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Increased Hepatic Expression of Endothelial Lipase Inhibits Cholesterol Diet-induced Hypercholesterolemia and Atherosclerosis in Transgenic Rabbits

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Abstract

Objective—Endothelial lipase (EL) is a key determinant in plasma HDL-C. However, functional roles of EL on the development of atherosclerosis have not been clarified. We investigated whether hepatic expression of EL affects plasma lipoprotein metabolism and cholesterol diet-induced atherosclerosis.

Approach and Results—We generated transgenic (Tg) rabbits expressing the human EL gene in the liver and then examined the effects of EL expression on plasma lipids and lipoproteins and compared the susceptibility of Tg rabbits to cholesterol diet-induced atherosclerosis with non-Tg littermates. On a chow diet, hepatic expression of human EL in Tg rabbits led to remarkable reductions in plasma levels of total cholesterol, phospholipids, and HDL-cholesterol compared with non-Tg controls. On a cholesterol-rich diet for 16 weeks, Tg rabbits exhibited significantly lower hypercholesterolemia and less atherosclerosis than non-Tg littermates. In Tg rabbits, gross lesion area of aortic atherosclerosis was reduced by 52%, and the lesions were characterized by fewer macrophages and smooth muscle cells compared with non-Tg littermates.

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*The first 2 authors contributed equally to this study.

Disclosure

None

Conclusions—Increased hepatic expression of EL attenuates cholesterol diet-induced hypercholesterolemia and protects against atherosclerosis.

Keywords

Endothelial lipase; High density lipoprotein; Atherosclerosis; Transgenic rabbits; Hypercholesterolemia

Introduction

Endothelial lipase (EL) (gene nomenclature, LIPG) was discovered by two independent groups in 1999[1, 2]. Although EL gene was initially cloned from endothelial cells, EL was also expressed in many other organs, such as liver, lung, thyroid, and kidney[3, 4]. Along with lipoprotein lipase and hepatic lipase, EL belongs to the triglyceride lipase family[5]. In contrast to lipoprotein lipase and hepatic lipase, EL exhibits high phospholipase activity but low triglyceride lipase activity[6]. Ample evidence has revealed that EL plays an important role in HDL metabolism. This phenomenon was found in both experimental animals and humans. Plasma HDL-C levels were increased in EL KO mice or in mice injected with EL antibody but reduced in EL-overexpressing transgenic (Tg) mice[7–9]. Clinical studies showed that plasma EL mass or activity was inversely associated with plasma HDL-C[10]. Loss-of-function EL gene variants are associated with increased plasma HDL-C[11]. Furthermore, increased plasma EL levels are associated with high risk of human cardiovascular disease[12], metabolic syndrome, obesity, and inflammation[13–15]. In addition to HDL metabolism, EL seems to participate in apoB-containing particle metabolism[16, 17]. Expression of human EL promotes the catabolism of apoB-containing lipoproteins in apoE-deficient, LDL receptor-deficient and human apoB transgenic mice[16]. Because EL is a key determinant in HDL metabolism, targeting EL has emerged as a novel strategy for the treatment and prevention of atherosclerosis through increasing plasma HDL-C levels[18–20]. Although this contention is very attractive and intriguing, it has not been clarified whether EL is definitely pro-atherogenic. The relationship between high plasma EL and increased cardiovascular risk cannot explain whether EL plays a causal role in atherosclerosis. On the other hand, conflicting results have been reported for the EL knock-out (KO) mice in terms of EL functions in the pathogenesis of atherosclerosis. In one report, EL inactivation protected against diet-induced atherosclerosis in apoE KO mice[21], but in another, there was no effect on atherosclerosis in both apoE and LDL receptor KO mice[22]. Because it is still unclear whether EL can affect the development of atherosclerosis, it remains unanswered whether targeting EL will be beneficial to cardiovascular disease patients. EL has multiple functions in lipoprotein metabolism in humans[23, 24]. Given the known differences in lipoprotein metabolism between mice and humans, it is necessary to study EL pathophysiological functions using animals that have lipoprotein metabolism features similar to humans. To this end, we generated Tg rabbits expressing human EL in the liver. The rationale of using rabbits for this undertaking was that rabbits have been widely used for the study of human lipid metabolism and atherosclerosis[25]. Like humans, but unlike mice, rabbits have abundant plasma cholesteryl ester transfer protein activity, an important regulator of cholesterol metabolism, and they are sensitive to a cholesterol diet and develop atherosclerosis rapidly because of hepatic apoB100 and intestinal apoB48 synthesis[25]. Our studies revealed that

hepatic expression of human EL protects against cholesterol diet-induced atherosclerosis in Tg rabbits.

Materials and Methods

Materials and methods are available in the online-only Data Supplement.

Results

Generation of hEL Tg rabbits

In a total, we implanted 770 microinjected-embryos into 29 surrogates and obtained 18 pups. Among them, two pups were found to have the human EL (hEL) transgene by Southern blotting. Tg founder (E18) showed detectable hEL expression (see below) and bred to generate F1 progeny for the current study. Tg rabbits showed no apparent abnormalities in terms of body weight and other organs. Transgenic hEL mRNA was expressed in the liver of Tg rabbits confirmed by real-time RT-PCR **(data not shown)**. Western blotting analysis revealed that hEL proteins were detected in both pre- and post-heparin plasma along with the liver. EL proteins in pre- and post-heparin plasma were ~40 kDa in size, similar to the N-terminal fragment of EL[26], whereas in the liver, there were two bands of EL proteins presumably representing full-length (~68 kDa) EL and N-terminal EL (~40 kDa) (Fig. 1A). The latter may be those of cleaved dimeric forms of EL[26].

Using hEL-specific ELISA kits, we further measured plasma levels of hEL and found that in pre-heparin plasma, hEL levels in Tg rabbits were 987.7 ± 355.7 pg/mL in male Tg and 1670.3 ± 469.4 pg/mL in female Tg rabbits (Fig. 1B), which is similar to normal human plasma levels of EL mass[27]. In human pre-heparin plasma, EL mass ranged up to 1387.7 pg/ml; therefore, transgenic hEL levels in Tg rabbits are equivalent to the upper portion of human EL levels. After heparin injection, plasma levels of hEL were increased by 1.5-fold in male and 1.4-fold in female Tg rabbits, suggesting that ~60% of EL proteins are in circulation, whereas the rest of them (~40%) are assumedly associated with heparan sulfate proteoglycans on the cellular surface[28], as shown in the human plasma[27].

