The official journal of the Japan Atherosclerosis Society and
the Asian Pacific Society of Atherosclerosis and Vascular Diseases

Original Article

J Atheroscler Thromb, 2021; 28: 157-168. http://doi.org/10.5551/jat.55244

Endothelial Lipase Exerts its Anti-Atherogenic Effect through Increased Catabolism of β-VLDLs

Haizhao Yan[1], Manabu Niimi[1], Chuan Wang[1, 2], Yajie Chen[1, 3], Huanjin Zhou[1], Fumikazu Matsuhisa[4], Kazutoshi Nishijima[5], Shuji Kitajima[4], Bo Zhang[6], Hiroshi Yokomichi[7], Katsuyuki Nakajima[8], Masami Murakami[8], Jifeng Zhang[9], Y. Eugene Chen[9] and Jianglin Fan[1, 3]

[1]Department of Molecular Pathology, Faculty of Medicine, Graduate School of Interdisciplinary Research, University of Yamanashi, Yamanashi, Japan
[2]Department of Pharmacology, College of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang, China
[3]School of Biotechnology and Health Sciences, Wuyi University, Jiangmen, China
[4]Analytical Research Center for Experimental Sciences, Saga University, Saga, Japan
[5]Animal Research Laboratory, Bioscience Education-Research Support Center, Akita University, Akita, Japan
[6]Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka, Japan
[7]Department of Health Sciences, University of Yamanashi, Yamanashi, Japan
[8]Department of Clinical Laboratory Medicine, Graduate School of Medicine, Gunma University, Maebashi, Japan
[9]Center for Advanced Models for Translational Sciences and Therapeutics, University of Michigan Medical Center, Ann Arbor, MI, USA

Aim: Endothelial lipase (EL) plays an important role in lipoprotein metabolism. Our recent study showed that increased hepatic expression of EL attenuates diet-induced hypercholesterolemia, thus subsequently reducing atherosclerosis in transgenic (Tg) rabbits. However, it is yet to be determined whether increased EL activity itself *per se* is anti-atherogenic or whether the anti-atherogenic effect of EL is exclusively dependent on its lipid-lowering effect.

Methods: To determine the mechanisms underlying EL-mediated anti-atherogenic effect, we fed Tg and non-Tg rabbits diets containing different amounts of cholesterol to make their plasma cholesterol levels similarly high. Sixteen weeks later, we examined their lipoprotein profiles and compared their susceptibility to atherosclerosis.

Results: With Tg and non-Tg rabbits having hypercholesterolemia, the plasma lipids and lipoprotein profiles were observed to be similar, while pathological examinations revealed that lesion areas of both aortic and coronary atherosclerosis of Tg rabbits were not significantly different from non-Tg rabbits. Moreover, Tg rabbits exhibited faster clearance of DiI-labeled β-VLDLs than non-Tg rabbits.

Conclusion: The results of our study suggest that the enhancement of β-VLDL catabolism is the major mechanism for atheroprotective effects of EL in Tg rabbits.

Key words: Endothelial lipase, Hypercholesterolemia, VLDL metabolism, Atherosclerosis, Transgenic rabbits

Introduction

Endothelial lipase (EL) belongs to a triglyceride lipase family, which also includes lipoprotein lipase (LPL) and hepatic lipase (HL)[1]. EL was originally cloned from endothelial cells[2, 3], but it is also expressed in the liver, lung, thyroid, and kidney[4, 5]. Although EL, LPL, and HL hydrolyze both triglycerides (TG) and phospholipids (PL) in the lipoproteins, they show different substrate selectivities. EL exhibits high phospholipase activity in high-density lipoprotein (HDL) but low triglyceride lipase activity, whereas LPL and HL mainly exercise triglyceride lipase activity[6]. Previous studies on mice[2, 7] and rabbits[8] have

Address for correspondence: Jianglin Fan, Department of Molecular Pathology, Faculty of Medicine, Graduate School of Interdisciplinary Research, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi, 409-3898, Japan E-mail: jianglin@yamanashi.ac.jp

Received: January 20, 2020 Accepted for publication: March 17, 2020

shown that overexpression of EL reduces HDL cholesterol (HDL-C) levels. On the contrary, plasma HDL-C levels are increased in mice with EL gene deficiency[7, 9] and EL-specific antibody injection[10]. Furthermore, human studies showed that EL mass or activity was inversely associated with plasma HDL-C levels[11, 12]. All these reports support the notion that EL is an important modulator of HDL metabolism. In addition to HDL, EL may be involved in the metabolism of apolipoprotein (apo)B-containing lipoproteins such as very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). For example, EL was reported to hydrolyze VLDL-TG and -PL *in vitro*[6]. Overexpression of human EL reduces apoB-containing lipoproteins in apoE-deficient, LDL receptor-deficient, and human apoB transgenic (Tg) mice[13] as well as cholesterol-fed Tg rabbits[8]. Besides, EL has been shown to exert non-catalytic function where it serves as a bridging molecule between plasma lipoproteins and vascular cells. Through this process, EL mediates the binding and uptake of HDLs and apoB-containing lipoproteins[14-16]. Although inhibition of EL was considered a new therapeutic strategy for the treatment and prevention of atherosclerosis through increasing plasma HDL-C[17], it is still unclear what precise roles EL plays in the development of atherosclerosis. Studies using EL-deficient mice generated conflicting results regarding EL functions in atherosclerosis. Ishida *et al.*[18] demonstrated that EL inactivation attenuated atherosclerosis in apoE knockout mice, whereas Ko *et al.*[19] reported no effect on atherosclerosis in both apoE and LDL receptor knockout mice. In human studies, the relationship between EL and HDL-C and coronary artery disease (CAD) risk remains unclear. Loss-of-function EL variant exhibited higher plasma HDL-C levels but did not show any association with reduced risk for CAD[20]. On the other hand, plasma EL concentrations or activity was correlated with the risks for CAD[12, 21].

Recently, we generated Tg rabbits expressing the human EL in the liver and showed that overexpression of EL attenuated diet-induced hypercholesterolemia and inhibited atherosclerosis[8]. Tg rabbits exhibited lower plasma HDL-C levels as well as remnant lipoproteins than non-Tg rabbits. However, it is unknown whether increased EL activity itself is anti-atherogenic or whether the anti-atherogenic effect of EL is dependent upon its lipid-lowering effect in Tg rabbits. To address this issue, we performed the following study using Tg rabbits which developed similar "high" hypercholesterolemia to non-Tg rabbits. Our studies revealed that anti-atherogenic effect in EL Tg rabbits mainly depends on its enhanced remnant lipoprotein clearance.

