Effects of lipid metabolism on mouse incisor dentinogenesis

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Tooth formation can be affected by various factors, such as oral disease, drug administration, and systemic illness, as well as internal conditions including dentin formation. Dyslipidemia is an important lifestyle disease, though the relationship of aberrant lipid metabolism with tooth formation has not been clarified. This study was performed to examine the effects of dyslipidemia on tooth formation and tooth development. Dyslipidemia was induced in mice by giving a high-fat diet (HFD) for 12 weeks. Additionally, LDL receptor-deficient (Ldlr−/−) strain mice were used to analyze the effects of dyslipidemia and lipid metabolism in greater detail. In the HFD-fed mice, incisor elongation was decreased and pulp was significantly narrowed, while histological findings revealed disappearance of predentin. In Ldlr−/− mice fed regular chow, incisor elongation showed a decreasing trend and pulp a narrowing trend, while predentin changes were unclear. Serum lipid levels were increased in the HFD-fed wild-type (WT) mice, while Ldlr−/− mice given the HFD showed the greatest increase. These results show important effects of lipid metabolism, especially via the LDL receptor, on tooth homeostasis maintenance. In addition, they suggest a different mechanism for WT and Ldlr−/− mice, though the LDL receptor pathway may not be the only factor involved.

A regular high-fat diet (HFD) has been shown to result in such lifestyle diseases as dyslipidemia, obesity, and diabetes12. Notably, dyslipidemia causes serious alterations of systemic tissue, including accumulation of lipids in blood vessel walls and the liver14. It has also been reported that lipid metabolism alteration produces changes in calcified tissue phenotypes5. Low-density lipoprotein (LDL) is known to transport cholesterol produced in the liver to peripheral cells6. However, as noted above, an increase in native LDL in blood induces dyslipidemia and atherosclerosis, which then induces typical cardiovascular events such as cardiac and cerebral infarction7. Increasing evidence shows that lifestyle, especially diet, can induce chronic disease. Furthermore, a previous study showed that patients with dyslipidemia can develop osteoporosis6–10. The gene disorder related to lipid metabolism known as familial hypercholesterolemia (FH) has been shown to be related to development of coronary heart disease as well as the LDL receptor, which is essential for lipid uptake11,12. Also, patients with FH have been reported to develop Achilles tendon (AT) xanthomas, resulting in an incrassate AT condition13,14, suggesting increased collagen in the tendon. However, detailed analysis findings regarding bone or teeth in FH patients are scarce. There are some reports of the relationship of lipids and teeth based on analyses of the components of enamel15–17 and dentin17–20, though details regarding the effects of lipid metabolism aberration on tooth

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results with those of other groups, the effects of dyslipidemia on tooth growth, histological alteration, tooth homeostasis maintenance, and bone metabolism.

A variety of mouse models are available for analysis of incisor tooth development and aberrations. Previous results have shown that in the cervical loop region, stem cells are fueled as the incisor elongates continuously throughout the life of a mouse. LDL receptor-deficient (Ldlr−/−) mice are generally used as animal models of dyslipidemia, atherosclerosis, and FH. Dyslipidemia is induced in these animals by feeding an HFD and findings obtained with a dyslipidemia model indicated increases in total cholesterol (T-CHO), low density lipoprotein−cholesterol (LDL-c), and triglycerides (TG), as well as decreased HDL as compared to Ldlr−/− mice fed normal chow. In addition, the aorta of Ldlr−/− mice fed an HFD narrows with accumulation of plaque. However, the influence of dyslipidemia on tooth growth has been scarcely reported. With this background in mind, we examined the effects of an HFD on tooth development and odontoblast activity. The aim of this study was to clarify the effects of dyslipidemia in mice induced by HFD consumption on tooth growth, histological alteration, tooth homeostasis maintenance, and bone metabolism.

Results
HFD-fed wild-type mice showed less incisor elongation and significantly narrowed incisor pulp as compared to chow-fed mice. After 12 weeks of feeding, 20-week-old mice were examined in this study. Measurements of incisor elongation were considered to demonstrate the effects of fatty ingredients in the diet (Fig. 1a–c; Supplementary Table 1). To examine the inner structure of the incisor, we performed μCT scanning of mandibular bone specimens. The incisor pulp was narrowed in HFD-fed as compared to chow-fed mice (Fig. 1d–f), while the molar pulp was not narrowed in mice given the HFD (Fig. 1g). Additionally, time-dependent representative μCT images showed an incisor pulp narrowing trend after 6 weeks of the feeding period in both HFD-fed wild-type (WT) and chow-fed Ldlr−/− mice, while the incisor pulp showed a narrowing trend after 3 weeks of the feeding period in HFD-fed Ldlr−/− mice (Supplementary Fig. 3). We also analyzed important hard tissue bone samples using μCT and noted that femur bone mass was decreased in HFD-fed mice (Supplementary Fig. 4a,b). These results suggested that the amount of fat consumed had effects on tooth growth and formation, as well as bone metabolism.