Effects of hEL expression on plasma lipids and lipoproteins

Analysis of plasma lipids revealed that expression of hEL led to a marked reduction of plasma lipids (Fig. 2). Plasma levels of TC were reduced by 67% in males and 47% in females, PL levels were reduced by 61% in males and 52% in females, HDL-C levels were reduced by 88% in males and 70% in females, and HDL-PL levels were reduced by 66% in males and 57% in females. Plasma TG levels were also reduced but were only statistically significant in males (36% decrease, $p<0.01$) compared with non-Tg rabbits.

Analysis of plasma lipoproteins by agarose gel electrophoresis revealed that both α-migrating (HDLs) and β-migrating lipoproteins (VLDLs and LDLs) in Tg rabbits were decreased compared with non-Tg rabbits (Fig. 3A). This change was more prominent in male Tg than that in female Tg rabbits. Western blotting analysis of the whole plasma showed that plasma apoE and apoAI contents were concomitantly reduced in Tg rabbits but apoB contents were unchanged compared with non-Tg rabbits (Fig. 3B). As shown in the

HPLC assay, HDL peaks surpassed VLDL/LDL peaks in non-Tg rabbits; however, in Tg rabbits, the heights of these two peaks were reversed, even though both became shorter (Fig. 3C). Analysis of lipoprotein fractions isolated by sequential gradient ultracentrifugation revealed that there was a marked reduction of HDL_2 (d=1.06~1.10 g/mL) and HDL_3 (d=1.10~1.21 g/mL) accompanied by decreased contents of apoAI and apoE in these fractions in Tg rabbits (Fig. 3D). ApoB-containing particles (VLDL and LDL) were not prominently changed in Tg rabbits. Reduction of apoAI and apoE contents in HDLs was also shown by SDS-PAGE using the same density fractions (Fig. 3E). Furthermore, $HDL_{2–3}$ fractions of Tg rabbits showed a marked reduction of both TC and TG contents in both male and female Tg rabbits compared with each counterpart non-Tg rabbit (Supplemental Fig. II).

Cholesterol-rich diet experiments

To investigate the response of Tg rabbits to a cholesterol-rich diet, Tg and non-Tg littermates were fed a cholesterol-rich diet for 16 weeks. Compared with those of non-Tg rabbits, Tg rabbits showed constantly and significantly “lower” hypercholesterolemia: lower TC and lower HDL-C levels than non-Tg rabbits throughout the experiment period (Fig. 4). TG levels of Tg rabbits were slightly (but not statistically significant) lower during the experiment period (Fig. 4). Analysis of lipoprotein profiles by agarose gel electrophoresis revealed that β-migrating lipoproteins (β-VLDLs and remnant lipoproteins) were remarkably reduced in Tg rabbits (Fig. 4, bottom panel).

We further analyzed lipoprotein fractions isolated from cholesterol-fed rabbits. There were two striking changes in the lipoproteins of cholesterol-fed Tg rabbits compared with non-Tg rabbits. First, there was a remarkable reduction of apoB-containing particles, including VLDLs, intermediate density lipoproteins (IDLs), and LDLs associated with reduced contents of apoB and apoE in Tg rabbits. Second, similar to the Tg rabbits on the chow diet, there was a prominent reduction of $HDL_{2–3}$ in which apoAI and apoE were also decreased (Fig. 5A-B). Quantitation of TC and TG in these fractions showed that all lipoproteins were reduced in Tg rabbits compared with non-Tg rabbits (Supplemental Fig. III).

Quantification of aortic and coronary atherosclerosis

Analysis of *en face* aortic lesion areas revealed that the whole aortic atherosclerotic lesions of Tg rabbits were significantly reduced by 52%, with a 42% reduction in the aortic arch, a 62% reduction in the thoracic aorta, and a 54% reduction in the abdominal aorta compared with non-Tg rabbits (Fig. 6). Histological examinations showed that the aortic lesions of both Tg and non-Tg rabbits were mainly composed of infiltrating macrophages and smooth muscle cells intermingled with extracellular matrix. The microscopic lesion size of the aortic arch was markedly deceased in Tg rabbits due to reduced numbers of both macrophages (53% decrease) and smooth muscle cells (63% decrease) compared with non-Tg rabbits (Fig. 7). Analysis of coronary atherosclerosis revealed that Tg rabbits had smaller lesions in both left and right coronary arteries (33% decrease in left and 42% decrease in right coronary arteries, $p>0.05$) than non-Tg rabbits (Supplemental Fig. IV).

Discussion

In the current study, we generated Tg rabbits expressing human EL in the liver and characterized the effects of overexpression of EL on plasma lipoproteins and cholesterol diet-induced atherosclerosis. Consistent with the previous studies[7, 15], hepatic expression of EL in Tg rabbits on a chow diet led to a remarkable reduction of plasma TC, PL, HDL-C, and HDL-PL, suggesting that EL indeed plays an important role in maintaining the HDL homeostasis. It should be noted that in Tg rabbits, about 60% of the EL proteins were present in pre-heparin plasma associated with lipoproteins, with rest of them bound to the luminal surface of endothelial surface heparan sulfate proteoglycans (HSPG) because they are releasable to the circulation by heparin injection. The presence of free EL immunoreactive proteins in the circulation has also been reported in WHHL rabbits[3] and humans[27], and measurement of the pre-heparin plasma EL activity showed that high EL activity is associated with high risk of coronary heart disease[10]. Besides its phospholipase activity, EL possesses a non-catalytic function[29] as lipoprotein lipase and hepatic lipase, which may facilitate binding of plasma lipoproteins to the HSPG, leading to enhancement of lipoprotein uptake and degradation in the arterial intima[28]. Taken together and based on our observations above, we initially postulated that hEL Tg rabbits should be extremely susceptible to cholesterol diet-induced atherosclerosis.