Methods

Rabbits

EL Tg rabbits along with sex- and age-matched littermates were used as reported previously[8]. In this experiment, male Tg and non-Tg rabbits aged 3 months were utilized. Rabbits were housed with a 12-hour light/12-hour dark cycle and fed a normal standard diet (CR-3M; CLEA Japan, Tokyo, Japan). The rabbits were allowed access to diets and water *ad libitum*. All animal experiments were conducted in accordance with the Animal Care Committee of the Universities of Yamanashi and Saga and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Induction of Similar Hypercholesterolemia

In the previous study, we found that Tg rabbits showed lower levels of plasma lipids than non-Tg rabbits on both normal and cholesterol-rich diets[8]. In order to make Tg and non-Tg rabbits to develop similarly "high" hypercholesterolemia, we fed Tg rabbits with a diet containing relatively high contents of cholesterol (≈1%), whereas non-Tg rabbits were fed a diet containing relatively low cholesterol (0.3%). To make this goal possible, we measured their plasma levels of total cholesterol (TC) weekly and then adjusted accordingly[22]. Six weeks later, plasma TC levels of both Tg and non-Tg rabbits reached about 800 mg/dL (arbitrary atherogenic levels in cholesterol-fed rabbits) and remained at such level until they were sacrificed at 16 weeks. Blood was collected from an auricular artery of fasted rabbits for analysis of plasma lipids weekly. Plasma TC, TG, PL, HDL-C, and HDL-PL were measured using enzymatic colorimetry assay kits (Wako Pure Chemical Industries, Osaka, Japan).

Plasma Lipoprotein Analysis

Plasma lipoproteins were isolated from rabbits fed with either a normal standard diet or a cholesterol-rich diet at 8 and 16 weeks using sequential ultracentrifugation with a Beckman Optima MAX-TL ultracentrifuge with a TLA120.2 rotor (Beckman, Brea, CA), as described previously[23]. Isolated individual density fractions contained the following lipoproteins: the $d < 1.006$ g/mL fraction contains VLDL or β-VLDL from cholesterol-fed rabbits; the $d = 1.02$ g/mL fraction contains intermediate-density lipoprotein (IDL); the $d = 1.04$ g/mL fraction contains large LDL; the $d = 1.06$ g/mL fraction contains both LDL and HDL_1; the $d = 1.08$ and 1.10 g/mL fractions are those of HDL_2; while the $d = 1.21$ g/mL fraction contains HDL_3. TC, TG, and PL contents in each fraction

were measured using Wako enzymatic colorimetry assay kits.

Lipoprotein Lipase Activity

Lipoprotein lipase (LPL) activity in post-heparinized plasma was measured using the method described previously[24, 25]. Briefly, blood was withdrawn from rabbits after heparin injection, and LPL activity in the post-heparin plasma was analyzed through an automated colorimetric method using the natural long-chain fatty acid 2-diglyceride as a substrate (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan).

Pathological Analysis of Atherosclerosis

At the end of the experiment, all rabbits were sacrificed for evaluation of atherosclerosis. The aortic gross and microscopic lesions were analyzed as described previously[26]. Briefly, aortas were stained with Sudan IV to evaluate the gross lesion size using an image analysis system. For microscopic evaluation of the lesion area, the aortic arch was dissected into eight segments, embedded in paraffin. Serial sections (3 μm thick) were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies (Abs) against macrophages RAM11 (Dako Inc., Carpinteria, CA) (1:400 dilution) and smooth muscle α -actin HHF35 (Dako Inc., Carpinteria, CA) (1:300 dilution). The microscopic lesion area along with macrophage and SMC staining area in the lesions were quantified with WinRoof image analysis system (Mitani Co., Tokyo, Japan). Coronary atherosclerosis was analyzed as described previously[26]. Paraffin-embedded left coronary artery trunk was sectioned at 50 μm intervals, and these sections were stained with HE and EVG. Four sections per rabbit heart were used for lesion analysis. The coronary lesion was expressed as stenosis percentage.

β-VLDL Clearance

Plasma β-VLDLs (d < 1.006) were isolated by ultracentrifugation from cholesterol-fed non-Tg rabbits. β-VLDLs were then labeled with fluorescence using the method described by Pitas RE *et al.*[27] and were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Wako Pure Chemical Industries, Osaka, Japan) at 37℃ for 9 hrs. Then, the labeled β-VLDLs were collected after ultracentrifugation for 18 hrs at 50,000 rpm with a 70.1Ti rotor (Beckman, CA). DiI-labeled β-VLDLs were dialyzed against saline and filtrated before use. For clearance studies, Tg and non-Tg rabbits were fasted overnight, and then the labeled β-VLDLs (3 mg protein/kg) were infused into an ear vein. Blood samples were collected at selected time points, and plasma fluorescent intensity was measured by a fluorescent microplate reader (Molecular Devices LLC., San Jose, CA). To evaluate the stability of DiI labeling within β-VLDLs *in vivo*, we measured the dynamic changes of DiI along with TC contents in VLDL, LDL, and HDL using wild-type rabbits. As shown in **Supplementary Fig. 1**, up to 80% of DiI-VLDL were cleared from the circulation, whereas about 20% of DiI were dissociated and transferred to HDL particles within 2 hr. Furthermore, the fractional catabolic rate was calculated as described previously[28].

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with SPSS software (IBM Co., Chicago, IL). T-test was used for comparison between two groups. A repeated measures ANOVA was used for comparing the time series of TC and HDL-C levels. $P < 0.05$ was considered statistically significant.

Results

Analysis of Plasma Lipids and Lipoproteins

Tg rabbits on a normal standard diet showed significantly lower plasma lipids than non-Tg rabbits, including 69% ↓ of TC, 85% ↓ of HDL-C, 51% ↓ of TG, 63% ↓ of PL, and 65% ↓ of HDL-PL **(Fig. 1A and Supplementary Table 1)**. When challenged with a 0.3% cholesterol diet for 1 week, both Tg and non-Tg rabbits showed elevated plasma levels of TC, but Tg rabbits exhibited less response to a cholesterol diet because their plasma TC levels reached to a half value of those of non-Tg rabbits while HDL-C remained at lower levels **(Fig. 1A)**. These results prompted us to investigate whether increased hepatic expression of EL affects VLDL catabolism in the liver in addition to hydrolyzing HDL-PL. To test this hypothesis, we compared the VLDL clearance capacity of Tg rabbits with non-Tg rabbits after injecting DiI-labeled β -VLDLs isolated from cholesterol-fed non-Tg rabbits. As shown in **Fig. 1B**, DiI-β-VLDLs were cleared faster in Tg rabbits than those in non-Tg rabbits after injection at 60 min. The fractional catabolic rate was measured but did not reach a significant difference (1.73 ± 0.08 in non-Tg vs 1.80 ± 0.09 pools/hour in Tg). We envisioned that increased EL expression led to enhancement of apoB-containing particle catabolism in the liver, thereby reducing atherogenic lipoproteins in the plasma.