Predentin in HFD-fed WT mice disappeared near central region of mandibular incisor. To perform histological examinations of narrowed incisor pulp and thickened dentin in mice given the HFD, histological sections of mandibular incisors were prepared. Sagittal section images showed that the pulp cavity of HFD-fed WT mice was significantly narrowed as compared to chow-fed WT mice (Fig. 2a), consistent with the μCT imaging findings. In addition, dentin thickness in the HFD group was significantly increased. The strong expansion shown in the images also indicated that predentin had disappeared in HFD-fed mice in the area near the central region (Fig. 2b ③⑦). In addition, the morphology of odontoblasts forming the odontoblast layer was not columnar, but rather round near the central region (Fig. 2b ③⑦). Also, the odontoblast layer on the apical side in HFD-fed mice showed a trend for greater density than that in chow-fed mice (Fig. 2b ⑧). Dentin formation was indicated by double labelling of calcein (Fig. 2c), and the width of formed dentin showed a decreasing trend on the incisor side and in the central region of the mandibular incisor in the HFD group (Fig. 2d,e). These results suggest that the amount of fat in the diet has effects on odontoblasts during tooth formation.

In Ldlr−/− mice, incisor elongation showed no significant differences, while incisor pulp in HFD-fed mice was significantly narrowed. Body weight was increased in both chow- and HFD-fed WT mice, while that showed a gradual decrease over time in HFD-fed Ldlr−/− mice. The body weight of HFD-fed WT mice was significantly increased as compared to that of HFD-fed Ldlr−/− mice (Supplementary Fig. 2). To further investigate the relationships of fat in the diet with tooth growth and dentin formation, we used Ldlr−/− mice. As compared to WT mice, incisor elongation amount and rate were decreased in the Ldlr−/− group when given the chow diet (Fig. 3a–c), indicating that lipid metabolism by the mediated LDL receptor and other factors has effects on tooth elongation. Furthermore, incisor pulp was narrowed in HFD-fed as compared to chow-fed Ldlr−/− mice (Fig. 3d–f). As for measurement results of molars in chow-fed WT and Ldlr−/− mice, pulp width, dentin width, crown width, and root length showed no significant differences (Fig. 3g). When diet types were compared, femur bone mass was decreased in the Ldlr−/− mice fed the HFD as compared to those given the chow diet (Supplementary Fig. 4a,b). In summary, these results suggest that the LDL receptor has some effects on tooth growth, incisor dentinogenesis, and bone metabolism.

Predentin near central region of mandibular incisor disappeared in HFD-fed Ldlr−/− mice. Examinations of sagittal histological sections showed narrowed incisor pulp with thickened dentin in both HFD- and chow-fed Ldlr−/− mice (Fig. 4a). On the other hand, strong expansion seen in imaging findings and predentin near the central region of the mandibular incisor had disappeared in the HFD-fed Ldlr−/− mice (Fig. 4b ⑦). In addition, the morphology of odontoblasts near the central region in chow-fed Ldlr−/− mice indicated a more
circular shape as compared to chow-fed WT mice (Fig. 2b③, Fig. 4b③). Also, the odontoblast layer on the apical side in both chow- and HFD-fed Ldlr−/− mice demonstrated a trend for greater density as compared to the WT mice given chow (Fig. 2b④, Fig. 4b④⑧). Dentin formation widths on the mandibular incisor side and in the central region of the mandibular incisor were nearly the same in the chow- and HFD-fed Ldlr−/− mice (Fig. 4c–e).
These results suggested that tooth constituent cells in the Ldlr−/− mice were also affected by fat in the diet and the LDL receptor.

**Ameloblasts near central region of mandibular incisor in HFD-fed WT mice, and chow- and HFD-fed Ldlr−/− mice promoted differentiation stage.** Images showing strong expansion indicated a cuboidal ameloblast morphology near the central region in the HFD-fed WT, and chow- and HFD-fed Ldlr−/− mice, as compared to the chow-fed WT mice (Fig. 5a②⑥, 5b②⑥). These results suggested that the ameloblast differentiation process was promoted in the HFD-fed WT, and chow- and HFD-fed Ldlr−/− mice.

