Low-density Lipoprotein Receptor Deficiency Causes Impaired Osteoclastogenesis and Increased Bone Mass in Mice because of Defect in Osteoclastic Cell-Cell Fusion

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Background: Dyslipidemia is associated with osteoporosis, atherosclerosis, and vascular calcification.

Results: Osteoclastogenesis in LDLR-deficient mice was reduced because of impaired cell fusion in preosteoclasts consistent with reduced Atp6v0d2 and DC-STAMP proteins, both of which regulate the fusion in the plasma membrane.

Conclusion: LDL uptake via LDLR is essential for osteoclastogenesis.

Significance: The findings improve the understanding of the correlation between osteoclast formation and lipids.

Osteoporosis is associated with both atherosclerosis and vascular calcification attributed to hyperlipidemia. However, the cellular and molecular mechanisms explaining the parallel progression of these diseases remain unclear. Here, we used low-density lipoprotein receptor knockout (LDLR−/−) mice to elucidate the role of LDLR in regulating the differentiation of osteoclasts, which are responsible for bone resorption. Culturing wild-type osteoclast precursors in medium containing LDL-depleted serum decreased receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation, and this defect was additively rescued by simultaneous treatment with native and oxidized LDLs. Osteoclast precursors constitutively expressed LDLR in a RANKL-independent manner. Osteoclast formation from LDLR−/− osteoclast precursors was delayed, and the multinucleated cells formed in culture were smaller and contained fewer nuclei than wild-type cells, implying impaired cell-cell fusion. Despite these findings, RANKL signaling, including the activation of Erk and Akt, was normal in LDLR−/− preosteoclasts, and RANKL-induced expression of NFATc1 (a master regulator of osteoclastogenesis), cathepsin K, and tartrate-resistant acid phosphatase was equivalent in LDLR-null and wild-type cells. In contrast, the amounts of the osteoclast fusion-related proteins v-ATPase V0 subunit d2 and dendritic cell-specific transmembrane protein 1 (DC-STAMP) in LDLR−/− plasma membranes were reduced when compared with the wild type, suggesting a correlation with impaired cell-cell fusion, which occurs on the plasma membrane. LDLR−/− mice consistently exhibited increased bone mass in vivo. This change was accompanied by decreases in bone resorption parameters, with no changes in bone formation parameters. These findings provide a novel mechanism for osteoclast differentiation and improve the understanding of the correlation between osteoclast formation and lipids.

Osteoclasts, which are the cells responsible for bone resorption, are of hematopoietic stem cell origin (1). During the last decade, the major molecular mechanisms of osteoclastogenesis have been elucidated. Osteoclast precursors have been demonstrated to share properties with the monocyte/macrophage cell lineage (2, 3). Although many systemic hormones and local cytokines participate in regulating osteoclast differentiation (4, 5), the receptor activator of NF-κB (RANK) is the most critical molecule in osteoclastogenesis, acting in cooperation with macrophage colony-stimulating factor (M-CSF) in the interaction between stromal cells and cells of the osteoclast lineage (6–8). Extensive studies have indicated that the induction of osteoclast differentiation by RANKL requires the activation of signaling pathways, such as the NF-κB and MAPK pathways (including Erk1/2, p38 MAPK, and JNK), via TNF receptor-associated factor family proteins associated with the RANKL receptor RANK and via calcium signaling associated with FcγRy and DAP12 (9–11). The activation of these signaling molecules leads to the expression of osteoclastogenesis-related transcription factors such as NFATc1, which is considered the master regulator of osteoclast differentiation (12, 13). NFATc1, in turn, induces osteoclast differentiation-related molecules, including tartrate-resistant acid phosphatase (TRAP), cathepsin K (14), v-ATPase V0 subunit d2 (Atp6v0d2) (15–17) and...
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dendritic cell-specific transmembrane protein (DC-STAMP) (18–20) that regulate cell-cell fusion of preosteoclasts.

An increasing number of epidemiological studies have demonstrated that dyslipidemia is a risk factor not only for atherosclerosis and vascular calcification, but also for osteoporosis (21–24). Both elevated levels of LDL and decreased levels of HDL in plasma have been found to be correlated with low bone mass, and high triglyceride levels are associated with a decreased incidence of vertebral fractures in postmenopausal women (23). Patients with low bone densities and osteoporosis are characterized by elevated plasma lipid levels, more severe coronary atherosclerosis, and an increased risk of stroke (25).

Furthermore, an in vivo study has shown that a mouse strain with susceptibility to atherosclerosis (C57BL/6) loses a greater amount of bone mass in response to a high-fat diet than atherosclerosis-resistant strains (26). In addition, several in vitro studies have demonstrated that oxidized LDL (ox-LDL) inhibits osteoblast differentiation (27, 28), whereas an absence of native LDL suppresses osteoclast formation in cocultures of spleen cells or bone marrow cells as osteoclast progenitors and osteoclast differentiation-supporting stromal or osteoblastic cells (29, 30). Such coculture systems, however, could not provide a evidence for direct action of LDL on osteoclast precursors. Nevertheless, osteoporosis associated with an abnormal plasma lipid level is likely to be attributed to decreased bone formation, increased bone resorption, or both. In contrast, there are fewer reports on the more detailed molecular mechanisms explaining the parallel progression of these diseases.