To our surprise, cholesterol-fed hEL Tg rabbits developed lower hypercholesterolemia and less aortic and coronary atherosclerosis than did non-Tg rabbits, suggesting that increased expression of EL is not pro-atherogenic but rather anti-atherogenic. Several mechanisms may be operative for EL anti-atherogenic effects. First, Tg rabbits had lower plasma TC levels with a remarkable reduction in apoB-containing lipoproteins in addition to low HDLs. When rabbits were fed with a cholesterol-rich diet, they develop hypercholesterolemia due to the elevation of hepatically and intestinally derived cholesteryl ester-rich remnant lipoproteins, called β-VLDLs[30]. It is these β-VLDLs that are atherogenic in cholesterol-fed rabbits. Even though plasma HDL levels (anti-atherogenic lipoproteins) were concomitantly lower in Tg rabbits, the net effects of EL overexpression were atheroprotective owing to lowering plasma β-VLDLs. Therefore, EL anti-atherogenic effects are basically dependent upon plasma β-VLDL or apoB levels. It has been reported that deficiency of EL led to increased small LDL levels in hepatic lipase KO mice whereas expression of EL in mouse models with elevated apoB-containing particles markedly reduces VLDL and LDL levels and accelerates the turnover rates of LDLs[16, 17]. However, whether increased hepatic EL expression in Tg rabbits enhances the clearance of apo-B containing particles awaits for the vigorous lipoprotein catabolism study in future. In addition, it is necessary to elucidate whether EL exerts such a function through either EL catalytic or non-catalytic mechanism. To examine this issue, we attempted to compare plasma triglyceride lipase and phospholipase activity of Tg rabbits with non-Tg rabbits. We found that although the majority (~ 60%) of hEL proteins exists in the pre-heparin plasma of Tg rabbits, their triglyceride lipase and phospholipase A1 activity was not significantly increased compared with non-Tg rabbits (Supplemental Fig. V). Because the current method for measuring phospholipase A1 activity was not specific for EL as other lipases such as hepatic lipase also exhibit phospholipase activity, it is still immature to conclude which EL (catalytic vs. non-

catalytic plays a major role in mediating lipoprotein metabolism in Tg rabbits including enhancement of hepatic uptake and clearance of apoB-containing particles. It remains unclear whether EL expressed by extrahepatic organs exhibits the same anti-atherogenic effects as hepatically expressed EL shown in this study.

A noteworthy finding in this study was that increased EL in Tg rabbits did not affect the particular cell types in the lesions because both macrophages and smooth muscle cells were similarly reduced in number compared with non-Tg rabbits. This strengthened the above notion that EL anti-atherogenic functions are virtually through lowering plasma atherogenic lipoproteins rather than mediating arterial wall macrophage infiltration or smooth muscle cell proliferation. Nevertheless, the current study using Tg rabbits along with our previous EL knockdown study[3] strongly suggests that therapeutic inhibition of EL expression may not be an appropriate strategy for the treatment of atherosclerosis. In our previous study, we investigated the effect of EL antisense oligonucleotides on HDL metabolism and atherosclerosis in both wild-type rabbits and WHHL rabbits[3]. Injection with rabbit EL antisense oligonucleotides (40 mg/kg) for 6 weeks resulted in 50% reduction of hepatic expression of EL but did not lead to a significant change in plasma total cholesterol and HDL-C levels. Although there was an increase of large-sized (>12 nm) phospholipid-rich HDL particles compared with mismatched oligonucleotide control, such a mild change in HDL particle components failed to affect the aortic lesion size in WHHL rabbits.

In conclusion, our results support the contention that EL functions in the metabolism of both HDL and apoB-containing lipoproteins, thereby playing a key role in plasma cholesterol homeostasis. Overexpression of EL in the liver protected against cholesterol-induced hypercholesterolemia and atherosclerosis. It remains to be verified whether inhibition of EL serves as a therapeutic target for the treatment of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations and Acronyms

Apo	Apolipoprotein
EL	Endothelial lipase
EVG	Elastica van Gieson

HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
H&E	Hematoxylin–eosin
LDL	Low-density lipoprotein
PL	Phospholipids
SDS-PAGE	SDS–polyacrylamide gel
Tg	Transgenic
TC	Total cholesterol
TG	Triglycerides
VLDL	Very low-density lipoprotein

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Highlights

- Increased hepatic expression of endothelial lipase in transgenic rabbits decreases plasma triglyceride and HDL-cholesterol levels.
- Endothelial lipase inhibits cholesterol-diet induced hypercholesterolemia in transgenic rabbits.
- Endothelial lipase protects against cholesterol-induced aortic and coronary atherosclerosis in transgenic rabbits.