**Ldlr−/− mice given HFD developed severe dyslipidemia.** In the HFD-fed WT mice, the levels of total-cholesterol (T-CHO), low density lipoprotein-cholesterol (LDL-C), and alkaline phosphatase (ALP) were increased by 2.6-, 10-, and 1.4-fold, respectively, as compared to WT mice given the chow diet, while the level of triglycerides (TG) was decreased in WT mice given the HFD (Fig. 6). On the other hand, the levels of high density lipoprotein-cholesterol (HDL-C), glucose (Glu), and calcium (Ca) in WT mice showed no significant differences between the diet groups (Fig. 6). In contrast, in the HFD-fed Ldlr−/− mice, the levels of T-CHO and LDL-C were significantly increased, while the level of HDL-C was decreased as compared to those given chow (Fig. 6), whereas the levels of TG, Glu, ALP, and Ca showed no significant differences between Ldlr−/− mice with the different diets (Fig. 6). Comparisons of chow-fed WT and Ldlr−/− mice showed that the levels of T-CHO (3.7-fold), LDL-C (24-fold), and TG (2-fold) were increased in the latter group (Fig. 6). These results indicated that increased fat in the diet induced an increase in T-CHO and LDL-C levels in both the WT and Ldlr−/− mice (Fig. 6), which resulted in narrowed pulp and thickened dentin in incisors.

**Discussion**

The present results showed that a diet high in fat has effects on tooth formation and growth, as well as bone metabolism, suggesting involvement of lipid components, especially cholesterol and LDL. In mice, incisors are different than molars, as they continue to grow and elongate throughout life. A previous study showed that measurement of incisor eruption rate is an appropriate method for evaluation of tooth physiological growth32, thus we mainly examined incisors in the present study. Our findings showed that the amount of elongation was decreased in WT mice fed the HFD (Fig. 1), while a decreasing trend was found in the HFD-fed Ldlr−/− mice (Fig. 3),

**Figure 2.** Histological analysis of WT mouse incisor dentin and pulp. (a) Sagittal histological section of WT mouse incisor. Scale bar = 1000 μm. (b) Magnified images show histological sections of pulp and odontoblasts from 20-week-old chow- and HFD-fed mice. Scale bar = 50 μm. (c) Calcein labeled histological section of incisal side. Scale bar = 100 μm. (d) Dentin formation width in mandibular incisal site (n = 5–6). (e) Dentin formation width in central region of mandibular incisor (n = 4–5). (d,e) Student’s t-test, NS; not significant. Error bars represent mean ± SD. D: dentin, P: pulp.
suggesting that a lipid metabolism disorder can delay tooth growth. In a previous study, a decrease in blood flow was found to induce a decreased incisor eruption rate in mice, while another reported that Ldlr−/− mice fed an HFD showed plaque adherence in the aorta. Together, these results suggest that peripheral blood vessels such as...
as those in pulp also have decreased blood flow, which impairs elongation of the incisor. To better understand the effects of lipid metabolism on tooth growth, a more detailed examination of molars will be necessary.

In the present experiments, the amount of dentin in the incisors of both WT and Ldlr−/− mice given the HFD was notably increased (Figs. 1 and 3), while a slight increase was seen in the chow-fed Ldlr−/− mice (Fig. 3). In addition, time-dependent μCT images showed that incisor pulp gradually narrowed in dyslipidemia model mice (Supplementary Fig. 3). In the chow-fed Ldlr−/− mice, the levels of T-CHO and LDL-C were increased as compared to chow-fed WT mice (Fig. 6), suggesting that increased cholesterol in blood induced narrowing of pulp with thickened dentin.

A physiological increase in dentin is known to normally occur throughout life, though some reports have noted increased dentin due to local or systemic pathological factors. Regarding local factors, attrition, abrasion, foreign materials, and caries, as well as other factors have been reported to cause an increase in dentin in both incisors and molars. As for systemic factors, some patients who continue to receive steroid administration for long periods after undergoing transplantation for renal disease have shown a significantly narrowed dental pulp cavity in both incisors and molar teeth, while those patients often develop dyslipidemia secondarily as well. A previous study reported that dyslipidemia model mice had a narrowed incisor dental pulp cavity. Others that investigated the relationship between leptin and the LDL receptor also showed that HFD consumption induced increased plasma leptin levels in both WT and Ldlr−/− mice, while it is known that leptin promotes odontoblastic differentiation and osteogenesis in alveolar bones in mice; thus Smpd3 is considered to be an important factor for dentinogenesis. We consider that the mechanism underlying the present results may involve additional factors other than leptin.