Cholesterol is one of the major components of biological membranes and lipoproteins. It affects the structure and function of biological membranes by defining the physicochemical characteristics of the membrane, such as membrane fluidity (31, 32). In addition, the sterol affects calcium uptake, cell migration, and cell proliferation (33–35). Lipid rafts and caveolae in plasma membranes are microdomains that contain abundant cholesterol and have diverse functions, including membrane trafficking, endocytosis, regulation of cholesterol and calcium homeostasis, and signal transduction involved in cell growth and function (36–39). More recently, we found that during osteoclastogenesis, RANKL induces the expression of caveolin-1 (Cav-1) (40), a principal scaffolding protein of lipid rafts and caveolae. Furthermore, depletion of exogenous LDL causes impaired NFATc1 activation and consequently reduces osteoclast formation (40), consistent with other studies (29, 30). These results suggest a tight correlation between osteoclast differentiation and cholesterol.

Intracellular cholesterol homeostasis is strictly controlled by cholesterol uptake from the extracellular space and its intracellular de novo biosynthesis (41–43). Reduced intracellular cholesterol induces the expression of 3-hydroxy-3-methylglutaryl-CoA reductase, which is a limiting enzyme of de novo cholesterol biosynthesis (44), and LDL receptor (LDLR) (45), which is involved in cholesterol endocytosis. Conversely, increased extracellular cholesterol causes the down-regulation of LDLR expression (43). Although numerous studies have demonstrated that statins, which inhibit 3-hydroxy-3-methylglutaryl-CoA reductase, also inhibit osteoclast formation (46–49), the stringent requirement for exogenous LDL (30, 40) indicates that de novo biosynthesis does not function in osteoclast lineage cells. Indeed, osteoclast lineage cells have been shown to express very low levels of 3-hydroxy-3-methylglutaryl-CoA reductase (50), and 3-hydroxy-3-methylglutaryl-CoA reductase expression is not up-regulated upon depletion of cholesterol from the plasma membrane (30). Therefore, the uptake of exogenous cholesterol plays a more important role in regulating osteoclast differentiation than its de novo biosynthesis. Thus, we focused on the involvement of LDLR in osteoclastogenesis.

In this study, we examined the effect of LDLR deficiency on osteoclast formation using LDLR knockout (LDLR−/−) mice and found that RANKL-induced osteoclast formation from LDLR−/− osteoclast precursors was reduced because of the impaired cell-cell fusion of preosteoclasts, whereas osteoclast differentiation-related transcription factors and functional proteins, such as NFATc1, cathepsin K, and TRAP, were expressed at normal levels in response to RANKL. Similarly, RANKL induced the expression of the osteoclast fusion regulators Atp6v0d2 and DC-STAMP at levels equivalent to those observed in wild-type osteoclast lineage cells. Despite these findings, the amount of Atp6v0d2 and DC-STAMP in the plasma membrane was greatly reduced in LDLR−/− osteoclast lineage cells. The decreased level of the fusion-related proteins in the plasma membrane may explain the observed osteoclastic cell-cell fusion defect. Furthermore, femora and tibiae from LDLR−/− mice exhibited increased bone mass and moderate osteopetrosis. Thus, the results of this study indicate that the uptake of LDL via LDLR is essential for osteoclastogenesis and provide a novel mechanism for osteoclast differentiation, thereby improving our understanding of the correlation between osteoclast formation and lipids.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Recombinant human M-CSF was kindly provided by the Morinaga Milk Industry Co. (Tokyo, Japan). Recombinant mouse soluble RANKL (sRANKL) was obtained from R&D Systems (Minneapolis, MN). Anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-Akt, and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-c-Fos and anti-NFATc1 antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA). Anti-cathepsin K, anti-DC-STAMP (clone 1A2), and anti-Atp6v0d2 antibodies were purchased from BioVision (Mountain View, CA), Millipore (Temecula, CA), and AVIVA Systems Biology (San Diego, CA), respectively. Anti-Cav-1 antibody was obtained from BD Biosciences. Alexa Fluor 555-conjugated cholera toxin subunit B (recombinant), Alexa Fluor 488-conjugated chicken anti-rabbit and anti-mouse IgG antibodies, and DAPI solution were purchased from Invitrogen. FBS and lipo-protein-reduced FBS (LR-FBS) were purchased from Intergen (Purchase, NY) and HyClone (South Logan, UT), respectively. Human native LDL and ox-LDL were obtained from Kalen Bio-medical, LLC (Montgomery Village, MA).

Mice—LDLR−/− mice from a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently backcrossed into a C57BL/6J (Japan SLC, Inc., Shizuoka, Japan) background for a minimum of 10 generations.
For in vitro osteoclast formation in medium containing LR-FBS, 4- to 8-week-old male ICR mice (Japan SLC) were used. All experimental animal procedures were reviewed and approved by the Meikai University School of Dentistry animal care committee.

In Vitro Osteoclastogenesis—Femora and tibiae were obtained from 4- to 8-week-old male mice, and soft connective tissues were removed from the bones. Bone marrow cells were flushed from the bone marrow cavity and cultured for 3 days in α-minimal essential medium (ICN Biomedicals, Aurora, OH) supplemented with 10% FBS, M-CSF (100 ng/ml), and 100 units/ml of penicillin in Petri dishes in a humidified atmosphere of 5% CO₂. After removal of nonadherent cells and stromal cells by washing the dishes with PBS and subsequent incubation for 5 min in 0.25% trypsin/0.05% EDTA, adherent monocytes were harvested for use as osteoclast precursors in α-minimal essential medium/10% FBS by vigorous pipetting. The harvested osteoclast precursors were seeded in various tissue culture dishes and plates at an initial density of 2.5 × 10⁴/cm² and cultured in α-minimal essential medium/10% FBS/M-CSF (20 ng/ml) with and without sRANKL (10 ng/ml). The culture medium was exchanged with fresh medium every 2 days. After culturing for the desired time, the cells were fixed in 10% formalin and stained for TRAP activity with a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO). TRAP-positive multinucleated cells (MNCs) with more than three nuclei were considered to be osteoclastic cells and were counted under a microscope.

