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Enteric lactoferrin attenuates the development of high-fat and high-cholesterol diet-induced hypercholesterolemia and atherosclerosis in Microminipigs

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Previously, we found that enteric lactoferrin (eLF) could reduce the visceral fat accumulation known to associate strongly with metabolic syndrome symptoms and consequently with an increased risk of atherosclerosis. In this study, the atherosclerosis-preventive potential of LF was assessed in a high-fat and high-cholesterol diet (HFCD)-induced hypercholesterolemia and atherosclerosis model using Microminipig™. Eight-week orally administered eLF remarkably reduced the HFCD-induced serum total and low-density lipoprotein cholesterol levels but not high-density lipoprotein cholesterol levels. A histological analysis of 15 arteries revealed that eLF systemically inhibited the development of atherosclerotic lesions. Pathway analysis using identified genes that characterized eLF administration in liver revealed significant changes in the steroid biosynthesis pathway (ssc00100) and all affected genes in this pathway were upregulated, suggesting that cholesterol synthesis inhibited by HFCD was recovered by eLF. In summary, eLF could potentially prevent the hypercholesterolemia and atherosclerosis through protecting homeostasis from HFCD-induced dysfunction of cholesterol metabolism.

Key words: lactoferrin; microminipig; atherosclerosis; LDL cholesterol; DNA microarray

Atherosclerosis is a characteristic risk factor of cardiovascular disease and is closely related to metabolic syndrome (MetS) symptoms such as high blood sugar, hyperlipidemia, and hypertension.[1,2)] Atherosclerosis is characterized by the accumulation of lipids within macrophages, leading to inflammation and the formation of atheroma plaques. A link between this process and oxidative modifications for circulating low-density lipoproteins (LDL) has been recognized, and often no symptoms are apparent until the arterial damage has become sufficiently severe to restrict blood flow. These silent disease characteristics increase the risk of atherosclerosis progression; therefore, it is important to establish therapeutic and preventive measures.

Lactoferrin (LF), a member of the transferrin family, is a well-known multi-functional glycoprotein with antibacterial, antiviral, immunostimulatory, antioxidant, and cancer-preventive potential.[3–7)] As a natural component of breast milk, LF is considered safe. Therefore, it has been categorized as "generally recognized as safe" in the USA and has been approved as a food additive in Japan.

We found that enteric LF (eLF) improved lipid metabolism disorders. An orally administered enteric-coated bovine LF tablet (300 mg/day as LF) significantly reduced visceral fat accumulation, a phenomenon that has been well established as the main cause of MetS in a double-blind clinical trial.[8)] From the results of *in vitro* experiments conducted in preadipocytes derived from rat mesenteric fat, the antiadipogenic and lipolytic effects of LF were proposed as possible effector mechanisms.[9,10)] Moreno-Navarrete et al. and Yagi et al. also demonstrated the antiadipogenic activity of LF in the MC3T3-G2/PA6 and 3T3-L1 cell line, respectively.[11,12)] In further investigations, we found that pepsin-degraded LF did not exhibit the antiadipogenic and lipolytic activities, suggesting that the enteric formulation was necessary for efficacy against lipid metabolism disorders.[9,10)]

We further evaluated the effect of LF on hypercholesterolemia in a preliminary animal study and found that administering LF via the drinking water inhibited the progression of high-cholesterol diet-induced

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hypercholesterolemia in mice (unpublished data). Takeuchi et al. also reported that LF supplementation yielded significant reductions in the serum total cholesterol and triglyceride (TG) levels in mice fed a normal diet.[13] These results suggest that LF may possess inhibitory activity against hypercholesterolemia-induced atherosclerosis. However, a previous report found that mice are generally resistant to high-cholesterol diet-induced atherosclerosis and that cholesterol metabolism differs considerably between mice and humans.[14] Therefore, experimental human atherogenesis studies will require an appropriate animal model that mimics human physiology and pathology because the pathogenesis of this disease includes both genetic and environmental factors.

Swine represent a potentially useful animal model because the anatomy, physiology, and feeding and sleep habits are very similar to those of humans.[15] Recently, it is reported the development of a hypercholesterolemia-induced atherosclerosis model involving the smallest available miniature pigs, "Microminipigs™" (MMPigs), which differ from experimental miniature pig strains such as Clawn and Göttingen.[16–18] A different study found that the expression of genes regulating cholesterol metabolism in liver such as low-density lipoprotein receptor (*LDLR*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), and sterol regulatory element-binding protein 2 (*SREBP-2*) were correlated and markedly downregulated following the consumption of high-fat and high-cholesterol diet (HFCD).[19] The regulation of these genes and the LDL cholesterol-rich lipid profile in MMPigs are therefore very similar to those observed in humans. In this study, we investigated the effect of eLF on HFCD-induced hypercholesterolemia and atherosclerosis in MMPigs to clarify the antiatherogenic potential of eLF.