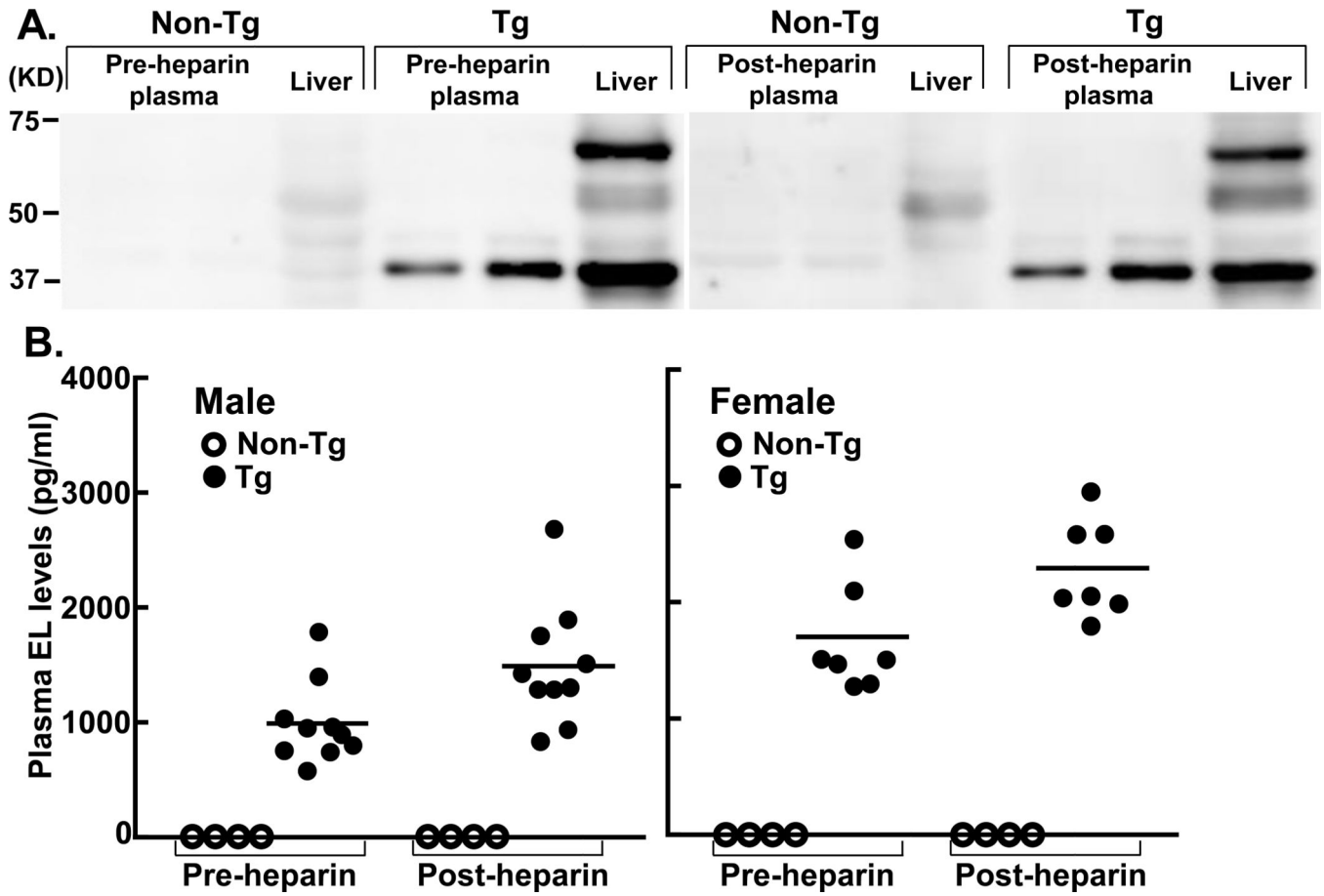


Figure 1.

Analysis of hEL in pre- and post-heparin plasma using Western blotting (A) and ELISA (B). Plasma fractions and liver proteins were fractionated by 10% SDS-PAGE under reducing conditions, followed by immunoblotting with hEL monoclonal Ab. Plasma concentrations of hEL protein in pre-heparin and post-heparin plasma were measured using ELISA kits.

