], and hepatic EpK secretory expression could reduce the progression of atherosclerotic lesions in apoE$^{-/-}$ mice through its significant anti-inflammatory effects without changing plasma cholesterol levels [21]. Based on the structure and function of EpK, we designed a novel apoE dual-domain peptide, hEp, which contains nearly the entire amphipathic helix 4 of the N-terminal domain and the major C-terminal lipid-binding region of apoE in order to enhance its receptor binding and lipid binding abilities. Previous work showed that

injection of hEp peptide could immediately reduce plasma cholesterol in mice, besides inhibiting hepatitis C virus infection by blocking virus binding [22]. Here, we used a lentivirus system to investigate the effects of hepatic hEp secretory expression on atherosclerosis. The present study shows that hEp could reduce macrophage cholesterol accumulation, inhibit macrophage inflammation, and have anti-oxidative and anti-inflammatory effects. We were excited to find that induction of hEp peptide expression by lentivirus injection reduced atherosclerotic lesion size in aged apoE$^{-/-}$ mice with established lesions.

Human apoE is a 34 kDa protein with atheroprotective properties, consisting of two distinct domains: the LDLR-binding globular domain (1-191 residues) and the lipid-binding domain (192-299 residues). Some studies on the LDLR-binding domain indicated that the prerequisite for receptor binding is the helicity, and the helix 4 (141-150 residues) region should be sufficient to confer LDLR-binding activity [16, 17]. As previously reported, the most extensively studied synthetic apoE mimetic peptide, Ac-hE18A-NH_2, which is composed of the LDLR-binding region of apoE (141-150 residues) and an 18-residue peptide 18A, mimicking the lipid-binding region of apoAI, was found to have multiple antiatherogenic functions including lowering plasma VLDL/LDL in hyperlipidemic rabbits and mice by mediating VLDL/LDL uptake. It also can mediate macrophage cholesterol efflux and inhibit cell inflammatory responses to LPS stimulation. The efficacy of this peptide has been demonstrated in several animal models and Phase 1 clinical trials for cardiovascular disease [10, 26]. We previously designed the LDLR-binding region of the EpK peptide based on this Ac-hE18A-NH_2 peptide, and linked it with a human apoE lipid binding region (234-254 residues). However, we found that EpK did not bind VLDL/LDL and did not lower plasma cholesterol even though EpK was shown to adopt an α-helical structure for lipid-binding activity [20, 21]. Interestingly, we elongated LDLR-binding region in designing hEp to include residues 131 to 162 of apoE, and found that hEp could mediate plasma cholesterol clearance in mice by enhanced LDLR-binding activity [22], and thus decreased plasma VLDL/LDL cholesterol but not HDL cholesterol levels.

The C-terminal lipid binding region of apoE may confer its cholesterol efflux property. Previous studies have confirmed that the lipid-binding region could associate with lipid complexes and promote lipid efflux from macrophages [15]. In contrast to the lipid-binding region (residues 234-254) of EpK, hEp contains more of the C-terminal lipid-binding region (residues 244-272) of apoE. In cell culture studies, we found that hEp significantly reduced lipid accumulation in cholesterol loaded primary murine macrophages, consistent with the properties of EpK and other apoE mimetic peptides [15, 16, 20]. The aforementioned data suggests that hEp could exert the function of apoE in attenuating macrophage lipid accumulation and lowering plasma cholesterol, contributing to a reduction of atherosclerosis severity in apoE$^{-/-}$ mice.

In addition to dyslipidemia, inflammation and oxidative stress are also well-known risk factors for atherosclerosis [27, 28]. It has been recognized that enhanced chronic inflammation and exaggerated acute immune responses to exogenous stimuli are also major pathological features of apoE$^{-/-}$ mice [2, 29]. Exogenous apoE and its mimetic peptides exhibit a wide range of anti-inflammatory and anti-oxidative activities which slow down the progression of atherosclerosis in apoE$^{-/-}$ mice, with the LDLR binding region conferring this anti-inflammatory function [18, 20, 21, 30]. In order to enhance the binding ability for cellular receptors, we designed the LDLR binding region of hEp to contain a larger portion of the LDLR binding region of apoE (131-162 residues), which differed from EpK (141-150 residues of apoE), and found that hEp inhibited macrophage inflammatory responses to LPS stimulation, and hEp expression induced by lentivirus injection in apoE$^{-/-}$ mice significantly decreased the expression of inflammatory cytokines IL-6 and TNFα in the liver. The plasma level of SAA, which is an acute phase reactant protein of systemic inflammation, was also reduced. The anti-inflammatory effect of hEp is similar to those of EpK and Ac-hE18A-NH_2 peptides [18-21]. Additionally, we tested plasma PON1 and MPO activities as indicators of anti-oxidative status in circulation. PON1 is anti-oxidative enzyme which is synthesized in the liver and mainly associated with HDL particles in circulation [31]. Our previous study indicated a negative correlation between plasma PON1 activity and the extent of atherosclerosis [25]. Here, our data showed that plasma PON1 activity as