Materials and methods

Materials. Commercially available bovine LF was purchased from FrieslandCampina DMV (LE, Amersfoort, Netherlands). According to the certificate of analysis, typical protein purity is 98%.

Animals and diet. Sixteen-week-old male MMPigs were maintained in a special facility with the following environmental conditions: room temperature, 24 ± 3 °C; relative humidity, 50% ± 20%; and light/dark cycle, 12-h each. Tap water was available *ad libitum*. The animals were fed 3% of its own body weight in a special diet once a day in the morning and administered 4 enteric capsules (E-capsule, major axis: 1.6 cm, minor axis: 0.6 cm; Sanseiiyaku, Shizuoka, Japan) filled with or without LF after the meal. Nine MMPigs were divided into 3 groups (3 animals per group). One group (Control group; $n = 3$) was fed a normal chow diet (Kodakara 73; Marubeni Nisshin Feed, Tokyo, Japan) and administered empty enteric capsules. A second group was fed a HFCD and administered empty enteric capsules (HFCD group). The third group was fed a HFCD and administered capsules filled with LF (500 mg LF/body/day, 125 mg LF/capsule; HFCD + eLF group). According to the product specification, normal chow diet was composed of less than 9.0% carbohydrate (w/w), greater than 15.0% protein (w/w), greater than 2.0% fat (w/w), and less than 9.0% fiber (w/w). HFCD comprised 6% lard (w/w) (Miyoshi Oil & Fat, Tokyo, Japan) and 0.5% cholesterol (w/w) (Wako Pure Chemical Industries, Osaka, Japan) combined with a normal chow diet. Body weight was measured every week. After 8 weeks, all MMPigs were anesthetized and sacrificed via bilateral auxillary artery exsanguination. All protocols were approved by the Ethics Committee of Animal Care and Experimentation, Kagoshima University (A09001), and all research was performed according to the institutional guidelines for animal experiments and in compliance with the Japanese law concerning the protection and control of animals (Law No. 105 and Notification No. 6). This study was also performed in accordance with the animal welfare bylaws of Shin Nippon Biomedical Laboratories Ltd., a facility with full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International and approval from the International Animal Care and Use Committee.

Biochemical analysis. Blood samples were collected every 2 weeks for lipid profiling. The total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, and TG levels were analyzed with an automated agarose gel electrophoresis apparatus (Epalyzer 2; Helena Laboratories, Saitama, Japan).

Extraction of hepatic lipids. Lipids were extracted from a portion of the liver according to the Folch method. Concentrated lipids were reconstituted in 2-propanol, and the TG and total cholesterol levels were analyzed using triglyceride E-Test Wako and total cholesterol E-Test Wako, respectively (Wako Pure Chemical Industries, Osaka, Japan).

Pathological examination. During the necropsy, the aorta, arteries, liver, spleen, heart, kidney, and omental fat were removed from each animal. The liver, spleen, heart, kidney, and omental fat were weighed. Each aorta and artery was cut into about 3 mm sections, which were fixed in 10% phosphate-buffered formalin and routinely processed as 4 μm thick paraffin-embedded tissue sections followed by staining with hematoxylin and eosin. The degree of atherosclerosis development in each animal was classified according to the Stary Type Classification.[20] Immunostaining for atherosclerotic lesions was performed on the paraffin-embedded sections using primary antibodies and Envision kit (Dako Cytomation, Kyoto, Japan). The primary antibodies and concentrations were as follows: anti-ionized calcium-binding adaptor molecule-1 (Iba-1) polyclonal antibody (1:250; Wako Pure Chemical Industries Ltd.) and mouse monoclonal α-smooth muscle actin (α-SMA) (1:100; clone 1A4; Dako Cytomation, Kyoto, Japan).

DNA microarray analysis. Total RNA was isolated from the liver samples of HFCD and HFCD + eLF

animals using TRIzol (Invitrogen Japan, Tokyo, Japan) and subsequently purified using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). The quality and quantity of the total RNA were evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies Japan, Tokyo, Japan). The total RNA integrity number exceeded 7. Total RNA samples from these animals were subjected to DNA microarray analysis as previously described.[21] The Affymetrix Porcine Genome Array™ (Affymetrix, Santa Clara, CA) was used, and fluorescence signals were scanned with the Affymetrix GeneChip System. Affymetrix GeneChip Command Console software was used to reduce the array images to the intensity values for each probe (CEL files).