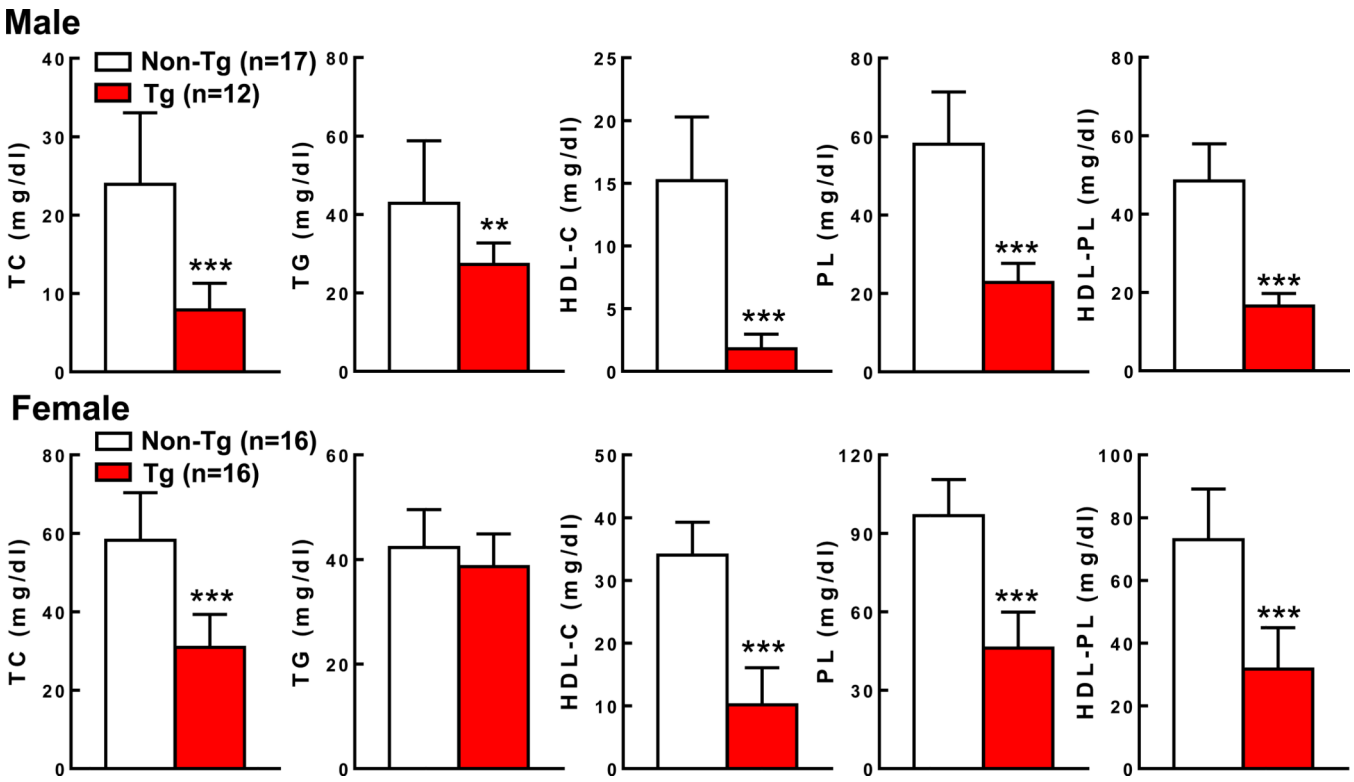
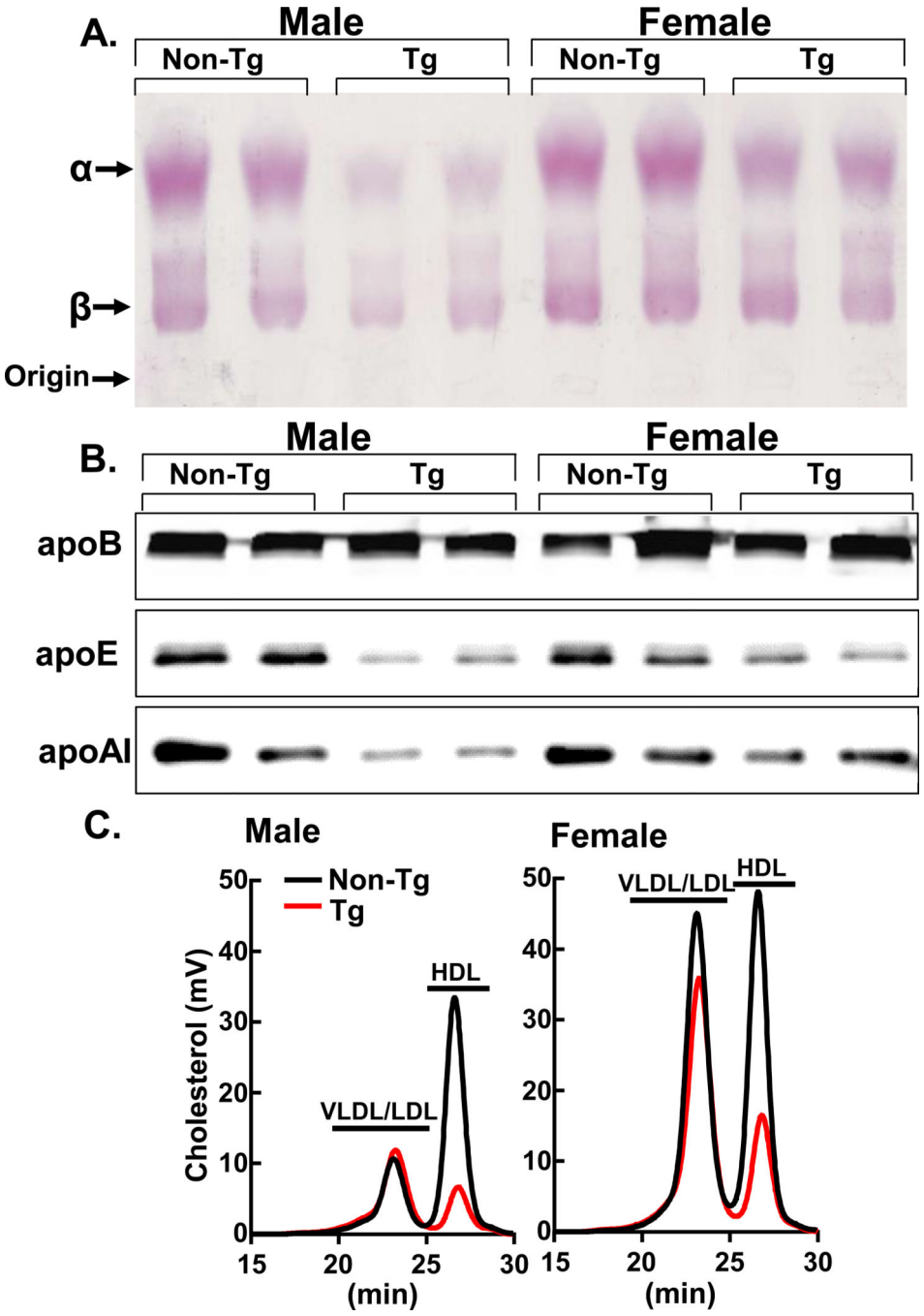


Figure 2.

Plasma lipids of hEL Tg and non-Tg rabbits. Plasma lipids were analyzed from fasting EDTA plasma of both male (top) and female rabbits (bottom). Data are expressed as mean ± SD. **p<0.01, ***p<0.001 vs. non-Tg rabbits.