well as PON1 levels were significantly increased, suggesting that hEp exerted anti-oxidative function in vivo. MPO, an abundant leukocyte enzyme that generates reactive oxygen species, contributing to inflammatory injury, is a risk marker for atherosclerosis [32, 33]. Our results suggest that decreased plasma MPO activity in Lv-hEp-GFP mice may also contribute to the anti-oxidative and anti-inflammatory function of hEp.

In summary, our study demonstrated that in aged apoE$^{-/-}$ mice, lentivirus-mediated hepatic hEp expression diminishes lipid accumulation, inhibits inflammation, and improves liver and plasma redox status, resulting in attenuated atherosclerosis. This study suggests that hEp can be developed as a promising new agent for the treatment of atherosclerosis-related cardiovascular diseases.

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Disclosure of conflict of interest

None.

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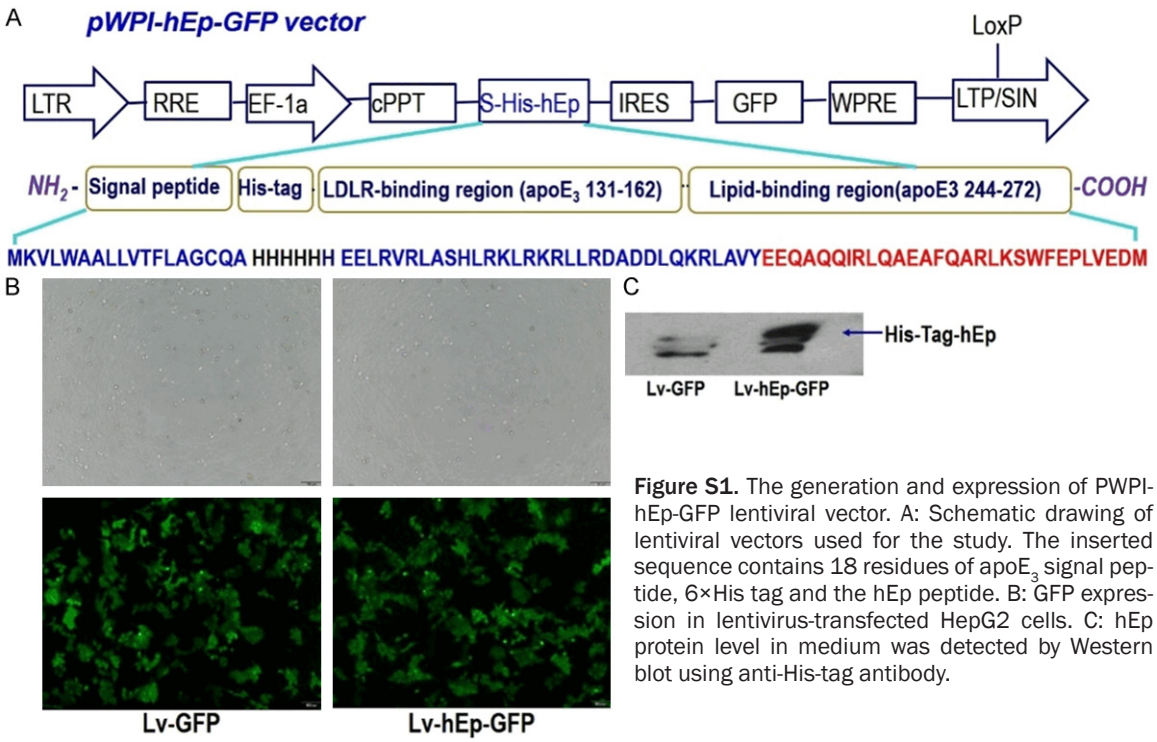

Figure S1. The generation and expression of PWPI-hEp-GFP lentiviral vector. A: Schematic drawing of lentiviral vectors used for the study. The inserted sequence contains 18 residues of apoE$_3$ signal peptide, 6×His tag and the hEp peptide. B: GFP expression in lentivirus-transfected HepG2 cells. C: hEp protein level in medium was detected by Western blot using anti-His-tag antibody.