To examine whether EL has any direct effect on the development of diet-induced atherosclerosis inde-

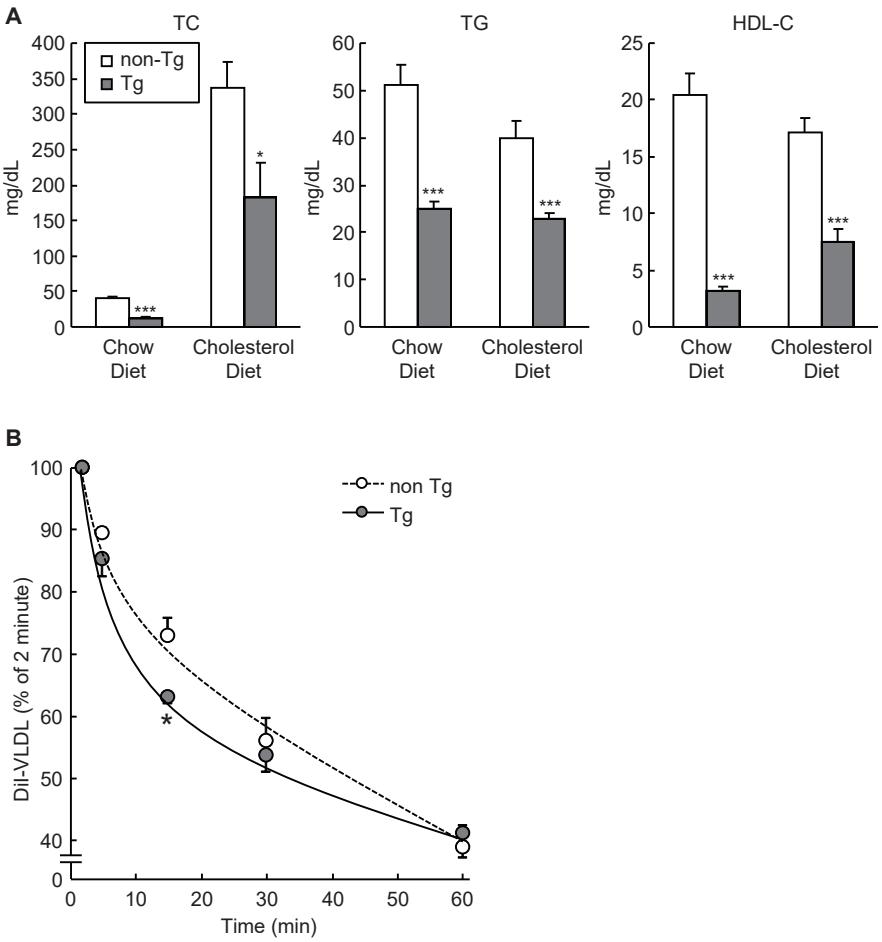


Fig. 1. Plasma lipids and β-VLDL clearance

(A) Plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) levels of transgenic (Tg) and non-Tg rabbits fed a normal standard and cholesterol-rich diet for 1 week. Data are expressed as mean ± SEM (n=8–12). $^{*}P<0.05$, $^{***}P<0.001$ vs non-Tg rabbits by t-test. (B) β-very-low-density lipoprotein (β-VLDL) clearance. Tg and non-Tg rabbits were intravenously injected with fluorescent-labeled β-VLDLs (DiI-β-VLDL). Blood samples were withdrawn at 2, 5, 15, 30, and 60 min after DiI-β-VLDL administration. Plasma fluorescent intensity was measured by a fluorescent microplate reader. Data are normalized to 2 min fluorescent intensity levels at 100%. Data are expressed as mean ± SEM (n=4). $^{*}P<0.05$ vs non-Tg rabbits by t-test.

pendent upon its lipid-lowering functions, we fed Tg rabbits with an adjusted cholesterol diet to match their plasma TC levels with those of non-Tg rabbits. After this manipulation, average plasma levels of TC and HDL-C of both Tg and non-Tg rabbits became close after 2–3 weeks **(Fig. 2)**. This similarly "high" cholesterol levels were maintained within a range of 600–1,200 mg/dL until 16 weeks **(Fig. 2)**. Plasma levels of TC remained at the same degree at 16 weeks because the area under the curve (AUC) of TC levels was almost identical between Tg and non-Tg rabbits (AUC 13,676 ± 774 in Tg vs. 12,593 ± 1,414 in non-Tg, $P>0.05$). In addition, plasma TG, PL, and HDL-PL levels were similar between Tg and non-Tg rabbits measured at 8 and 16 weeks **(Supplementary Table 1)**.

Analysis of lipoproteins revealed that on a normal standard diet, Tg rabbits exhibited a marked decrease of TC, TG, and PL levels in all lipoproteins (VLDL, IDL, and HDL_{2-3}) except LDLs (d=1.06 g/mL) **(Fig. 3A)**. In contrast, under similarly "high" cholesterol levels, plasma lipoprotein profiles of Tg rabbits were essentially comparable to those of non-Tg rabbits **(Fig. 3B)**. Both groups showed a marked increase of apoB-containing lipoproteins, including β-VLDL, IDL, and large LDL. TC, TG, and PL contents in these fractions were all increased compared with when fed a normal diet **(Fig. 3)**. Cholesterol diet

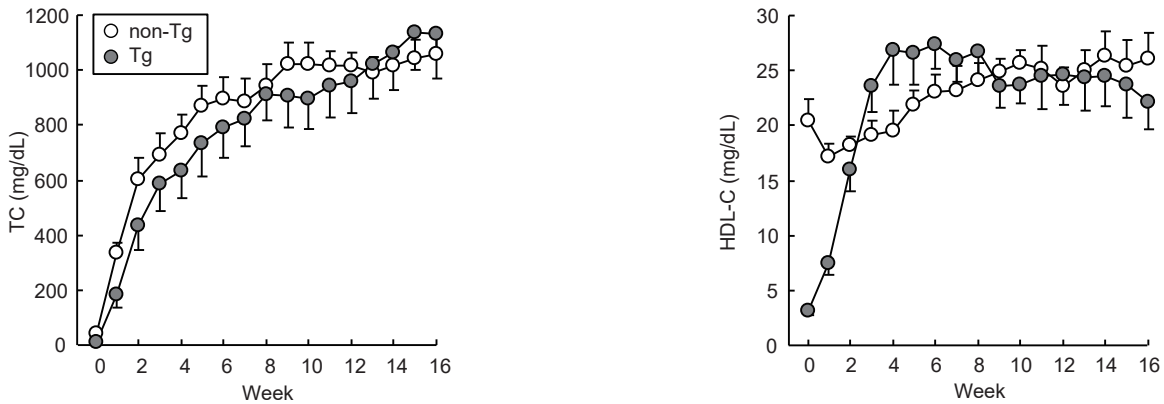


Fig. 2. Plasma total cholesterol and HDL-C levels of rabbits after feeding a cholesterol diet for 16 weeks

Plasma total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels of transgenic (Tg) and non-Tg rabbits fed with an adjusted cholesterol-rich diet for 16 weeks. Data are expressed as mean ± SEM (n=8–12). $P>0.05$ vs non-Tg rabbits by two-way repeated measure ANOVA.