It is thus suggested that increased lipid biomarkers, including cholesterol, as well as hormones related to lipid metabolism, including leptin, in blood induce tooth pulp narrowing. Narrowed pulp cavities have been found in both incisors and molars in humans, whereas only incisors were affected in the present mice, likely because of histological and anatomical differences between humans and rodents. As noted above, a rodent incisor shows growth throughout life, whereas dentin in humans increases because of physiological reasons or when stimulated. Also, active mesenchymal stem cells providing new tooth constituent cells to maintain growth have been found in mouse incisors.
Figure 5. Histological analysis of incisor ameloblasts in WT and Ldlr<sup>−/−</sup> mice. (a) Sagittal histological section of incisor from 20-week-old WT mouse (upper panel). Scale bar = 1000 μm. Magnified image of ameloblast (lower panel). Scale bar = 30 μm. (b) Sagittal histological section of incisor from 20-week-old Ldlr<sup>−/−</sup> mouse (upper panel). Scale bar = 1000 μm. Magnified image of ameloblast (lower panel). Scale bar = 30 μm. E: enamel, Es: enamel space, Ab: ameloblast.

Figure 6. WT and Ldlr<sup>−/−</sup> mouse blood analysis. Blood was obtained from 20-week-old WT and Ldlr<sup>−/−</sup> mice (n = 4–12), and examined. T-CHO: total cholesterol, LDL-C: low density-cholesterol, HDL-C: high density-cholesterol, TG: triglyceride, Glu: glucose, ALP: alkaline phosphatase, Ca: calcium. One-way ANOVA with Dunnett’s test. **p < 0.01, *p < 0.05. NS, not significant. Error bars represent mean ± SD.
It is also considered that reactive cells are more susceptible to an increased fat component in the diet as compared to quiescent cells. In the present study, calcine labeling showed that dentin formation on the incisal side and in the central region was decreased in teeth considered to be in an active formation state (Figs. 2d,e and 4d,e), suggesting that dentin formation ability was declining at 12 weeks after beginning the special diet. Histological sections also showed that odontoblast density in dentin was increased in the HFD-fed WT and Ldlr−/− mice, as well as chow-fed Ldlr−/− mice (Figs. 2b and 4b), indicating that odontoblast differentiation and/or function was increased in those mice. Therefore, it is suggested that the mechanism of dentin formation promotion differs between WT and Ldlr−/− mice. However, the pathway involving the LDL receptor might not be exclusive and different pathways may also be involved.

Histological findings showed that pre-dentin in the HFD-fed WT and Ldlr−/− mice disappeared (Figs. 2 and 4), and the odontoblast morphology was circular within that region, while pre-dentin was unclear in Ldlr−/− mice given chow. In addition, ameloblast morphology was cuboidal near the central region in the HFD fed WT, and chow- and HFD- fed Ldlr−/− mice (Fig. 5a(2/6), 5b(2/6)). These results suggest that increased fat in the diet induces morphological changes in odontoblasts and ameloblasts, as well as dentin formation ability and ameloblast differentiation, while a deficiency of the LDL receptor has effects on dentin formation and the process of ameloblast differentiation. μCT imaging of femurs showed that bone mass was decreased in WT mice fed the HFD as compared to chow, while the Ldlr−/− mice showed no significant difference regardless of diet (Supplementary Fig. 4). However, the bone volume/tissue volume (BV/TV) ratio in Ldlr−/− mice fed chow had a decreasing trend as compared to the chow-fed WT mice, while that ratio was equally decreased in the HFD-fed Ldlr−/−and WT mice. A previous study demonstrated that LDL is associated with hard tissue metabolism, especially oxidized LDL, which promotes bone loss by facilitating adipogenic differentiation more than osteogenic differentiation in bone marrow stromal cells. Our results indicate that increased cholesterol in blood induces a decrease in bone mass, while histological examinations of hind limbs revealed fatty bone marrow (data not shown), thus suggesting a mechanism of decreased bone formation in mice with increased cholesterol. Achilles tendon thickness, calcification, and FH are also known to have associations. In addition, ultrastructural analysis of odontoblasts, ameloblasts, dental pulp and extracellular matrices in the teeth is necessary to clarify the mechanisms for the effects of lipid metabolism on tooth development. In the present mice, teeth and bones showed changes, indicating that tissue subjected to stress has increased collagen.

In summary, the present results provided clarification regarding the role of the LDL receptor in dentin formation and bone metabolism. In addition, effects of cholesterol and the LDL receptor on dentin formation and bone metabolism were suggested.