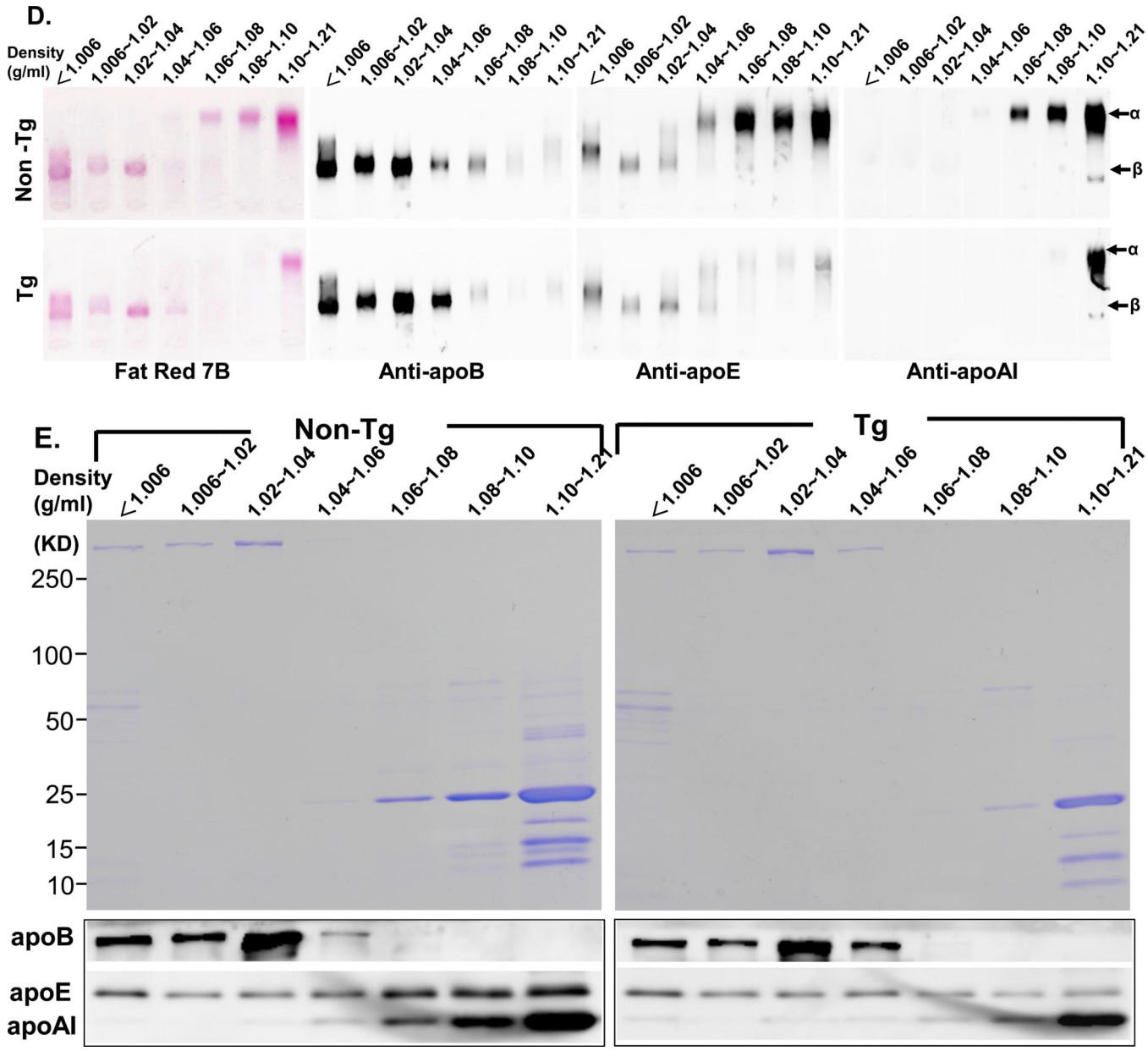


Figure 3.

Plasma lipoproteins and apolipoproteins.

Plasma (4 μL) was electrophoresed on a 1% agarose gel and stained for neutral lipids with Fat Red 7B (A). Plasma lipoprotein profiles were analyzed by HPLC (B). Plasma (0.5 μL) was resolved by 4~20% SDS-PAGE, followed by immunoblotting with apoB, apoE, and apoAI Abs (C). Plasma lipoproteins were separated by sequential gradient ultracentrifugation. An equal volume (8 μL) of each fraction was resolved by electrophoresis in a 1% agarose gel. Lipoproteins were visualized using Fat Red 7B staining, and apolipoproteins were identified by immunoblotting with apoB, apoE, and apoAI Abs (D). An equal volume of each fraction (5 μL) was resolved by electrophoresis by 4~20% SDS-

PAGE. Apolipoproteins were visualized using either CBB staining or immunoblotting with apoB, apoE, and apoAI Abs (E).

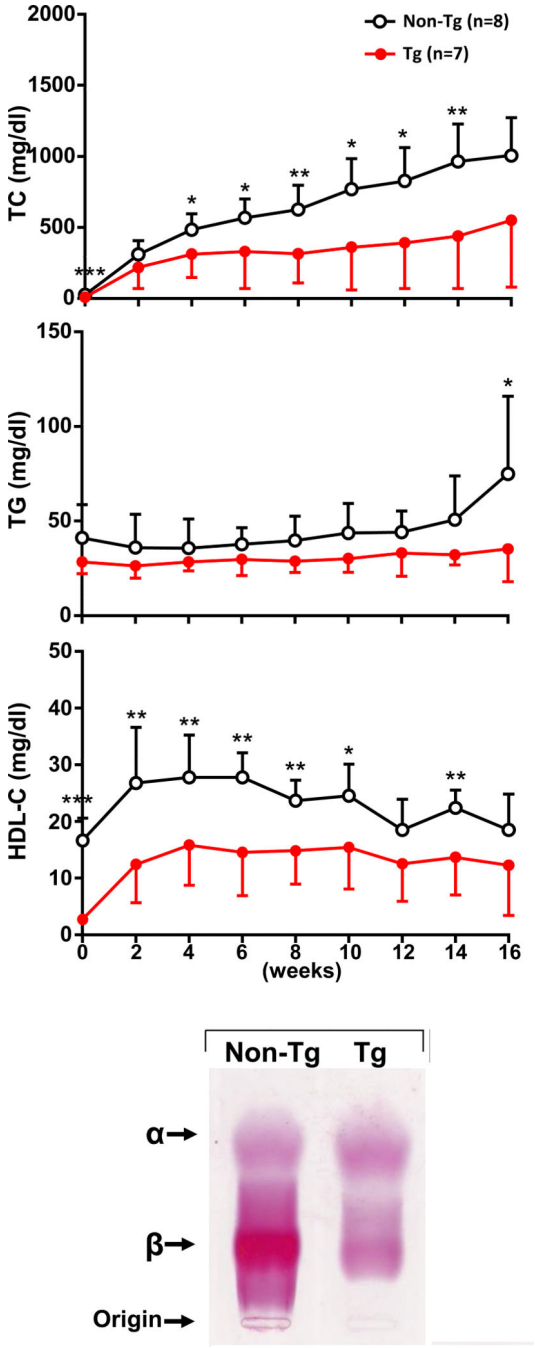


Figure 4.

Plasma TC, TG, and HDL-C levels of Tg and non-Tg rabbits fed a cholesterol-rich diet. All rabbits were male at the age of 4~5 months. Data are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 vs. non-Tg rabbits. Plasma (4 μL) was electrophoresed on a 1% agarose gel and stained for neutral lipids with Fat Red 7B (bottom panel).