DNA microarray data analysis. Using the R software (ver. 2.7.1; The R Project, http://www.r-project.org), the CEL files were quantified according to the distribution free weighted method (DFW).[22] A statistical comparison of HFCD and HFCD + eLF groups was performed with the Rank Products and Benjamini and Hochberg false discovery rate (FDR) corrections.[23] The Porcine Genome Array annotation file was downloaded from the Affymetrix website [Porcine Annotations, CSV format, Release 33 (06/22/12)]. The selected probe sets were classified according to gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the functional annotation tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID).[24] The GO analysis was performed on the basis of the biological process in GOTERM_BP_ALL. The related statistical analysis was performed using Fisher's exact test and FDR corrections. A significant difference was defined as an FDR-corrected P-value < 0.05.

Statistical analysis. The measured values have all been presented as means with standard deviations. The data between HFCD and HFCD + eLF group were compared using Welch's test by assuming that the data obtained in this experiment were normally distributed as well as those in many cholesterol-administered animal experiments. Statistical significance was set at a P-value < 0.05. Data were analyzed using JMP version 11. 2. 0 (SAS Institute Inc., Cary, NC, USA).

Results

Effect of eLF on HFCD-induced hypercholesterolemia and atherosclerosis

The growth curve in HFCD + eLF group was slightly lower than that in HFCD group (Fig. 1(A)). However, the difference was not statistically significant at week 8. Diet intake was also not significantly different at any time point (Fig. 1(B)). There were no organs whose weights were significantly different between HFCD and HFCD + eLF group (Table 1). The hepatic TG and cholesterol levels also did not exhibit the significant difference between HFCD and HFCD + eLF groups. However, eLF treatment tended to reduce HFCD-induced cholesterol levels (35% decrease). Serum total, LDL, and HDL cholesterol levels in HFCD group but not TG levels were remarkably increased and reached plateau at week 2 (Fig. 2(A)–(D)). eLF treatment exhibited the decrease of total (52%) and LDL cholesterol levels (42%) at week 2. These effects were observed during an experimental period. Because serum total, LDL, and HDL cholesterol levels reached plateau at week 2, statistical analysis for the hypercholesterolemia preventive efficacy of eLF was conducted at week 2 (Table 2). eLF treatment significantly decreased the serum total and LDL cholesterol levels ($p < 0.05$). Serum total and LDL cholesterol levels were positively correlated with hepatic cholesterol levels, respectively (Fig. 3(A) and (B)). During the necropsy, no atherosclerotic lesions were observed in the right coronary artery (RCA) from the Control

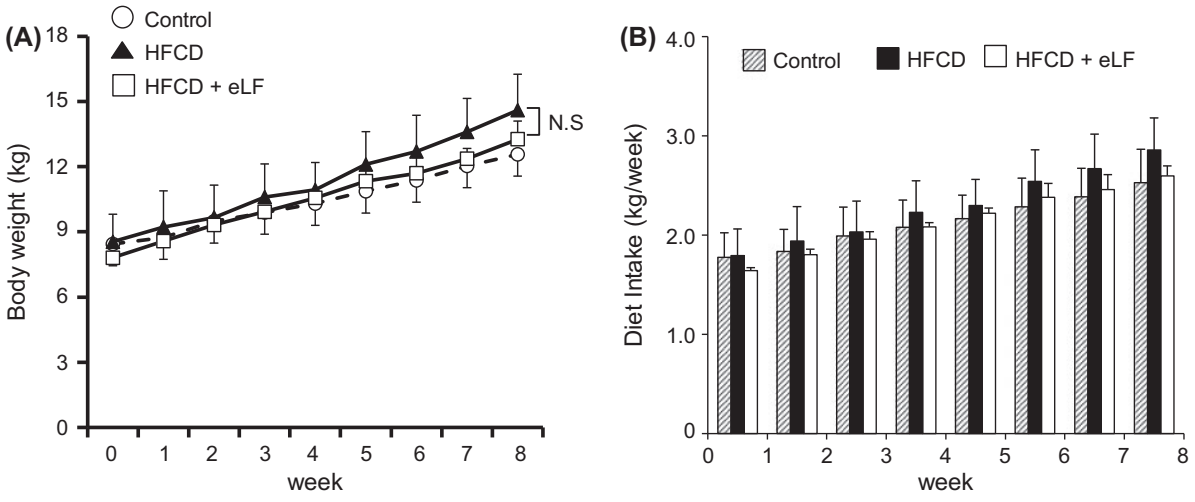

Fig. 1. Changes of body weight and diet intake during an 8-week period.