Bone Resorption Assay—Osteoclast precursors (8 × 10⁴ cells) isolated from wild-type and LDLR-null mice were seeded and cultured on a dentine slice (6 mm in diameter) in the presence of M-CSF and sRANKL for the desired time. Following culture, the cells were scraped off the dentine slices, and the slices were stained with acid hematoxylin (Sigma-Aldrich). The stained pit areas were viewed under a microscope using the image analysis software Sensiv MEASURE (Mitani, Tokyo, Japan) to evaluate osteoclastic dentine resorbing activity. For TRAP staining, after culturing the cells on dentine slices, the osteoclasts were fixed and stained for TRAP activity with the leukocyte acid phosphatase kit, and TRAP-positive MNCs were counted.

Preparation of Cellular Membrane Proteins—Osteoclast progenitors derived from LDLR−/− and wild-type mice were treated with M-CSF and sRANKL for the desired culture periods, the cells were washed with PBS and lysed in whole-cell lysis buffer (10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM p-aminooxyethyl-benzensulfonyl fluoride (p-ABSF), 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml aprotinin). The cell lysates were centrifuged at 12,000 × g for 10 min, and the supernatants were used as whole-cell lysates for the analysis of protein expression. The preparation of cytosol and total cellular membrane proteins, including organelle and plasma membrane proteins, was carried out using the ProteoJET™ membrane protein extraction kit (Fermentas Life Sciences, St. Leon-Rot, Germany) according to the instructions of the manufacturer. Plasma membrane proteins from LDLR−/− and wild-type preosteoclasts were isolated using a plasma membrane protein extraction kit (BioVision) following the instructions of the manufacturer.

Real-time RT-PCR—Total RNA (1 µg) extracted from cultured cells was used as the template for cDNA synthesis using a Superscript III preamplification system (Invitrogen). Real-time PCR was performed using the TaqMan universal PCR master mix on a GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA). TagMan primers for the indicated genes were obtained from Applied Biosystems. Relative quantification of target mRNA expression was calculated and normalized to the amount of 18 S rRNA and compared with the levels expressed in wild-type osteoclast precursors before treatment with sRANKL.

Western Blot Analysis—Samples of whole-cell lysates and cellular fractions containing equal amounts of protein were subjected to 12% SDS-PAGE, and the proteins separated in the gel were subsequently electrotransferred onto polyvinylidene difluoride membranes. After blocking with 5% skim milk, the membranes were incubated with the indicated antibodies followed by a peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody. Immunoreactive proteins were visualized using Western blot chemiluminescence reagents (Amer sham Biosciences) in accordance with the instructions of the manufacturer.

Confocal Laser Microscopy—The localization of Atp6v0d2 and DC-STAMP was examined by immunofluorescence histochemistry using a confocal laser microscope (LSM5, Carl Zeiss, Stuttgart, Germany). Osteoclast precursors were cultured in glass-bottom culture dishes in the presence of sRANKL and/or M-CSF for 2 days. The cells were then incubated with α-MEM/10% FBS containing 1 µg/ml Alexa Fluor 555-conjugated cholera toxin subunit B (Invitrogen) at 37 °C for 3 min and fixed in 4% paraformaldehyde/PBS at 4 °C for 40 min. Free aldehyde groups were quenched with 100 mM glycine, and the cells were washed three times with PBS. The cells were then permeabilized with 0.1% saponin in PBS for 10 min and incubated in Image-iT™ (Invitrogen) for 30 min followed by incubation in 5% BSA/10% normal chicken serum/PBS for an additional 60 min to block nonspecific binding. Next, the cells were incubated with 2 µg/ml rabbit polyclonal anti-Atp6v0d2 and 5 µg/ml mouse monoclonal anti-DC-STAMP antibodies in 5% BSA/PBS for 60 min at room temperature. Thereafter, the cells were washed with PBS and stained with Alexa Fluor 488-conjugated chicken anti-rabbit and anti-mouse IgG antibodies (Invitrogen) for 60 min. DAPI solution (300 nM, Invitrogen) was used for the counterstaining of nuclei.

Histological and Morphometric Analyses—Eight-week-old male LDLR−/− and wild-type littermates or gender/age-matched C57BL/6 mice were intraperitoneally injected with calcine (16 mg/kg, Dojindo Co., Kumamoto, Japan) 4 days and 1 day before being sacrificed to evaluate their bone formation rate. Their hind limbs were removed and fixed with 70% ethanol. Undecalcified titiae were embedded in glycol methacrylate, and longitudinal slices (3 µm) were cut with a microtome (Leica RM2255), used for toluidine blue and TRAP staining, and subjected to an analysis of bone histomorphometric parameters (bone volume (BV/TV), osteoid volume (OV/BV), osteoblast surface (Ob.S/BS), osteoclast number (Oc.N/B.Pm),...
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osteoclast surface (Oc.S/BS), eroded surface (ES/BS), bone formation rate (BFR/BS), mineral apposition rate (MAR), and mineralizing surface (MS/BS)). Furthermore, the maximum diameter of the TRAP-positive osteoclasts was measured, and the number of nuclei per TRAP-positive osteoclast on the cross-sections was counted.