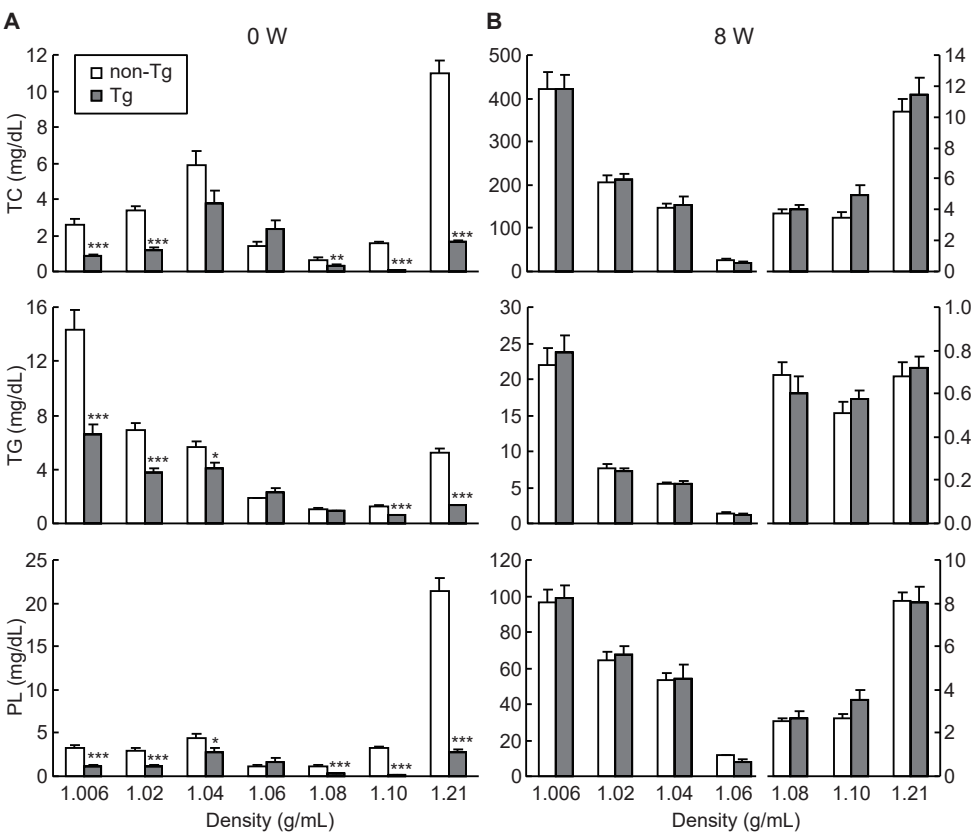


Fig. 3. Plasma lipoprotein profiles of rabbits on a chow diet and a cholesterol diet

Plasma lipoproteins were separated by sequential gradient ultracentrifugation from plasma of rabbits on a normal (A) and an adjusted cholesterol diet for 8 weeks (B). Total cholesterol (TC), triglycerides (TG), and phospholipids (PL) in lipoproteins were measured. Data are expressed as mean ± SEM (n=6). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs non-Tg rabbits by t-test.

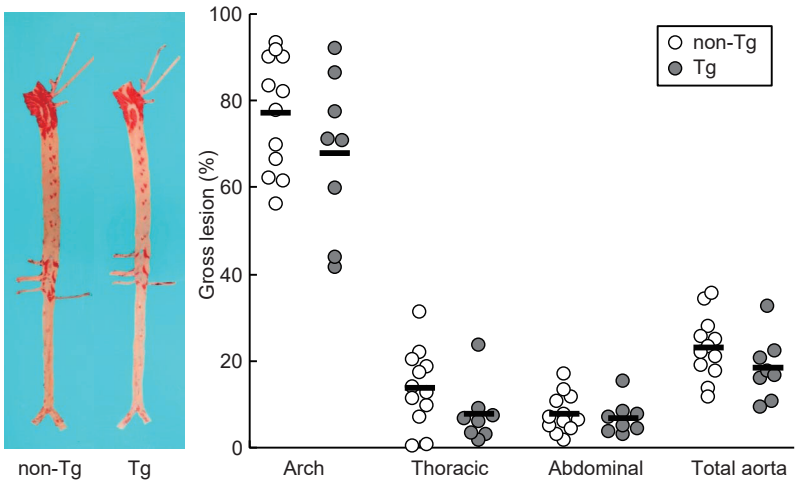


Fig. 4. Analysis of gross aortic lesions

Transgenic (Tg) and non-Tg rabbits were fed an adjusted cholesterol diet for 16 weeks, and then the aortic lesions were quantified. Representative pictures of aortas stained with Sudan IV are shown (left). The lesion area, defined by the sudanophilic area, was quantified (right). Each dot represents the lesion area of an individual animal ($n=8$–12). $P>0.05$ vs non-Tg rabbits by t-test.

feeding led to the elevation of HDL-C in Tg rabbits: 22-fold increase in HDL_2 (d=1.08–1.10 g/mL) and 7-fold increase in HDL_3 (d=1.21 g/mL) over normal diet fed Tg rabbits. Eventually, HDL-C levels along with TG and PL in Tg rabbits became similar to those in non-Tg rabbits after cholesterol diet feeding **(Fig. 3 and Supplementary Fig. 2A)**. We also evaluated apolipoprotein distribution in each lipoprotein fraction using SDS-PAGE stained with Coomassie brilliant blue and found that apoB, apoE, and apoA-I contents in these lipoproteins were similar between two groups **(Supplementary Fig. 2B)**. These results indicate that having "high" hypercholesterolemia saturated the capacity of EL-mediated apoB-containing particle clearance.