Notes: Control group was fed a normal chow diet and administered empty enteric capsules. HFCD group was fed a HFCD and administered empty enteric capsules. HFCD + eLF group was fed a HFCD and administered capsules filled with LF (500 mg LF/body/day). (A) Growth curves of Control group (○), HFCD group (▲), and HFCD + eLF group (□), (B) diet intakes of HFCD group (black bar) and HFCD + eLF group (open bar). All data are expressed as mean ± SD ($n = 3$). Statistical analysis between HFCD and HFCD + eLF group was conducted by Welch's test ($p < 0.05$).

Table 1. Organs weight and hepatic lipid concentration at week 8.

	Control	HFCD	HFCD + eLF	P^*
Organs				
Liver (g)	224.1 ± 9.6	275.3 ± 13.0	256.3 ± 29.2	N.S
Spleen (g)	34.0 ± 6.7	33.1 ± 3.8	32.8 ± 4.5	N.S
Heart (g)	60.8 ± 3.1	61.8 ± 4.6	64.2 ± 3.8	N.S
Kidney (g)	57.6 ± 5.0	57.8 ± 11.0	62.6 ± 6.5	N.S
Omental fat (g)	16.6 ± 4.5	20.6 ± 4.7	15.6 ± 1.6	N.S
Hepatic lipid concentration				
Triglyceride (mg/g-liver)	2.4 ± 0.3	4.2 ± 0.8	4.5 ± 1.0	N.S
Cholesterol (mg/g-liver)	1.8 ± 0.2	9.1 ± 2.8	5.9 ± 2.2	N.S

Notes: Values are expressed as mean ± standard deviation.
*Statistical analysis between HFCD and HFCD + eLF group was conducted by Welch's test ($p < 0.05$).

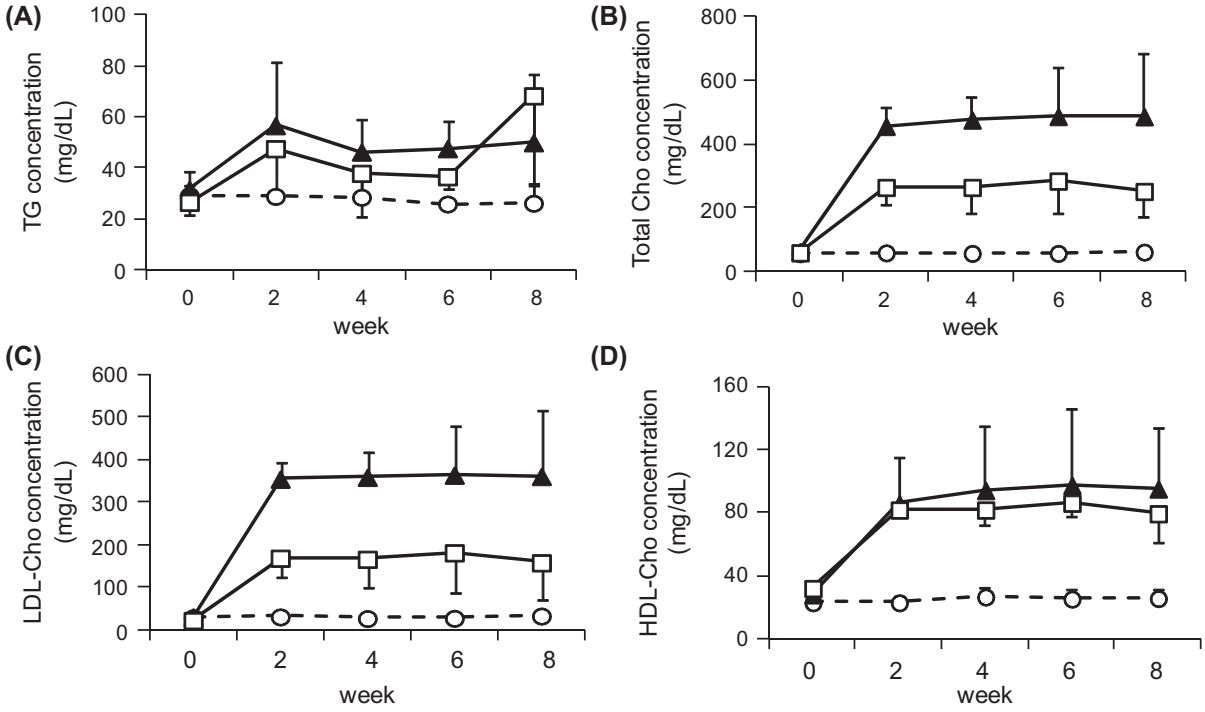

Fig. 2. Changes in the serum lipid concentrations during the 8-week period.
Notes: Blood samples of Control group (○), HFCD group (▲), and HFCD + eLF group (□) were collected and (A) TG, (B) total cholesterol, (C) LDL cholesterol, and (D) HDL cholesterol concentrations in serum were analyzed. All data are expressed as mean ± SD ($n = 3$).