Bone structural parameters (bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular spacing (Tb.Spac)) of LDL−/− and wild-type femora were measured using micro-computed tomography (μCT, Scan Xmate-L900, Comscantecno Co., Yokohama, Japan).

Statistical Analysis—The mean values of groups were compared by analysis of variance, and the significance of the observed differences was determined by post-hoc testing using Bonferroni’s method.

RESULTS

Stringent Dependence of Osteoclast Differentiation on Exogenous LDL.—To examine how exogenous cholesterol influences osteoclastogenesis, we cultured osteoclast precursors with sRANKL in medium containing normal FBS and LR-FBS. When osteoclast precursors were cultured in medium containing normal FBS, TRAP-positive mononuclear preosteoclast cells appeared in culture and began to fuse with each other to form multinucleated osteoclasts on day 2 after the addition of sRANKL, and osteoclast formation reached a maximum within 3–4 days. In contrast, when cells were cultured in medium containing LR-FBS, in vitro osteoclast formation greatly decreased, even in the presence of RANKL. When various doses of LDL were added to medium containing LR-FBS, osteoclast formation was partially restored in the culture in a dose-dependent manner (Fig. 1A), as reported previously (40). Similar to the effect of exogenous addition of native LDL, ox-LDL also dose-dependently rescued the reduced osteoclast formation (Fig. 1A). The most effective doses of each lipoprotein were between 12.5 and 25 μg/ml. In comparison to the degree of osteoclast formation observed when cells were cultured in medium containing normal FBS, however, this rescue was incomplete. Simultaneous addition of LDL and ox-LDL additively stimulated in vitro osteoclast formation, and the number of TRAP-positive osteoclasts observed was equivalent to that observed in cultures containing normal FBS (Fig. 1B). However, the osteoclasts formed in cultures containing LDL and ox-LDL were smaller than those formed in cultures containing normal FBS (Fig. 1C). The additive effect of LDL and ox-LDL on osteoclastogenesis suggested that native and denatured LDL were incorporated independently into the cells via individual receptors. Indeed, as shown in Fig. 1D, osteoclast precursors expressed LDLR and scavenger receptor class A (SR-A), which recognize and internalize native and oxidized LDL, respectively. The gene expression level of LDLR did not change during osteoclast formation, implying its constitutive and RANKL-independent expression. In contrast, although osteoclast precursors expressed SR-A mRNA, the expression level decreased with the differentiation into mature osteoclasts in response to RANKL. Taken together, these results indicate that osteoclastogenesis intrinsically requires exogenous LDL.

Impaired in Vitro Osteoclastogenesis from LDLR−/− Osteoclast Progenitors—In this study, we focused on the physiological roles of LDLR in osteoclastogenesis and analyzed LDLR-deficient (LDLR−/−) mice (51). The formation of osteoclasts from LDLR−/− osteoclast precursors was clearly delayed compared with the formation of osteoclasts from wild-type littermate-derived osteoclast precursors (Fig. 2A). In addition, although the number of TRAP-positive LDLR−/− osteoclasts equaled that of wild-type osteoclasts 3.5 days after the addition of RANKL, small osteoclasts were abundant in the culture of LDLR−/− precursors (Fig. 2A). The reduced osteoclast formation from LDLR−/− osteoclast precursors was also confirmed in cultures of osteoclast precursors on dentine slices (Fig. 2B). Both the number of TRAP-positive osteoclasts and the size of the pit areas formed on the dentine slices were greatly reduced in LDLR−/− cultures, and there was a larger reduction in osteoclast formation from the osteoclast precursors cultured on dentine slices than those grown on culture plates. To address the molecular mechanism involved in the impaired osteoclastogenesis from LDLR−/− osteoclast precursors, we examined the activation of Erk and Akt, which are involved in signaling pathways that play important roles in initial osteoclast differentiation and survival (52–54), respectively. Osteoclast precursors pre cultured with M-CSF alone and preosteoclasts pre cultured with M-CSF/sRANKL were starved in serum-free media for 3 h and then retreated with sRANKL. The RANKL treatment induced comparable activation of both signal transduction molecules in LDLR−/− and wild-type preosteoclasts, whereas the activation of Erk in LDLR−/− osteoclast precursors appeared to be greater than that in wild-type osteoclast precursors (Fig. 3A). Moreover, the temporal gene and protein expression profiles of c-Fos and NFATc1, which are crucial transcription factors for osteoclast differentiation, did not differ between the LDLR−/− and wild-type osteoclast lineage cells during osteoclastogenesis (Fig. 3, B and C). Furthermore, we consistently found that the expression of the osteoclast function-related molecules TRAP and cathepsin K was equivalent in both genotypes (Fig. 3, B and C). These results suggest that the impaired osteoclast formation observed in LDLR−/− mice does not result from altered signal transduction pathways or expression of osteoclast differentiation-associated molecules.