Comparison of Aortic and Coronary Atherosclerosis

Because cholesterol diet feeding can completely abolish the lipid-lowering effect of EL, we were able to investigate whether EL can affect atherosclerosis without considering plasma lipid levels. Analysis of gross aortic lesion areas defined by Sudan IV staining revealed that there was no significant difference between Tg and non-Tg rabbits, although average aortic lesion areas of Tg rabbits were 21% less than those of non-Tg rabbits (n.s.) **(Fig. 4)**. Histological examinations showed that the aortic lesions of both Tg and non-Tg rabbits were mainly composed of infiltrating macrophages with relatively less smooth muscle cells **(Fig. 5)**. The intimal microscopic lesion sizes along with macrophages and smooth muscle cells were not significantly different between Tg and non-Tg rabbits, suggesting that increased EL did not affect lesion size including its cellular components. We also analyzed coronary atherosclerosis but did not find any difference between Tg and non-Tg rabbits **(Fig. 6)**.

Discussion

EL has been postulated to be involved in HDL metabolism through its phospholipase hydrolysis activity, yet its roles in apoB-containing lipoprotein metabolism and atherosclerosis have not been fully defined. Our previous study showed that on a 0.3% cholesterol diet for 16 weeks, Tg rabbits exhibited lower plasma levels of TC (48% decrease of AUC over non-Tg) and subsequently had 55% reduction of aortic atherosclerosis compared with non-Tg rabbits[8]. Although several mechanisms may be involved in EL-mediated anti-atherogenic functions, it is likely that EL may attenuate diet-induced hypercholesterolemia through enhanced hepatic catabolism of apoB-containing lipoproteins. In support of this notion, the current study showed that DiI-labeled β-VLDLs were cleared faster in the circulation of Tg rabbits than of non-Tg rabbits. This result is also consistent with the finding by Broedl *et al.* who showed that EL is capable of hydrolyzing VLDL and LDL lipids *ex vivo* and promotes ^{125}I-LDL clearance in mice[13]. Apparently, EL exerts its functions on apoB-containing particles mainly dependent upon its catalytic activity[13]. In spite of this, it is necessary to generate Tg rabbits expressing an inactive EL transgene in the future. In the current study, we examined the β-VLDL clearance

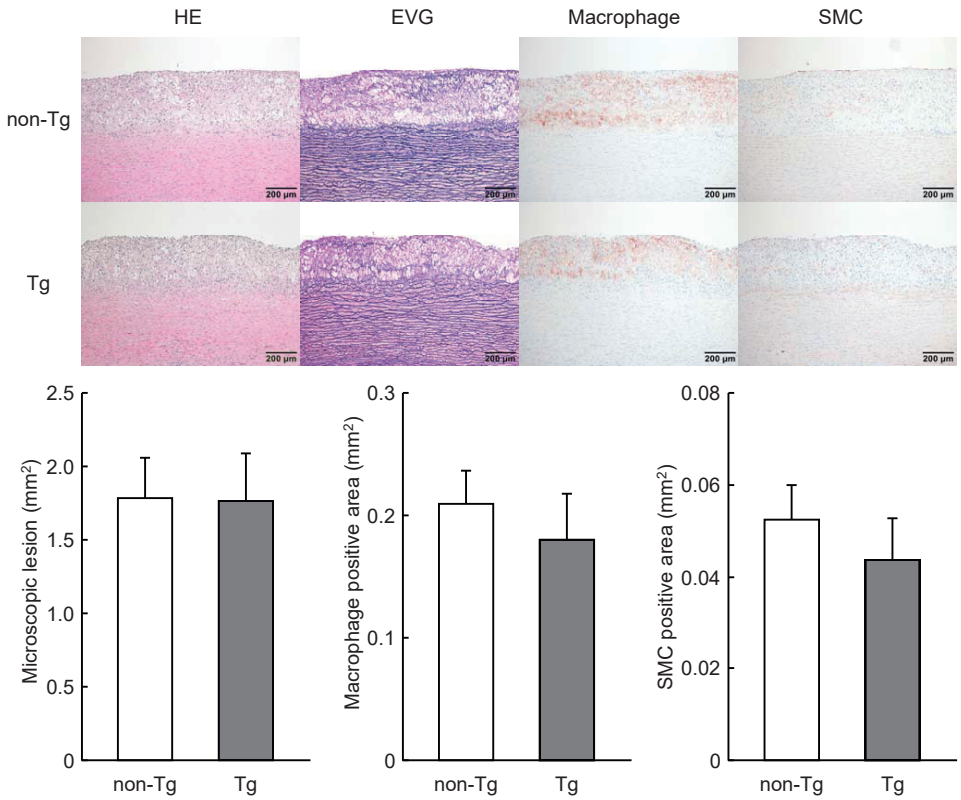


Fig. 5. Microscopic analysis of aortic atherosclerosis

Representative micrographs of the aortic arch lesions from transgenic (Tg) and non-Tg rabbits are shown (top). Serial paraffin sections of the aortic arch were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies against either macrophages or smooth muscle α-actin for smooth muscle cells (SMC). Intimal lesions on EVG stained sections and positively stained areas of macrophage and SMC were quantified (bottom). Data are expressed as mean ± SEM (n=8–12). $P>0.05$ vs non-Tg rabbits by t-test.

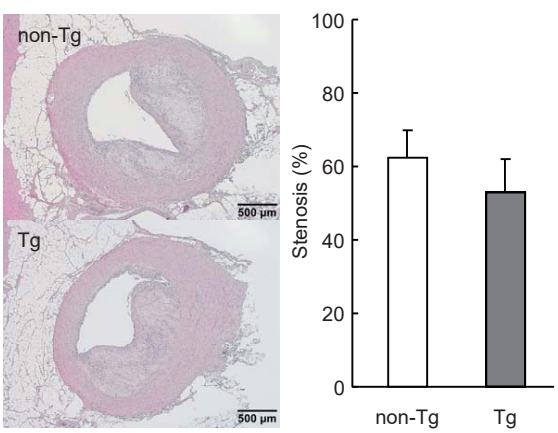


Fig. 6. Analysis of coronary atherosclerosis

Representative micrographs of the coronary lesions stained with hematoxylin-eosin from transgenic (Tg) and non-Tg rabbits are shown (left). The lesion size, expressed as stenosis % of coronary arteries, was quantified (right). Data are expressed as mean ± SEM (n=7–11). $P>0.05$ vs non-Tg rabbits by t-test.

in normal diet fed Tg and non-Tg rabbits in which hepatic LDL receptors along with other receptors are functional; however, it is currently unknown whether LDL receptor function is essentially required for EL-mediated clearance of β-VLDLs in the liver. To address this issue, we need to crossbreed Tg rabbits with WHHL rabbits in the future.

Since it is not known whether anti-atherogenic effect of EL is totally dependent upon its lipid-lowering effects, we conducted experiments using Tg rabbits fed with an adjusted cholesterol-rich diet to make them to similarly develop hypercholesterolemia with non-Tg rabbits. In this situation, plasma lipids, lipoprotein profiles, and apoB were almost identical between the two groups as EL lipid-lowering effect was totally overwhelmed by extra cholesterol feeding in Tg rabbits and it is possible to elucidate whether EL affects the development of aortic and coronary atherosclerosis without considering plasma atherogenic lipoproteins levels[29, 30].