group (Fig. 4(A) and (B)). The arteries from the HFCD group exhibited intimal thickening with foam cell infiltration (Fig. 4(C) and (D)). Immunohistochemistry also confirmed the presence of macrophage-derived ionized calcium-binding adaptor molecule 1-positive cells and α-smooth muscle actin-positive cells in the intima of RCA (Fig. 5(A) and (B)). In the HFCD + eLF group (Animal No. 8, Table 3), no atherosclerotic lesions were observed except for very slight focal regions (Fig. 1(E) and (F)). The type of RCA atherosclerosis was classified according to the Stary Type classification (Table 3). The atherosclerosis type in the Control group could not be classified because no lesions had developed. All three MMPigs from the HFCD group were classified as having atherosclerosis Type II. In the HFCD + eLF group, 1 MMPig was classified as Type I and the other 2 MMPigs could not be classified. The classifications of the other 14 arteries revealed the remarkable inhibitory effect of eLF in terms of atherosclerosis development (Table 3).

Effect of eLF on hepatic gene expression profiles

The statistical comparison of the HFCD and HFCD + eLF group data according to the RankProducts method with multiple testing corrections using the Benjamini and Hochberg FDR ($p < 0.05$) resulted in the selection of 444 probes (data not shown). In these probe sets, *LDLR* and *HMGCR* were both found to be upregulated (Table 4) and the *SREBP-2*, a key transcriptional factor for these genes, also displayed the tendency toward upregulation (Ssc_16976.1.S1_at, $p = 0.055$; data not shown). Subsequently, we used the online DAVID software module to identify the overrepresented GO terms and metabolic pathways in the selected genes. A GO analysis of the selected 444 probes demonstrated that the effects of eLF administration indicated significant involvement in the oxidation reduction (GO: 005514) biological process (Supplemental Table S1, S2). A KEGG pathway analysis revealed that seven significantly affected pathways (Table 5). The affected genes in steroid biosynthesis (ssc00100) category were

Table 2. Effect of eLF on the serum lipid concentration at week 2.

Parameter	Group	Δvalue at week 2 Mean SD	*P
Triglyceride	HFCD	25.0 ± 28.7	N.S
	HFCD + eLF	21.0 ± 13.0	
Total cholesterol	HFCD	392.3 ± 52.6	<0.01
	HFCD + eLF	205.0 ± 55.1	
HDL cholesterol	HFCD	57.7 ± 24.7	N.S
	HFCD + eLF	49.7 ± 2.9	
LDL cholesterol	HFCD	323.7 ± 37.8	<0.01
	HFCD + eLF	146.3 ± 48.7	

Notes: Values are expressed as mean ± standard deviation.
*Statistical analysis between HFCD and HFCD + eLF group was conducted by Welch's test ($p < 0.05$).

CYP family 51, subfamily A, polypeptide 1 (*CYP51*), NAD(P)-dependent steroid dehydrogenase-like (*NSDHL*), squalene epoxidase (*SQLE*), and methylsterol monooxygenase 1 (*MSMO1*), all of which encode enzymes that promote cholesterol synthesis (Table 6). All these genes were upregulated in the HFCD + eLF group compared with the HFCD group.

Discussion

In this study, we proved that eLF administration could attenuate the development of HFCD-induced hypercholesterolemia and atherosclerosis in MMPigs (Tables 2 and 3 and Fig. 2). Although the effects of LF on lipid metabolism have been reported in clinical trials and animal studies,[8,13,25–27] no previous reports clarified the beneficial effects of LF with respect to hypercholesterolemia and atherosclerosis in MMPigs, which are very similar to humans in various aspects such as the regulation of cholesterol homeostasis.