Defective Cell-Cell Fusion of LDLR−/− Preosteoclasts—As the size of osteoclasts formed from LDLR−/− osteoclast precursors was small (Fig. 2A), we measured the maximum diameter and area of LDLR−/− osteoclasts randomly selected from the cultures (Fig. 4A). After culturing the cells for 3 days, the mean maximum diameter and area of LDLR−/− multinucleated osteoclasts decreased by 40 and 60%, respectively, when compared with osteoclasts derived from wild-type littermates. Furthermore, the fusion index, which was expressed as the mean number of nuclei per multinucleated osteoclast (55), in cultures of LDLR−/− osteoclast precursors was ~20% of that observed in cultures of wild-type osteoclast precursors (Fig. 4D). Because the fusion index indicates the frequency of cell fusion to form multinucleated osteoclasts, the decreased fusion index indicated that the decreased osteoclast formation of the LDLR−/− mice was due to impaired cell-cell fusion of mononuclear preosteoclasts into mature multinucleated osteoclasts.
Therefore, to address the mechanism underlying the fusion defect, we examined the LDLR/H11002/H11002 preosteoclast expression levels of Atp6v0d2 and DC-STAMP, which are essential for the cell-cell fusion of preosteoclasts (15–20). The messenger RNA expression of both fusion proteins in LDLR/H11002/H11002 osteoclast lineage cells was comparable with that observed in wild-type cells during osteoclastogenesis (Fig. 5A), and similar results were observed for the osteoclast differentiation-related molecules TRAP and cathepsin K (Fig. 3B). The induction of these mRNAs began on day 1 after the addition of sRANKL and reached a maximum on day 3 (mature osteoclast stage). The RANKL-induced protein levels of Atp6v0d2 and DC-STAMP in LDLR−/− osteoclast lineage cells were consistently equivalent to those observed in wild-type osteoclast lineage cells (Fig. 5B).

**Decreased Amounts of the Osteoclast Fusion Proteins Atp6v0d2 and DC-STAMP in the Plasma Membrane in LDLR−/− Preosteoclasts—**Cell-cell fusion events occur on the plasma membrane of cells. Thus, we determined the localization of Atp6v0d2 and DC-STAMP in osteoclast lineage cells. Osteoclast precursors derived from LDLR−/− and wild-type mice were treated with sRANKL for 2 days, and the whole-cell lysates, cytosolic fractions, and total cellular membranes, including intracellular organelle membranes and plasma mem-

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**Figure 1.** Suppression of *in vitro* osteoclast formation in the absence of exogenous LDL. Osteoclast precursors obtained from bone marrow cells treated with M-CSF for 3 days were cultured in medium containing normal FBS or lipoprotein-reduced FBS (LR-FBS) with or without various doses of LDL or ox-LDL (A) or a fixed dose (15 μg/ml) of LDL and/or ox-LDL (B) in the presence of M-CSF (20 ng/ml) and sRANKL (10 ng/ml) for 3 days. After culturing, the cells were stained for TRAP activity, and the number of TRAP-positive MNCs per well was counted. The experiments were performed in triplicate, and reproducibility was confirmed. The values presented are the mean ± S.E. of four cultures from a representative experiment. *, p < 0.05 versus cells cultured in medium containing LR-FBS without LDL or ox-LDL; **, p < 0.05 versus cells cultured with LDL or ox-LDL alone. C, photographs of TRAP-stained osteoclastic cells in cultures shown in B. Scale bar = 500 μm. D, osteoclast precursors were cultured in medium containing normal FBS with or without sRANKL in the presence of M-CSF for the indicated times, and total RNA was then prepared and subjected to real-time RT-PCR for LDLR, SR-A, or 18 S rRNA.
branes, were then fractionated (Fig. 5C). To determine the cross-contamination in each total cellular membrane and cytosolic fraction, we employed an immunoblot of Cav-1, which is a membrane-bound principal scaffolding protein of lipid rafts in the plasma membrane (39). Cav-1 was detected only in whole-cell lysate and total cellular membranes but not in the cytosolic fraction, which indicated that there was no cross-contamination between the fractions (Fig. 5C). The amounts of Atp6v0d2 in the whole-cell lysates and total cellular membranes were equivalent in LDLR−/− and wild-type preosteoclasts (Fig. 5C). On the other hand, despite its original property as a membrane protein, DC-STAMP was not detected in the total cellular membranes, and most of the protein was abundant in the cytosolic fraction, whereas the amounts of DC-STAMP in the whole-cell lysates and cytosolic fraction were comparable between LDLR−/− and wild-type preosteoclasts (Fig. 5C). Confocal immunofluorescence consistently showed that most DC-STAMP remained in the cytoplasm (supplemental Fig. S1).

Thus, we prepared plasma membrane proteins excluding organelle membrane proteins from both preosteoclasts of both genotypes and compared the amounts of the fusion proteins. The plasma membranes derived from LDLR−/− preosteoclasts contained less Atp6v0d2 than those derived from wild-type preosteoclasts (Fig. 5D). In contrast, the amount of Cav-1 in the plasma membranes of the LDLR−/− preosteoclasts was compa-
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Expression and localization of the osteoclast fusion proteins Atp6v0d2 and DC-STAMP in LDLR−/− osteoclast lineage cells. A, osteoclast precursors derived from LDLR−/− mice and wild-type littermates were cultured with sRANKL for 6 h or the indicated number of days (D). After culturing, the cells were extracted, and total RNA and cell lysates were subjected to real-time RT-PCR (A) and Western blot analysis (B), respectively, to determine the expression levels of Atp6v0d2 and DC-STAMP. The experiments were performed in triplicate, and reproducibility was confirmed. C and D, osteoclast precursors derived from both genotypes were cultured with M-CSF and sRANKL for 2 days. Thereafter, whole-cell lysates, total cellular membranes (TCM) containing proteins from both plasma membranes and cellular organelle membranes, cytosol fractions (C), and plasma membranes (PM) excluding cellular organelle membranes (D) were prepared as described under “Experimental Procedures.” Samples of each fraction were subjected to Western blot analysis for Atp6v0d2, DC-STAMP and Cav-1 protein levels. The experiments were performed four times, and reproducibility was confirmed.