Methods

Mice and feeding. WT mice with a C57BL/6J genetic background were purchased from Sankyo Labo Service Corporation, Inc. Tokyo, Japan. The Ldlr−/− (B6. Ldlr<sup>tm1Her</sup>) mouse strain was purchased from The Jackson Laboratory (stock no. 2207). At 8 weeks old, all mice were separated into those fed regular chow (CRF-1; 24.8% protein, 36% fat, 36% carbohydrate; Oriental Yeast Corp., Tokyo, Japan) or F2HFD1 (HFD; 22% protein, 13.7% fat, 61.5% carbohydrate; Oriental Yeast Corp., Tokyo, Japan) or F2HFD2 (HFD; 22% protein, 36% fat, 42% carbohydrate; Oriental Yeast Corp., Tokyo, Japan). Details regarding the compositions of the diets are shown in Supplementary Table 1. After being fed chow or the HFD for 12 weeks, blood, skull bone, and mandibular bone samples were collected following administration of a mixed anesthetic (midazolam, medetomidine, ketamine, and xylazine). The mice were labeled with calcein via a subcutaneous injection at 5 days and again 1 day before death. A mixed anesthetic (midazolam, medetomidine, and xylazine) was administrated by intraperitoneal injection. The mice were then euthanized with an intracardiac injection of ketamine and xylazine. The experimental design is shown in Supplementary Fig. 1. All animal experiments were approved by the ethical guidelines of that institution.

Incisor elongation rate. At the age of 18 weeks, the mandibular right incisor cervix surface in each mouse was defaced using a power pack (Minimo ONE SERIES ver. 2; MINITOR CO., Ltd. Tokyo, Japan), standard rotary (M212H; MINITOR CO., Ltd. Tokyo, Japan), and 1-mm round bur, with the mice given the same mixed anesthetic noted above. Tooth elongation process X-ray photography was performed with an in vivo μCT system (R_mCT2; Rigaku Co., Ltd., Tokyo, Japan) at 1 and 2 weeks after defacement. Incisor elongation rate was determined by measuring from the alveolar bone crest to middle of the round lesion using the Image J software package, version 1.52a (National Institutes of Health, Maryland, United States).

Micro-computed tomography (μCT) analysis. Mandibular bone and femur specimens obtained from the mice were scanned using a ScanXmate-L090H (Comscantecno, Co., Ltd., Yokohama, Japan), as previously described. Three-dimensional distal images were reconstructed with a TRI/3D-Bon-PCS system (RATOC System Engineering Co., Ltd., Tokyo, Japan). Following reconstruction, the coronal plane at the mesial root of the first molar was selected to determine enamel, dentin, and tooth full-width thicknesses, and pulp cavity diameter, with the values calculated using Image J 1.52a.

Mandibular incisor histological sections. Mandibular bone specimens obtained from the mice were fixed in a 4% paraformaldehyde phosphate buffer solution (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) or 10% formalin neutral buffer solution (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan), then demineralized for 2 weeks in decalcification solution (OSTEOSOFT; Merck KGaA, Corp., Darmstadt, Germany). Thereafter, they were embedded in paraffin wax and cut into sagittal sections, and subjected to hematoxylin and eosin staining. All sections were analyzed using an all-in-one fluorescence microscope (BZ-X710; KEYENCE, Corp., Osaka, Japan).

Serum examinations. After completion of the 12-week special feeding period, all mice were euthanized and blood samples were collected from the heart. Serum levels of total cholesterol (T-CHO), low density lipoprotein cholesterol (LDL-C), triglyceride (TG), and glucose (GLC) were measured using a high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Kyoto, Japan) or an enzyme-linked immunoabsorbent assay (ELISA) kit (Kanto Chemical Co. Inc., Tokyo, Japan).
cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglyceride (TG), calcium (Ca), inorganic phosphorus (IP), glucose (Glu), and alkaline phosphatase (ALP) were determined using routine laboratory methods (Oriental Yeast Corp., Tokyo, Japan).

**Statistical analysis.** Statistical analyses were performed with the IBM PASW statistical software package, version 18.0 (IBM, Chicago IL, USA). Values are expressed as the mean ± SD (standard deviation). All analyses were performed using one-way ANOVA or Student's t-test, with p values less than 0.05 considered to indicate statistical significance.

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Author contributions

Y.K., N.S., and M.T. designed the study and wrote the main manuscript. T.M., A.K., M.C., M.M., T.S., T.N.-K., R.K., A.M., and Y.M. contributed to the experiments and data interpretation. M.H. and Y.S. contributed to data acquisition and interpretation. All of the authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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