In mammals, cholesterol homeostasis is primarily controlled in the liver, which regulates the blood cholesterol concentration and cholesterol synthesis–catabolism balance. Therefore, in this study, the hepatic gene expression profiles were analyzed via DNA microarray to clarify the effects of eLF on hepatic function. Recently, the RankProducts method with a DFW preprocessing algorithm was reported to be among the best analytical combinations for the accurate detection of differentially expressed genes.[22] Therefore, we applied DFW as a quantification method for our DNA microarray data. In this experiment, eLF administration increased the expression of genes related to the cholesterol metabolism (*LDLR* and *HMGCR*) and cholesterol synthesis-promoting enzymes (*CYP51*, *MSMO1*, *NSDHL*, and *SQLE*) (Tables 4–6).[28–31] We confirmed that the expression levels of these genes were significantly downregulated in the HFCD group relative to the Control group (data not shown). These results suggest that eLF restores the cholesterol metabolism and synthesis inhibited by HFCD intake. Furthermore, eLF administration exhibited 35% decrease of hepatic cholesterol levels (Table 1), and serum total and LDL cholesterol levels were strongly correlated with hepatic cholesterol levels, respectively (Fig. 3(A) and (B)). Combined with these results, it is suggested that eLF protected the homeostasis from HFCD-induced cholesterol metabolism dysregulation in liver through the decrease of serum cholesterol levels. As one of the action mechanisms, eLF would inhibit cholesterol absorption from the small intestine. Because LF is a cationic protein (isoelectric point: 8.2–8.9)[32] and bile acids are anionic substances, LF may interact with bile acids to decrease the micellar solubility of cholesterol. The cationic resin cholestyramine promotes fecal cholesterol excretion[33] and has been approved for use as a lipid-lowering agent in several countries. Furthermore, β-conglycinin, a representative cationic protein from soybeans, was shown to promote reduced total blood cholesterol levels in mice and rats,[34,35] and β-conglycinin-derived peptides were found to interact with bile acids.[8] Takeuchi et al.[36] reported that LF supplementation did not promote the bile acid and cholesterol excretion in fecals in mice fed a HFCD. However, unlike our experiment, mice were administered normal LF but not eLF, and high concentration of bile acid (0.25%) was added to the diet. Because experimental conditions are quite different between 2 experiments, the effect of LF on cholesterol and bile acid excretion is still unclear. Therefore, further studies are needed to validate this hypothesis and to clarify the inhibitory potential of LF with respect to cholesterol absorption.

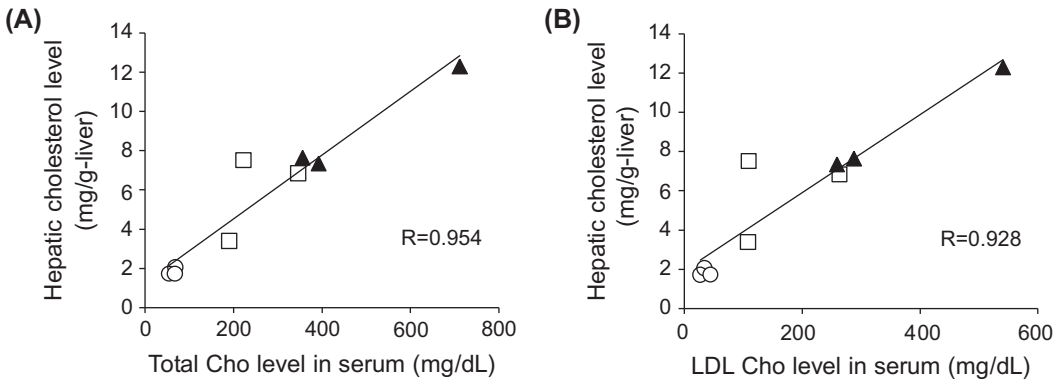

Fig. 3. Correlation between the serum cholesterol and hepatic cholesterol levels at week 8. Control group (○), HFCD group (▲), and HFCD + eLF group (□).