Bone Mass Increases in LDLR−/− Mice because of Decreased Osteoclast Numbers—Finally, we analyzed femora and tibiae from 8-week-old wild-type and LDLR−/− mice histomorphometrically and microradiographically. Two- and three-dimen-sional μCT results clearly showed that the trabecular bone mass of femora and tibiae from LDLR−/− mice increased compared with wild-type mice (Fig. 7A). We consistently observed significant increases in the bone volume to tissue volume ratio (BV/TV) and the trabecular number (Tb.N) in the LDLR null mice, whereas trabecular thickness did not differ between the mice of the two genotypes (Fig. 7B). In contrast, decreased trabecular separation was observed in LDLR−/− mice. Fig. 7, C and D, shows toluidine blue and TRAP stainings of mesial tibiae. Bone morphometric analysis showed that the surface area char-
FIGURE 6. Determination of the localization of Atp6v0d2 in osteoclast lineage cells by confocal laser microscopy. Osteoclast precursors derived from wild-type (WT, a–h and q–s) and LDLR−/− (i–p) mice were cultured in the presence of sRANKL for 2 days. The cells were then treated with (a–p) or without (q–s) Alexa Fluor 555-conjugated CT-B prior to fixation. The fixed cells were incubated with an anti-Atp6v0d2 antibody (a–p) or normal rabbit IgG (q–s) and subsequently with DAPI solution. Fluorescence images for DAPI (blue), anti-Atp6v0d2 (green), and CT-B (red) were merged. Scale bars in (a–d, i–l) and (e–h, m–p, and q–s) = 10 μm and 20 μm, respectively.

ROLE OF LDL AND LDL RECEPTOR IN OSTEOCLASTOGENESIS

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FIGURE 6. Determination of the localization of Atp6v0d2 in osteoclast lineage cells by confocal laser microscopy. Osteoclast precursors derived from wild-type (WT, a–h and q–s) and LDLR−/− (i–p) mice were cultured in the presence of sRANKL for 2 days. The cells were then treated with (a–p) or without (q–s) Alexa Fluor 555-conjugated CT-B prior to fixation. The fixed cells were incubated with an anti-Atp6v0d2 antibody (a–p) or normal rabbit IgG (q–s) and subsequently with DAPI solution. Fluorescence images for DAPI (blue), anti-Atp6v0d2 (green), and CT-B (red) were merged. Scale bars in (a–d, i–l) and (e–h, m–p, and q–s) = 10 μm and 20 μm, respectively.
characterized by bone resorption compared with the total bone surface area in LDLR−/− mice was attenuated compared with that observed in wild-type mice (Fig. 7E). Similar to our observations during in vitro osteoclast formation, decreases in osteoclast number/bone perimeter and osteoclast surface/bone surface were particularly obvious in the LDLR−/− mice. In

FIGURE 7. Increased bone mass in LDLR-deficient mice. Femora of 8-week-old male LDLR−/− and wild-type mice were radiologically analyzed by μCT (A and B). The two left panels in A show two-dimensional reconstructions of the epiphysis and metaphysis of distal femora from LDLR−/− and control wild-type (+/+ ) mice, and the two right panels in A show three-dimensional reconstructions of secondary spongy bone of the metaphysis. B, histograms represent bone structural parameters: bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th). Mesial tibiae from LDLR−/− and wild-type (+/+ ) mice were stained with toluidine blue (C) and for TRAP activity (D), and histomorphometric parameters (E), including osteoid volume (OV/BV), osteoblast surface (Ob.S/BS), bone formation rate (BFR/BS), mineral apposition rate (MAR), mineralizing surface (MS/BS), osteoclast number (Oc.N/B.Pm), eroded surface (ES/BS), and osteoclast surface (Oc.S/BS) were determined. Values are the mean ± S.E. for five bones each from LDLR−/− and wild-type mice. *p < 0.05 versus wild-type mice. NS, not significant. F, the maximum diameter of TRAP-positive osteoclasts (n > 800) and nuclei per TRAP-positive osteoclast (n ≥ 100) on the cross-sections were measured and counted, respectively. *p < 0.01 versus wild-type mice. Scale bars = 1 mm, 800 μm, 500 μm, and 100 μm in the left panels in A and the right panels in A, C, and D, respectively.
addition, the average number of nuclei per TRAP-positive osteoclast and the maximum diameter of the osteoclasts in cross-sections of LDLR−/− mice were reduced to approximately half of those observed in wild-type mice, suggesting the impaired in vivo cell-cell fusion of LDLR−/− preosteoclasts (Fig. 7F). In contrast, bone formation parameters such as osteoid volume/bone volume, osteoblast surface/bone surface, bone-forming rate/bone surface, mineral apposition rate, and mineralizing surface/bone surface did not differ between wild-type and LDLR−/− mice. Taken together, these results indicate that LDLR−/− mice exhibit moderately increased bone mass because of decreased osteoclast formation. However, LDLR deficiency did not influence bone formation.