Analysis of both aortic and coronary atherosclerosis showed that there were no differences in the lesion size and lesional cellular components between Tg and non-Tg rabbits, suggesting that increased EL expression cannot inhibit the development of atherosclerosis under the similar hypercholesterolemia. In other words, EL does not directly influence arterial wall cells during atherogenesis. This result further strengthened the contention that anti-atherogenic functions of EL are totally attributed to its lipid-lowering effect, namely, enhanced hepatic catabolism of apoB-containing particles[2, 13]. In this aspect, EL exhibits significant phospholipase activity with less triglyceride lipase activity, which is different from other lipases such as LPL because LPL mainly hydrolyzes TG in VLDLs and chylomicrons. Our previous studies have shown that overexpression of LPL reduced plasma lipids and atherosclerosis[31]. However under similar hypercholesterolemia, LPL, resembling hepatic lipase, enhances the conversion of VLDL/IDL particles to small LDLs, which are more atherogenic[22]. Unlike LPL, EL does not confer a prominent function on the conversion of VLDLs/IDLs to LDLs. In addition, EL overexpression did not interfere with LPL activity because there was no difference in LPL activity between Tg and non-Tg rabbits **(Supplementary Fig. 3)**. Takiguchi *et al.* recently reported that hepatic EL overexpression in mice maintained reverse cholesterol efflux despite of hypoalphalipoproteinemia[32]. In the current study, HDL-C levels were also similar between Tg and non-Tg rabbits, but it is unlikely that reverse cholesterol efflux mediated by EL plays any role in atherosclerosis under similar hypercholesterolemia.

In summary, increased EL expression enhances β-VLDL catabolism, thereby inhibiting cholesterol diet-induced hypercholesterolemia and atherosclerosis in Tg rabbits. Abolishment of EL lipid-lowering function eliminates anti-atherogenic effects of EL. We concluded that overexpression of EL in the liver protects against diet-induced atherosclerosis via enhanced β-VLDL clearance.

Acknowledgements

This work was supported in part by JSPS KAKENHI (JP17K08783, JP15H04718), the National Natural Science Foundation of China (No.8194100101, 81770457 and 81570392), the JSPS-CAS Bilateral Joint Research Program (JPJSBP120187204), and NIH grants (R01HL117491 and R01HL129778).

Conflict of Interest

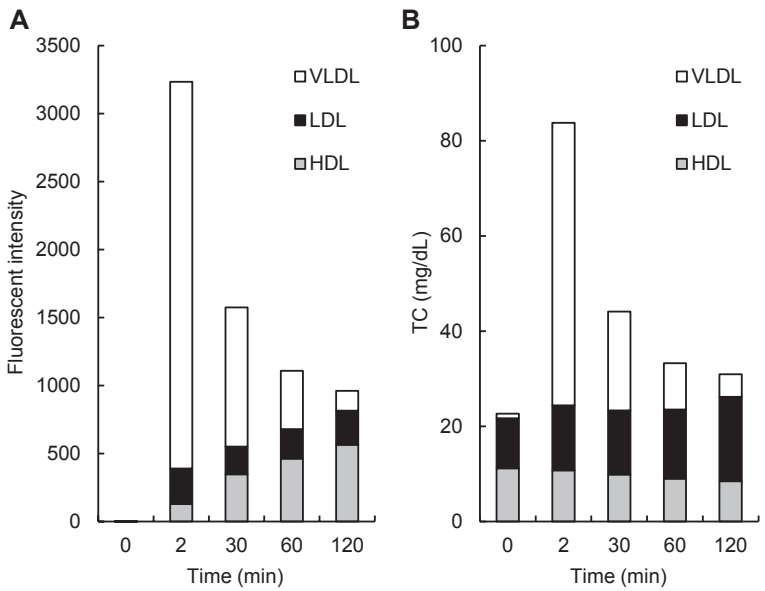
The authors declare that they have no conflict of interest.

References

1) Rader DJ, Jaye M: Endothelial lipase: a new member of the triglyceride lipase gene family. Curr Opin Lipidol, 2000; 11: 141-147
2) Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, South V, Amin D, Perrone M, Rader DJ: A novel endothelial-derived lipase that modulates HDL metabolism. Nat Genet, 1999; 21: 424-428
3) Hirata K, Dichek HL, Cioffi JA, Choi SY, Leeper NJ, Quintana L, Kronmal GS, Cooper AD, Quertermous T: Cloning of a unique lipase from endothelial cells extends the lipase gene family. J Biol Chem, 1999; 274: 14170-14175
4) Zhang J, Yu Y, Nakamura K, Koike T, Waqar AB, Zhang X, Liu E, Nishijima K, Kitajima S, Shiomi M, Qi Z, Yu J, Graham MJ, Crooke RM, Ishida T, Hirata K, Hurt-Camejo E, Chen YE, Fan J: Endothelial Lipase Mediates HDL Levels in Normal and Hyperlipidemic Rabbits. J Atheroscler Thromb, 2012; 19: 213-226
5) Yu KC-W, David C, Kadambi S, Stahl A, Hirata K-I, Ishida T, Quertermous T, Cooper AD, Choi SY: Endothelial lipase is synthesized by hepatic and aorta endothelial cells and its expression is altered in apoE-deficient mice. J Lipid Res, 2004; 45: 1614-1623
6) McCoy MG, Sun G-S, Marchadier D, Maugeais C, Glick JM, Rader DJ: Characterization of the lipolytic activity of endothelial lipase. J Lipid Res, 2002; 43: 921-929
7) Ishida T, Choi S, Kundu RK, Hirata K-I, Rubin EM, Cooper AD, Quertermous T: Endothelial lipase is a major determinant of HDL level. J Clin Invest, 2003; 111: 347-355
8) Wang C, Nishijima K, Kitajima S, Niimi M, Yan H, Chen Y, Ning B, Matsuhisa F, Liu E, Zhang J, Chen YE, Fan J: Increased Hepatic Expression of Endothelial Lipase Inhibits Cholesterol Diet-Induced Hypercholesterolemia