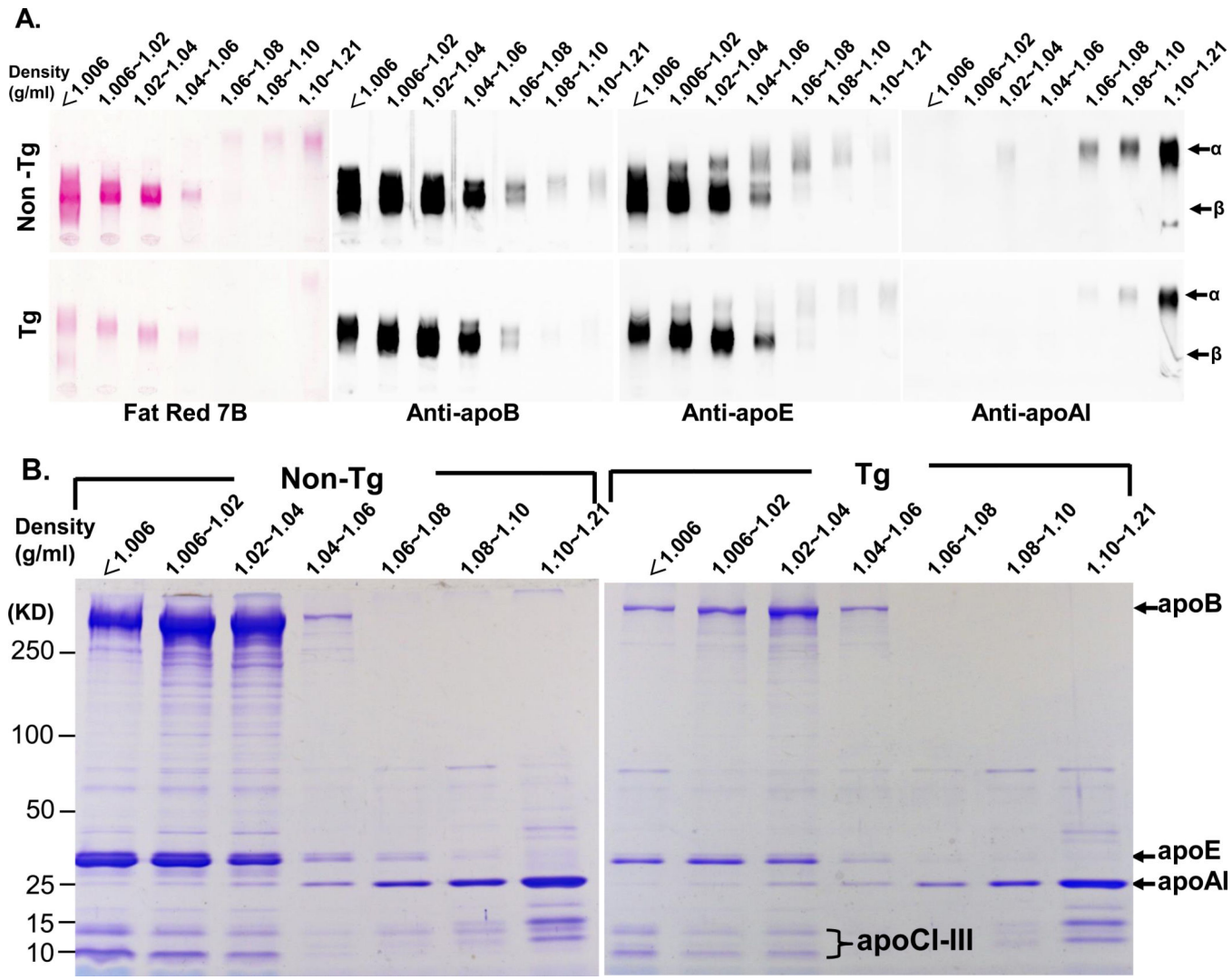


Figure 5.

Analysis of plasma lipoproteins isolated from rabbits at 15 weeks after cholesterol diet feeding. An equal volume (2 μL) of each fraction was resolved by electrophoresis in a 1% agarose gel. Lipoproteins were visualized using Fat Red 7B staining, and apolipoproteins were identified by immunoblotting with apoB, apoE, and apoAI Abs (A). An equal volume of each fraction (5 μL) was resolved by electrophoresis by 4~20% SDS-PAGE. Apolipoproteins were visualized using CBB staining (B). *Size of apoCI-III (between 10~15 KD) shown on SDS-PAGE is larger than those predicted possibly due to glycosylation.