**DISCUSSION**

In this study, we showed that osteoclastogenesis stringently required exogenous native LDL and modified LDLs, which are integral carrier proteins involved in cholesterol uptake into cells. The impairment in osteoclast formation because of the removal of exogenous LDL was additively rescued by simultaneous treatment with LDL and ox-LDL, indicating the existence of distinct incorporation pathways for native and modified LDLs. In support of this hypothesis, osteoclast precursors constitutively expressed both LDLR, which is specific for native LDL, and SR-A, which is specific for modified LDL. In this study, we focused on the physiological roles of LDLR in regulating osteoclastogenesis and attempted to elucidate its roles using LDLR-deficient mice. Deletion of the LDLR gene caused a decrease in osteoclast formation because of the impaired cell-cell fusion of preosteoclasts. Nevertheless, Atp6v0d2 and DC-STAMP, which are essential cell fusion proteins for the production of multinucleated osteoclasts (15–20), were expressed at levels equivalent to those observed in wild-type osteoclast lineage cells. Furthermore, no differences were observed in the signal transduction pathways necessary for osteoclast differentiation, such as RANKL-induced activation of Erk and Akt, between LDLR−/− and wild-type preosteoclasts. In addition, the expression of c-Fos and NFATc1, which are integral transcription factors involved in osteoclast differentiation, and the osteoclast differentiation-related proteins TRAP and cathepsin K did not differ between osteoclast lineage cells of the two genotypes. In contrast, the amount of Atp6v0d2 and DC-STAMP in the plasma membranes of LDLR−/− preosteoclasts was clearly less than that observed in wild-type preosteoclasts, suggesting that the reduced amounts of Atp6v0d2 and DC-STAMP in the plasma membrane result in the cell fusion defect observed in the LDLR−/− preosteoclasts. Consistent with the results of in vitro experiments, LDLR−/− mice showed increased bone mass because of a reduction in osteoclast numbers but did not exhibit altered bone formation in vivo.

Numerous studies have indicated a critical involvement of cholesterol in the differentiation and function of a variety of cell types, including endothelial cells, cardiac muscle cells, macrophages, and osteoblasts (57–59). It is well known that cholesterol is a major component of the plasma membrane, and especially of lipid rafts (36–39). Cholesterol is intrinsically produced in the liver and transferred to individual cells via the blood (60). For individual cells, there are two sources of cholesterol: cholesterol synthesized in the endoplasmic reticulum (61) and cholesterol acquired from the extracellular space via LDLR-mediated endocytosis (45). Both pools of cholesterol require proper intracellular transport to reach their final destinations. Therefore, intracellular cholesterol homeostasis is tightly controlled by the de novo synthesis and uptake of cholesterol. However, the facts that intracellular de novo cholesterol biosynthesis did not function well in osteoclast lineage cells (30, 50) and that osteoclastogenesis highly depended on exogenous LDL (as shown in this study) suggest that LDLR plays important roles in the uptake of cholesterol into osteoclast lineage cells and in regulating osteoclast differentiation.

The study presented here demonstrated constitutive RANKL-independent expression of LDLR mRNA in osteoclast precursors during osteoclastogenesis. Using LDLR−/− mice, we found that the osteoclastogenesis of LDLR−/− osteoclast precursors was obviously delayed compared with that of wild-type osteoclast precursors. In addition, osteoclasts formed from LDLR-deficient precursors were smaller than wild-type osteoclasts, and the LDLR-deficient osteoclasts contained fewer nuclei, indicating impaired cell-cell fusion of osteoclast lineage cells. One possible mechanism underlying this cell fusion defect is that the lack of the LDLR gene results in abnormalities in signal transduction pathways necessary for osteoclast formation, as we reported previously that methyl-β-cyclodextrin-mediated depletion of cholesterol from the plasma membrane and destruction of lipid rafts caused alterations in signal transduction related to osteoclastogenesis, such as hyperactivation of Erk1/2 and insensitivity of Akt to RANKL stimulation (40). Removal of exogenous lipoproteins from the culture medium recapitulated this abnormal signaling and also resulted in reduced osteoclast formation because of the delayed expression of NFATc1 in preosteoclasts (40). However, no abnormalities were observed in these signaling pathways in LDLR−/− osteoclast lineage cells. Therefore, the impaired cell-cell fusion observed in LDLR−/− osteoclast lineage cells is not due to this type of abnormality. Although the cause of the discrepancies between studies using LDLR−/− mice and those using MCD-treated cells is not understood at present, it is likely that MCD is able to remove large amounts of cholesterol from the plasma membrane (62), and this cholesterol depletion destroys lipid rafts, resulting in intense disorder in the molecular microenvironment around RANK, including certain adaptor proteins or suppressors. In contrast, the uptake, intracellular degradation and reutilization of modified LDL through SR-A remain intact in LDLR−/− osteoclast precursors and preosteoclasts, and thus, the integrity of the plasma membrane might be partially maintained. In addition, NFATc1, a master regulator of osteoclast differentiation, and the osteoclast functional molecules cathepsin K and TRAP were expressed at normal levels in the LDLR−/− cells in response to RANKL, suggesting other mechanisms explaining the impaired cell-cell fusion of preosteoclasts.