and Atherosclerosis in Transgenic Rabbits. Arterioscler Thromb Vasc Biol, 2017; 37: 1282-1289
9) Ma K, Cilingiroglu M, Otvos JD, Ballantyne CM, Marian AJ, Chan L: Endothelial lipase is a major genetic determinant for high-density lipoprotein concentration, structure, and metabolism. Proc Natl Acad Sci U S A, 2003; 100: 2748-2753
10) Jin W, Millar JS, Broedl U, Glick JM, Rader DJ: Inhibition of endothelial lipase causes increased HDL cholesterol levels in vivo. J Clin Invest, 2003; 111: 357-362
11) Ishida T, Miyashita K, Shimizu M, Kinoshita N, Mori K, Sun L, Yasuda T, Imamura S, Nakajima K, Stanhope KL, Havel PJ, Hirata K: ELISA system for human endothelial lipase. Clin Chem, 2012; 58: 1656-1664
12) Sun L, Ishida T, Miyashita K, Kinoshita N, Mori K, Yasuda T, Toh R, Nakajima K, Imamura S, Hirata K: Plasma activity of endothelial lipase impacts high-density lipoprotein metabolism and coronary risk factors in humans. J Atheroscler Thromb, 2014; 21: 313-321
13) Broedl UC, Maugeais C, Millar JS, Jin W, Moore RE, Fuki I V, Marchadier D, Glick JM, Rader DJ: Endothelial lipase promotes the catabolism of ApoB-containing lipoproteins. Circ Res, 2004; 94: 1554-1561
14) Strauss JG, Zimmermann R, Hrzenjak A, Zhou Y, Kratky D, Levak-Frank S, Kostner GM, Zechner R, Frank S: Endothelial cell-derived lipase mediates uptake and binding of high-density lipoprotein (HDL) particles and the selective uptake of HDL-associated cholesterol esters independent of its enzymic activity. Biochem J, 2002; 368: 69-79
15) Fuki I V, Blanchard N, Jin W, Marchadier DHL, Millar JS, Glick JM, Rader DJ: Endogenously produced endothelial lipase enhances binding and cellular processing of plasma lipoproteins via heparan sulfate proteoglycan-mediated pathway. J Biol Chem, 2003; 278: 34331-34338
16) Broedl UC, Maugeais C, Marchadier D, Glick JM, Rader DJ: Effects of nonlipolytic ligand function of endothelial lipase on high density lipoprotein metabolism in vivo. J Biol Chem, 2003; 278: 40688-40693
17) Degoma EM, Rader DJ: Novel HDL-directed pharmacotherapeutic strategies. Nat Rev Cardiol, 2011; 8: 266-277
18) Ishida T, Choi SY, Kundu RK, Spin J, Yamashita T, Hirata K, Kojima Y, Yokoyama M, Cooper AD, Quertermous T: Endothelial lipase modulates susceptibility to atherosclerosis in apolipoprotein-E-deficient mice. J Biol Chem, 2004; 279: 45085-45092
19) Ko KWS, Paul A, Ma K, Li L, Chan L: Endothelial lipase modulates HDL but has no effect on atherosclerosis development in apoE-/- and LDLR-/- mice. J Lipid Res, 2005; 46: 2586-2594
20) Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Hólm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JF, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart AFR, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, Stoll M, Berger K, Martinelli N, Girelli D, McKeown PP, Patterson CC, Epstein SE, Devaney J, Burnett M-S, Mooser V, Ripatti S, Surakka I, Nieminen MS, Sinisalo J, Lokki M-L, Perola M, Havulinna A, de Faire U, Gigante B, Ingelsson E, Zeller T, Wild P, de Bakker PIW, Klungel OH, Maitland-van der Zee A-H, Peters BJM, de Boer A, Grobbee DE, Kamphuisen PW, Deneer VHM, Elbers CC, Onland-Moret NC, Hofker MH, Wijmenga C, Verschuren WMM, Boer JMA, van der Schouw YT, Rasheed A, Frossard P, Demissie S, Willer C, Do R, Ordovas JM, Abecasis GR, Boehnke M, Mohlke KL, Daly MJ, Guiducci C, Burtt NP, Surti A, Gonzalez E, Purcell S, Gabriel S, Marrugat J, Peden J, Erdmann J, Diemert P, Willenborg C, König IR, Fischer M, Hengstenberg C, Ziegler A, Buysschaert I, Lambrechts D, Van de Werf F, Fox KA, El Mokhtari NE, Rubin D, et al.: Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet (London, England), 2012; 380: 572-580
21) Yu X, Lu J, Li J, Guan W, Deng S, Deng Q, Ye H, Han W, Yu Y, Zhang R: Serum Triglyceride Lipase Concentrations are Independent Risk Factors for Coronary Artery Disease and In-Stent Restenosis. J Atheroscler Thromb, 2019; 26: 762-774
22) Ichikawa T, Kitajima S, Liang J, Koike T, Wang X, Sun H, Okazaki M, Morimoto M, Shikama H, Watanabe T, Yamada N, Fan J: Overexpression of lipoprotein lipase in transgenic rabbits leads to increased small dense LDL in plasma and promotes atherosclerosis. Lab Invest, 2004; 84: 715-726
23) Niimi M, Yang D, Kitajima S, Ning B, Wang C, Li S, Liu E, Zhang J, Chen YE, Fan J: ApoE knockout rabbits: A novel model for the study of human hyperlipidemia. Atherosclerosis, 2016; 245: 187-193
24) Nakajima K, Machida T, Imamura S, Kawase D, Miyashita K, Fukamachi I, Maeda M, Muraba Y, Koga T, Kobayashi J, Kimura T, Nakajima K, Murakami M: An automated method for measuring lipoprotein lipase and hepatic triglyceride lipase activities in post-heparin plasma. Clin Chim Acta, 2018; 487: 54-59
25) Kimura N, Kikumori A, Kawase D, Okano M, Fukamachi K, Ishida T, Nakajima K, Shiomi M: Species differences in lipoprotein lipase and hepatic lipase activities: comparative studies of animal models of lifestyle-related diseases. Exp Anim, 2019; 68: 267-275
26) Zhang J, Niimi M, Yang D, Liang J, Xu J, Kimura T, Mathew A V, Guo Y, Fan Y, Zhu T, Song J, Ackermann R, Koike Y, Schwendeman A, Lai L, Pennathur S, Garcia-Barrio M, Fan J, Chen YE: Deficiency of Cholesteryl Ester Transfer Protein Protects Against Atherosclerosis in RabbitsHighlights. Arterioscler Thromb Vasc Biol, 2017; 37: 1068-1075
27) Pitas RE, Innerarity TL, Weinstein JN, Mahley RW: Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. Arteriosclerosis, 1981; 1: 177-185
28) Shimizu T, Miura S, Tanigawa H, Kuwano T, Zhang B, Uehara Y, Saku K: Rosuvastatin activates ATP-binding cassette transporter A1-dependent efflux ex vivo and promotes reverse cholesterol transport in macrophage cells in mice fed a high-fat diet. Arterioscler Thromb Vasc Biol, 2014; 34: 2246-2253
29) Mahley RW, Innerarity TL, Brown MS, Ho YK, Goldstein JL: Cholesteryl ester synthesis in macrophages: stim-