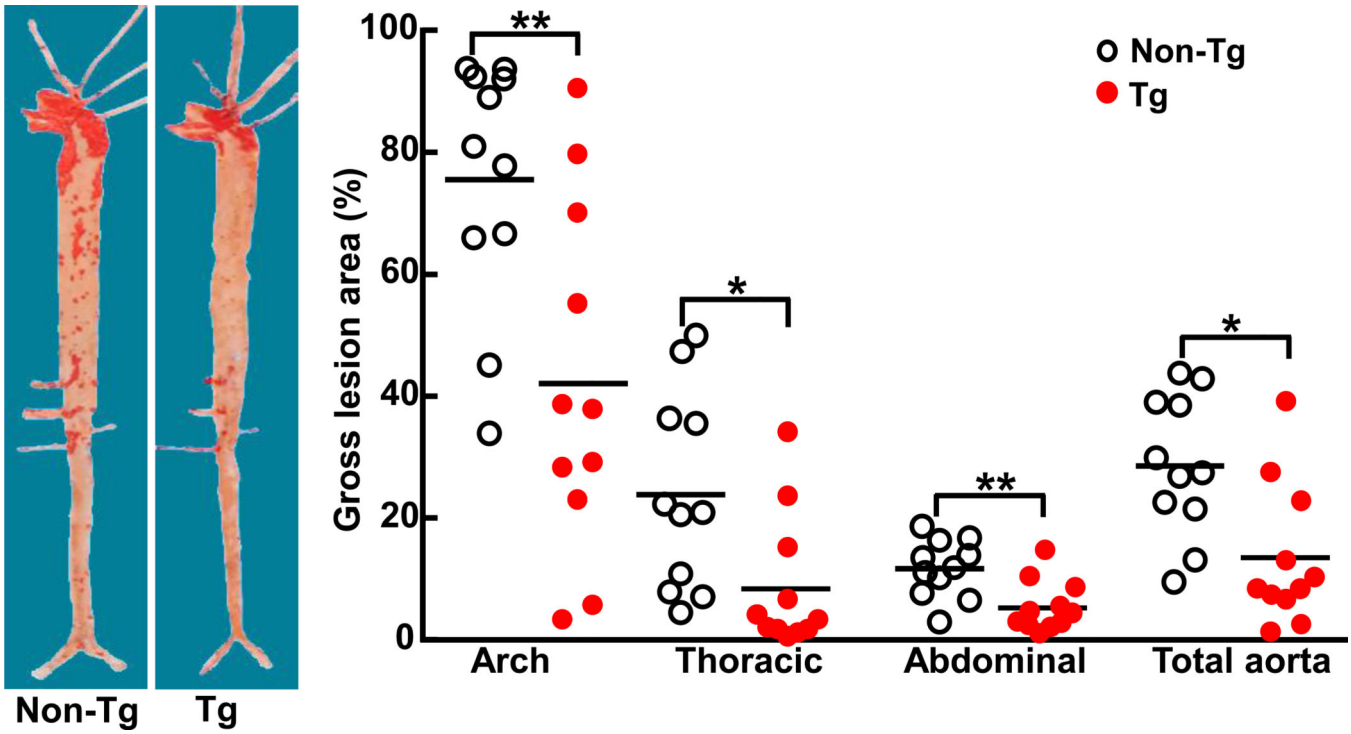


Figure 6.

Analysis of atherosclerotic lesions of aorta. Male Tg and non-Tg rabbits were fed a cholesterol diet for 16 weeks and then the aortic lesions were quantified. Representative pictures of aortas stained with Sudan IV are shown on the left. The lesion area (defined by the sudanophilic area) was quantified using an image analysis system. Each dot represents the lesion area of an individual animal. *p<0.05, **p<0.01 vs. non-Tg rabbits.

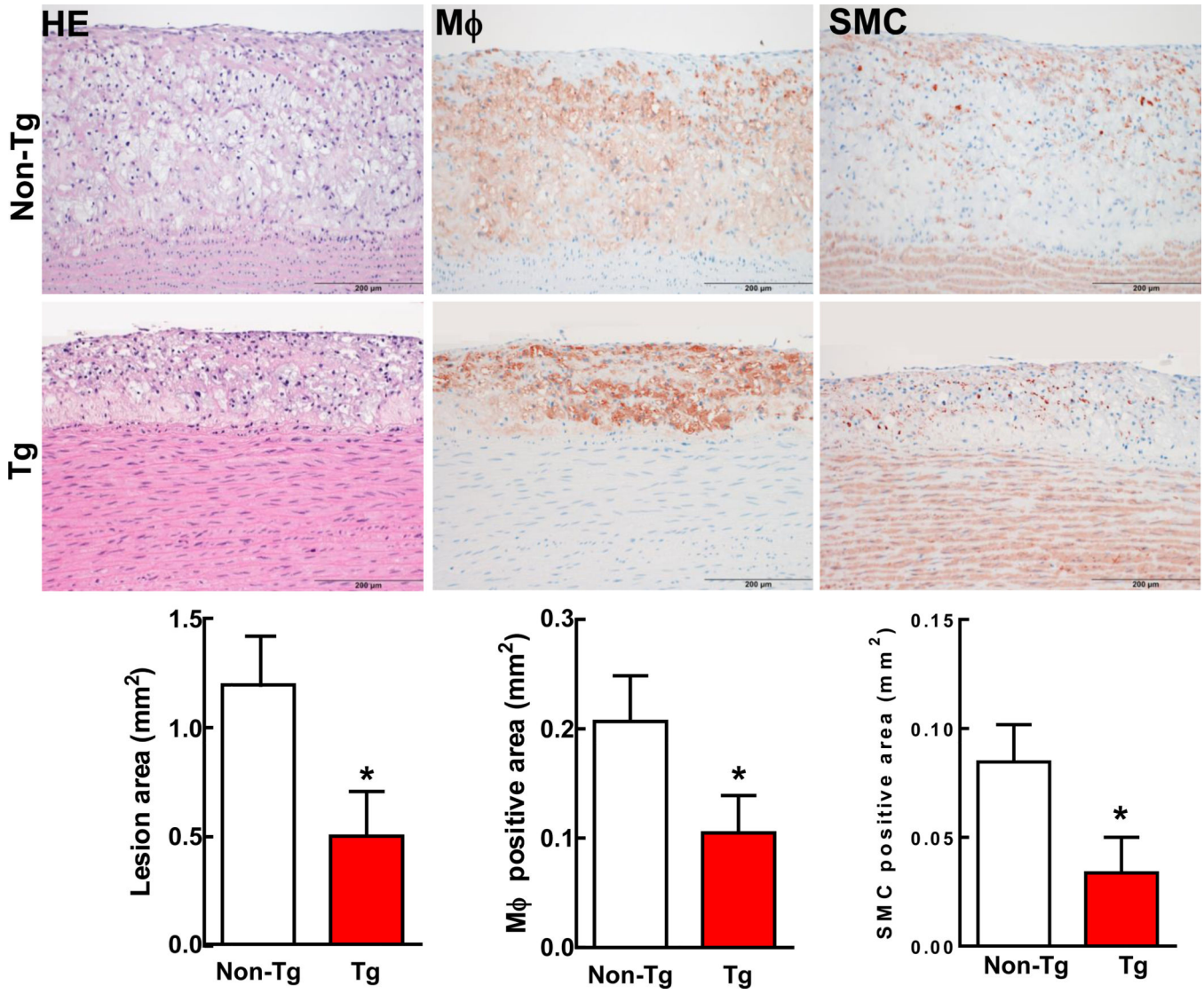


Figure 7.

Representative micrographs of the aortic arch lesions from male Non-Tg and Tg rabbits. Serial paraffin sections of the aortic arch were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies (mAbs) against either macrophages (Mφ) or α-smooth muscle actin for smooth muscle cells (SMC). Intimal lesions on EVG-stained sections and positively stained areas of Mφ and SMC were quantified with an image analysis system. (n=11 for Tg and n=12 for non-Tg). *p<0.05 vs. non-Tg rabbits.