In this study, we found that osteoclast formation was decreased in LDLR−/− mice because of the impaired cell-cell fusion of osteoclast precursors. Similar to our study, Luegmayr et al. (30) demonstrated that osteoclast formation was reduced in LDLR−/− mice and that the osteoclasts formed in culture...
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Atp6v0d2 and DC-STAMP have been identified as essential regulators of osteoclast fusion on the basis of studies using knockout mice for these genes (15, 18). It has been reported recently that Atp6v0d2 interacts with adhesion-regulating molecule 1 (ADAM1) protein and regulates osteoclast multinucleation (16) and that DC-STAMP cooperates with osteoclast stimulatory transmembrane protein (OC-STAMP) in promoting cell-cell fusion of preosteoclasts (63). In addition, Atp6v0d2 has been shown to possess the differentiation-specific dual functions as a regulator of cell-cell fusion at the early preosteoclast stage and as an essential component of the osteoclast-specific proton pump in bone resorption (17). However, the detailed molecular mechanism underlying the involvement of the proteins in the cell-cell fusion of preosteoclasts remains to be clarified.

Atp6v0d2- and DC-STAMP-deficient mice display a common phenotype with respect to osteoclastogenesis; namely, the multinucleation of preosteoclasts is intrinsically impaired, whereas expression of the osteoclast master regulator NFATc1 and the osteoclast differentiation-related proteins TRAP and cathepsin K is comparable with that of wild-type osteoclast lineage cells. Thus, mice deficient in both genes exhibited the phenotype of LDLR−/− preosteoclasts was very low and was equivalent to the level in wild-type preosteoclasts (data not shown), suggesting that the induction of apoptosis might not be an important cause of the observed decrease in osteoclast formation. In addition, these authors did not report on the impairment of cell-cell fusion of osteoclast lineage cells in LDLR−/− mice. Our finding that the numbers of nuclei per osteoclast formed in vitro and per TRAP-positive osteoclast in cross-sections of tibiae in vivo were reduced in LDLR−/− mice obviously indicates that the impaired cell-cell fusion because of LDLR deficiency plays a more significant role in reducing osteoclast formation.

With respect to DC-STAMP function as a fusion protein, it has been reported recently that RANKL induces two distinct preosteoclast populations expressing high and low levels of DC-STAMP (DC-STAMPh and DC-STAMPlo, respectively) on the cell surfaces, that DC-STAMPlo cells act as master fusogens and that DC-STAMPh cells function as mononuclear donors (19). In addition, the mixed cultures of DC-STAMPlo and DC-STAMPh cells effectively formed larger and more nucleated osteoclasts than cultures of each pure cell population (19). Furthermore, the level of cell surface DC-STAMP on osteoclast lineage cells was down-regulated during osteoclast differentiation, possibly because of increased internalization of DC-STAMP (19, 20). In this study, we did not detect DC-STAMP in the total membrane protein fractions, whereas the proteins were abundant in cytosolic fractions. At present, we do not know the reason why DC-STAMP was undetectable in the total membrane fractions, which included intracellular organelle membranes such as lysosomes and Golgi apparatus. Furthermore, a larger amount of plasma membrane protein was required for the detection of surface DC-STAMP than for the detection of Atp6v0d2 in plasma membrane fractions, implying the down-regulation of cell surface DC-STAMP. Nevertheless, the detectable level of DC-STAMP proteins in the plasma membranes in LDLR−/− preosteoclasts was greatly reduced compared with that of wild-type cells, as was the level of Atp6v0d2 protein. These results suggest that the reduced levels of the fusion proteins in plasma membranes might be a major cause of the impaired cell-cell fusion of the LDLR−/− preosteoclasts. Although the mechanism underlying the reduction remains unclear, the reduction might be caused by the decreased transport of the fusion proteins to the plasma membranes or the increased clearance of the proteins from the plasma membranes, or both. Consequently, the phenotype of LDLR−/− mice mimicked that of Atp6v0d2−/− and DC-STAMP−/− mice (15, 18) with respect to osteoclastogenesis and the impaired cell-cell fusion of osteoclast lineage cells. Furthermore, despite the decrease in Atp6v0d2 and DC-STAMP in the plasma membranes of the knockout mice, the amount of Cav-1 in the membranes did not differ between osteoclast lineage cells of the two genotypes, suggesting a mechanism for Atp6v0d2- and DC-STAMP-specific localization associated with intracellular cholesterol content. Further studies will be required to elucidate the molecular mechanisms.

In conclusion, LDLR−/− mice exhibited increased bone mass in vivo. This change was accompanied by decreases in bone resorption parameters with no changes in bone formation parameters. Consistently, in vitro osteoclastogenesis was highly dependent on exogenous LDL, and LDLR deficiency impaired osteoclast formation because of the reduced cell-cell fusion of preosteoclasts. However, the osteoclast differentiation-related transcription factor NFATc1, the related functional proteins cathepsin K and TRAP, and the osteoclast cell fusion proteins Atp6v0d2 and DC-STAMP were expressed in LDLR−/− osteoclast lineage cells at levels equivalent to those observed in wild-type cells. In contrast, the levels of Atp6v0d2 and DC-STAMP proteins at the plasma membrane were significantly
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decreased in the LDLR−/− osteoclast lineage cells. Taken together, the results presented here provide novel insight regarding the correlation between bone and lipid metabolism.

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Low-density Lipoprotein Receptor Deficiency Causes Impaired Osteoclastogenesis and Increased Bone Mass in Mice because of Defect in Osteoclastic Cell-Cell Fusion

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