ulation by beta-very low density lipoproteins from cholesterol-fed animals of several species. J Lipid Res, 1980; 21: 970-980
30) Holvoet P, Collen D: beta-VLDL hypercholesterolemia relative to LDL hypercholesterolemia is associated with higher levels of oxidized lipoproteins and a more rapid progression of coronary atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol, 1997; 17: 2376-2382
31) Fan J, Unoki H, Kojima N, Sun H, Shimoyamada H, Deng H, Okazaki M, Shikama H, Yamada N, Watanabe T: Overexpression of Lipoprotein Lipase in Transgenic Rabbits Inhibits Diet-induced Hypercholesterolemia and Atherosclerosis. J Biol Chem, 2001; 276: 40071-40079
32) Takiguchi S, Ayaori M, Yakushiji E, Nishida T, Nakaya K, Sasaki M, Iizuka M, Uto-Kondo H, Terao Y, Yogo M, Komatsu T, Ogura M, Ikewaki K: Hepatic Overexpression of Endothelial Lipase Lowers High-Density Lipoprotein but Maintains Reverse Cholesterol Transport in Mice. Arterioscler Thromb Vasc Biol, 2018; 38: 1454-1467



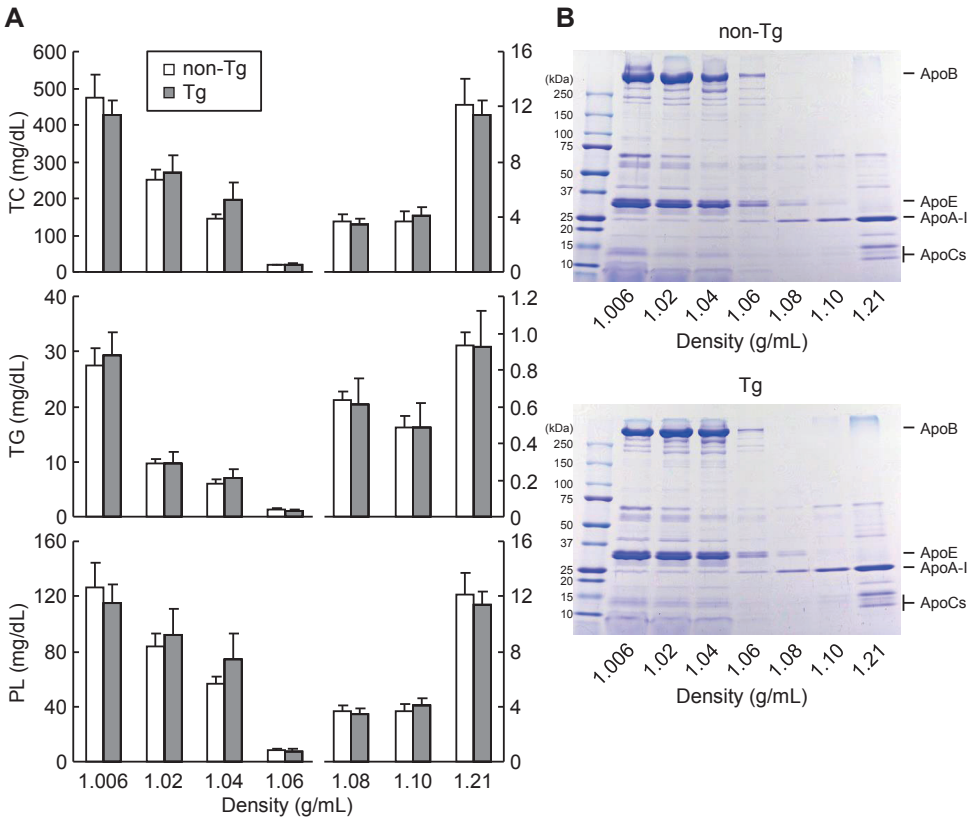
Supplementary Fig. 1. Validation of DiI distribution in lipoproteins

WT rabbits fed a normal diet were intravenously injected with fluorescent labeled β-VLDLs (DiI-β-VLDL). Blood samples were withdrawn at 0, 2, 30, 60 and 120 min after DiI-β-VLDL administration. Plasma lipoproteins were isolated using sequential ultracentrifugation. Density fractions contained following lipoproteins: VLDL (d<1.006 g/mL), LDL (d=1.006~1.063 g/ml), and HDL (d=1.063~1.21 g/ml). (A) Fluorescent intensity and (B) total cholesterol (TC) were measured. Two rabbits were evaluated for the validation of DiI-β-VLDL stability and representative data are shown.

Supplementary Table 1. Plasma TG, PL, and HDL-PL levels

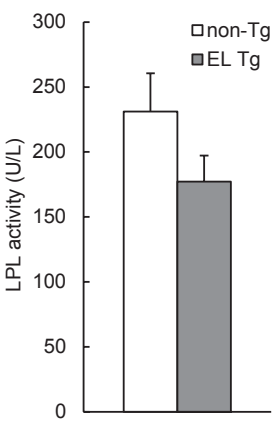
		non-Tg	Tg
TG (mg/dL)	0 W	51±4	25±2***
	8 W	51±4	46±3
	16 W	55±5	65±14
PL (mg/dL)	0 W	79±3	29±3***
	8 W	306±22	292±29
	16 W	363±27	413±64
HDL-PL (mg/dL)	0 W	58.7±2.4	20.5±1.7***
	8 W	40.9±1.6	40.3±1.5
	16 W	42.5±1.6	41.3±1.6

TG, triglycerides; PL, phospholipids; HDL-PL, high-density lipoprotein phospholipids. Data are expressed as mean±SEM (n=8~12). ***P<0.001 vs non-Tg rabbits by t-test.



Supplementary Fig. 2. Plasma lipoprotein and apolipoprotein analysis of rabbits on a cholesterol diet for 16 week

Plasma lipoproteins were separated by sequential gradient ultracentrifugation. (A) Total cholesterol (TC), triglycerides (TG) and phospholipids (PL) in the lipoprotein fractions were measured. Data are expressed as mean ± SEM ($n=6$). $P>0.05$ vs non-Tg rabbits by t-test. (B) Apolipoprotein distribution in the lipoprotein fractions were analyzed by 4-20% SDS-PAGE stained with Coomassie Brilliant Blue. Representative gel images from each group are shown.



Supplementary Fig. 3. Analysis of lipoprotein lipase activity

Post-heparinized plasma from non-Tg and Tg fed a cholesterol diet for 16 weeks were collected and lipoprotein lipase (LPL) activity was measured using a commercial colorimetric assay kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). Data are expressed as mean ± SEM ($n=8$-12).