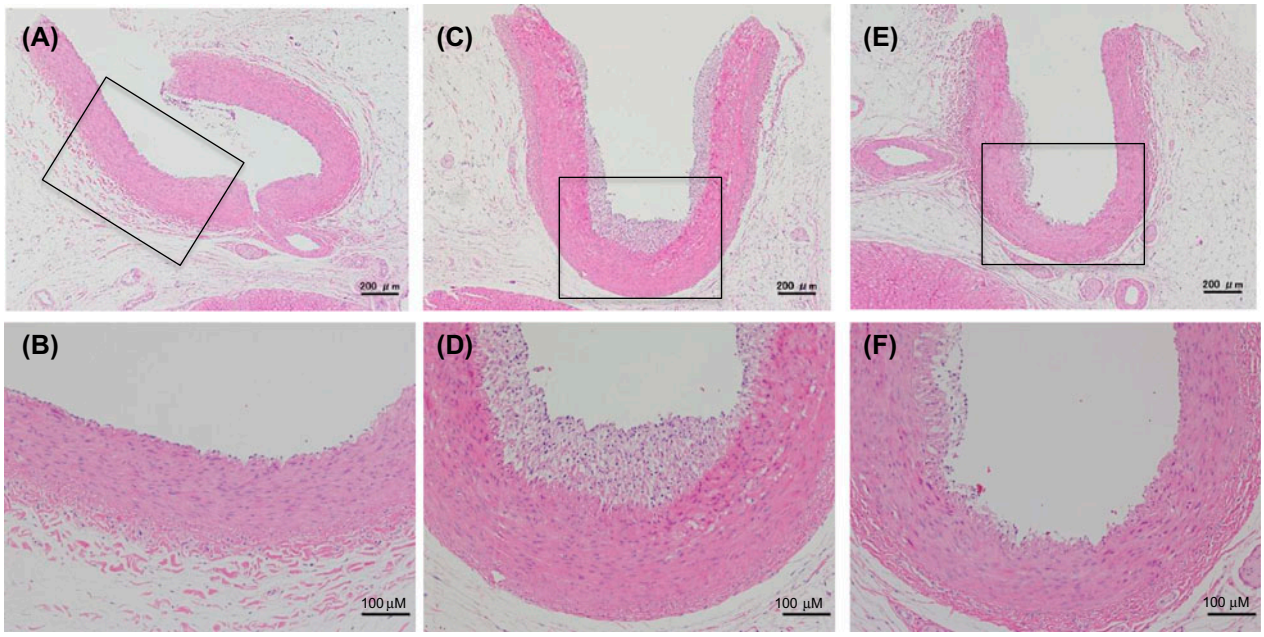

Fig. 4. Histological analysis of the right coronary artery at week 8.

Notes: The histological analysis was performed on hematoxylin and eosin-stained right coronary arteries. Representative results from each group are shown in this figure. (A) and (B), Control group; (C) and (D), high-fat and high-cholesterol diet (HFCD) group; and (E) and (F), HFCD + enteric lactoferrin (eLF) group. The bars on (A), (C), and (E) represent 200 μm and the bars on (B), (D), and (F) represent 100 μm.

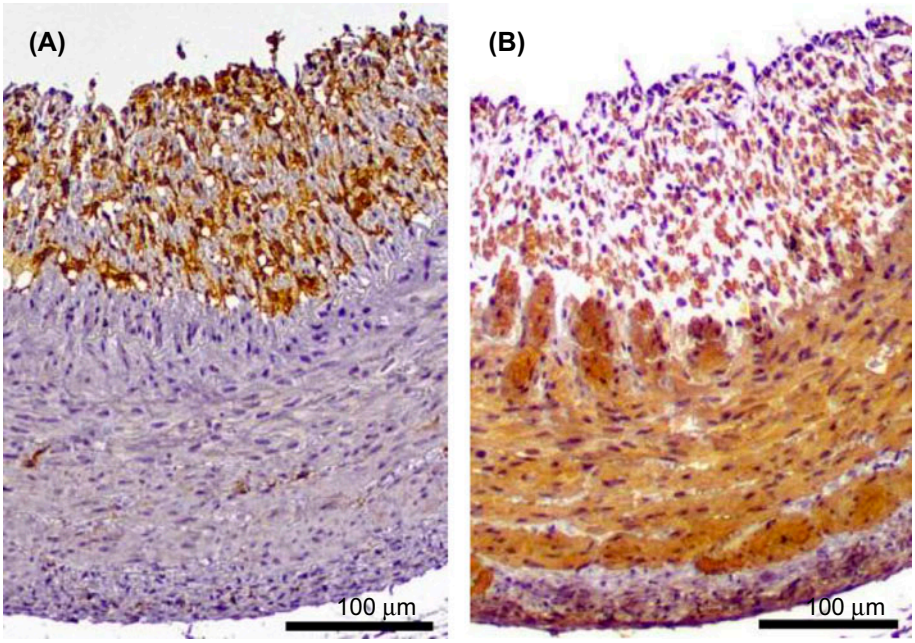

Fig. 5. Immunohistochemistry of atherosclerotic lesions in the high-fat and high-cholesterol diet (HFCD) group. The right coronary artery was analyzed via immunohistochemistry with primary antibodies against the ionized calcium-binding adaptor molecule 1 (Iba-1) and a-smooth muscle actin (α-SMA) and Envision Kit. The sections exhibit positive (A) Iba-1 and (B) α-SMA expression. All bars indicate 100 μm.

The prevention of hypercholesterolemia is one of the major mechanisms of the inhibition of the atherosclerosis. We hereby propose additional possible action mechanism of eLF in a circulation. Reportedly, endothelial cell injury causes the infiltration of smooth muscle cells and macrophages into the intima in the early stage of atherosclerosis.[37] As the next step, macrophages have been reported to take up oxidized LDL deposits from the blood vessel walls via scavenger receptors and to subsequently develop into foam cells.[38,39] Reportedly, bovine LF (1 μg/mL) promotes the tube formation and cell proliferation of human aortic endothelial cells.[40] Kajikawa et al. reported that bovine LF (0.2–1 mg/mL) remarkably inhibited scavenger receptor-mediated cholesteryl ester accumulation in macrophages.[41] Fischer et al.[42] reported that administered bovine LF by intragastric intubation to mice could be detected in the plasma as an intact form. Therefore, inhibition of endothelial cell dysfunction and foam cell development from macrophages may be an antiatherogenic effect of eLF. By contrast, in this study, eLF was administered to MMPigs during study

Table 3. Atherosclerotic scores of 15 arteries according to the Stary type classification.

Group	Control			HFCD			HFCD + eLF		
Animal no	1	2	3	4	5	6	7	8	9
LAD coronary artery	–	–	–	II	II	II	–	I	–
Right coronary artery	–	–	–	II	II	II	–	I	–
Pulmonary artery	–	–	–	I	I	I	–	–	–
Aortic arch	–	–	–	II	II	II	–	–	–
Common carotid artery	–	–	–	I	–	–	–	–	–
Thoracic aorta	–	–	–	II	I	II	–	–	–
Abdominal aorta	–	–	–	II	II	II	–	–	–
External iliac arteries	–	–	–	II	II	I	–	–	–
Internal iliac arteries	–	–	–	–	II	–	–	I	–
Renal artery	–	–	–	II	II	–	–	–	–
Pancreatic artery	–	–	–	–	–	–	–	–	–
Rostral cerebral artery	–	–	–	I	–	–	–	–	–
Internal carotid artery	–	–	–	–	–	–	–	–	–
Caudal communicating artery	–	–	–	II	–	–	–	–	–
Basilar artery	–	–	–	–	–	–	–	–	–

Notes: –: No observed symptom development.
I: Observed isolated macrophage foam cells.
II: Observed formation of multiple foam cell layers.

Table 4. Significantly affected cholesterol metabolism-related gene expression.

Affy_ID	Ref_seq	Change	Gene title	Gene symbol
Ssc.16088.1.S1_at	NM_001122988	UP	3-Hydroxy-3-methylglutaryl-CoA reductase	HMGCR
Ssc.21926.1.S1_at	NM_001206354	UP	Low-density lipoprotein receptor	LDLR

Table 5. Significantly affected pathways.

Pathway_ID	Term	FDR-corrected *p*-value
ssc00980	Metabolism of xenobiotics by cytochrome P450	9.33E−05
ssc00982	Drug metabolism	1.29E−04
ssc00830	Retinol metabolism	1.54E−03
ssc00100	Steroid biosynthesis	1.94E−02
ssc00900	Terpenoid backbone biosynthesis	2.64E−02
ssc00380	Tryptophan metabolism	3.20E−02
ssc00480	Glutathione metabolism	3.14E−02

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis via Fisher's exact test and false discovery rate (FDR) correction ($p < 0.05$).

Table 6. Significantly affected steroid biosynthesis genes.

Affy_ID	Ref_seq	Change	Gene title	Gene symbol
SSC.3253.1.S1_AT	NM_001167636	UP	NAD(P) dependent steroid dehydrogenase-like	NSDHL
SSC.5712.1.S1_AT	NM_214432	UP	Cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51
SSC.8385.1.A1_AT	NM_001101026	UP	Squalene epoxidase	SQLE
SSC.8774.1.S1_AT	NM_213752	UP	Methylsterol monooxygenase 1	MSMO1
SSC.8774.2.A1_AT		UP		

periods in the dosage of 37–65 mg/kg/body same range as the Fischer's report (about 35 mg/kg/body), in which the maximum detected LF was about 50–60 ng/mL in serum.[14] Therefore, it is an important issue to clarify whether LF of lower concentration exhibits the inhibitory effect on endothelial cell injury and cholesteryl ester accumulation in macrophages or not. In this study, we demonstrated that eLF exerted inhibitory effects on hypercholesterolemia and atherosclerosis development in MMPigs. Together with these results and the existing food safety profile of LF, eLF appears to be a promising food additive for the prevention of hypercholesterolemia and atherosclerosis.

Authors contributions

H. Kawaguchi, T. Ono, M. Murakoshi, K. Sugiyama, A. Tanimoto, and H. Nishino conceived and designed the experiments. S. Morishita, H. Kawaguchi, T. Ono, and A. Tanimoto performed the experiments. S. Morishita, H. Kawaguchi, T. Ono, and N. Miura

analyzed the data. H. Kato contributed DNA microarray reagents/materials/analysis tools. S. Morishita, H. Kawaguchi, T. Ono, M. Murakoshi, and A. Tanimoto wrote the manuscript. All authors reviewed and approved the final manuscript.

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Disclosure statement

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Supplemental material

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