Bone Formation Is Coupled to Resorption Via Suppression of Sclerostin Expression by Osteoclasts

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ABSTRACT

Bone formation is coupled to bone resorption throughout life. However, the coupling mechanisms are not fully elucidated. Using Tnfrsf11b-deficient (OPG–/–) mice, in which bone formation is clearly coupled to bone resorption, we found here that osteoclasts suppress the expression of sclerostin, a Wnt antagonist, thereby promoting bone formation. Wnt/b-catenin signals were higher in OPG–/– and RANKL-transgenic mice with a low level of sclerostin. Conditioned medium from osteoclast cultures (Ocl-CM) suppressed sclerostin expression in UMR106 cells and osteocyte cultures. In vitro experiments revealed that osteoclasts secreted leukemia inhibitory factor (LIF) and inhibited sclerostin expression. Anti-RANKL antibodies, antiresorptive agents, suppressed LIF expression and increased sclerostin expression, thereby reducing bone formation in OPG–/– mice. Taken together, osteoclast-derived LIF regulates bone turnover through sclerostin expression. Thus, LIF represents a target for improving the prolonged suppression of bone turnover by antiresorptive agents. © 2017 The Authors. Journal of Bone and Mineral Research Published by Wiley Periodicals Inc.

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Introduction

Bone is remodeled by bone resorption and formation to maintain calcium homeostasis.1,2 Osteoclasts, bone-resorbing multinucleated cells, differentiate from the monocyte/macrophage-lineage cells. The differentiation of osteoclasts is regulated by receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG), a decoy receptor of RANKL, both of which are secreted from osteoblast-lineage cells such as osteoblasts and osteocytes.3,4 OPG-deficient (OPG–/–) mice exhibited severe osteopenia with enhanced bone resorption and formation.5,6 The administration of bisphosphonates, anti-bone-resorbing agents, into OPG–/– mice successfully inhibited bone resorption, and the enhanced bone formation was also suppressed.7 Furthermore, the administration of an anti-RANKL antibody into wild-type (WT) mice also inhibited bone resorption followed by the suppression of bone formation.8–10 These findings demonstrated that bone resorption is linked to bone formation by factors facilitating the transition from bone resorption to formation, so-called coupling factors.

Currently, several factors are considered to act as coupling factors between bone resorption and formation. Transforming growth factor beta (TGF–b) and insulin-like growth factor I (IGF-I) released from bone matrix during bone resorption led to bone formation by inducing the migration of osteoblast precursors to bone resorption sites.9,10 Soluble factors secreted from osteoclasts such as cardiotrophin-1 (CT-1), Wnt10b, Sphingosine-1-phosphate (S1P), BMP-6, collagen triple-helix repeat-containing 1 (Cthr1), and platelet-derived growth factor (PDGF)-BB were reported to promote the differentiation of osteoblasts and bone formation.1,11–15 EphrinB2, expressed by

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osteoclasts, also promotes the differentiation of osteoblasts through EphB4 receptors. In contrast, Semaphorin 4D, expressed by osteoclasts, directly inhibits the differentiation of osteoblasts through Plexin-B1. Thus, bone remodeling is a complicated process, in which osteoclasts and osteoblasts are involved. Currently, it has not been fully elucidated how osteocytes contribute to the coupling process between bone resorption and formation.

Sclerostin (encoded by the Sost gene), an antagonist of Wnt/β-catenin signaling, is secreted from osteocytes and inhibits bone formation. The administration of an anti-sclerostin-neutralizing antibody has been shown to increase bone mass. The expression of sclerostin was reportedly suppressed by mechanical stimulation, parathyroid hormone (PTH), prostaglandin E2 (PGE2), and IL-6 family members, such as oncostatin M (OSM), leukemia inhibitory factor (LIF), and CT-1. OSM, LIF, and osteoclast-derived CT-1 promoted bone formation in vitro and in vivo. Thus, it has been proposed that osteoclast-derived CT-1 as a coupling factor suppresses sclerostin expression in osteocytes to promote transitions from bone resorption to formation. Furthermore, several studies in humans and mice demonstrated that the expression of sclerostin was decreased in conditions that enhanced bone resorption such as osteoporosis. TGF-β induced the expression of LIF in osteoclasts. However, it remains to be clarified how bone resorption regulates the expression of sclerostin during bone remodeling.

Here, we found that osteoclast-secreted factors, including LIF, suppress the expression of sclerostin, thereby promoting bone formation. Thus, osteoclast-derived LIF as well as CT-1 suppresses the expression of sclerostin to regulate bone remodeling.

Materials and Methods

Mice and reagents

Seven-week-old male mice and newborn mice of the ddY strain were obtained from Japan SLC (Shizuoka, Japan) for in vitro experiments. RANKL-Tg mice (C57BL/6 background), which express a soluble form of RANKL using the human serum amyloid P component promoter, were generated in one of the authors' laboratories. RANKL-Tg mice were bred with WT mice (strain C57BL/6), which were purchased from Japan SLC. RANKL-Tg and WT littermates were used in this study. OPG−/− mice (C57BL/6 background) were purchased fromCLEA Japan (Tokyo, Japan). OPG−/− and OPG+/− littermates (WT) were used. Twelve-week-old male mice were used in this study. For the animal experiments, no statistical method was used to predetermine sample size. The number of mice in the respective group was determined according to our previous study. Mice used for experiments were randomly chosen from each line. The mice were housed at maximum 5 mice per cage. All mice were fed a normal diet (CLEA, CE-2). All mice were housed in a specific-pathogen-free facility in Matsumoto Dental University at 24°C ± 2°C and 50% to 60% humidity with a 12-hour light/dark cycle, and were provided with sterilized water and diets ad libitum. All procedures for animal care were approved by the Animal Management Committee of Matsumoto Dental University and performed accordingly. Neutralizing antibodies against mouse RANKL (clone OYC1) and recombinant GST-RANKL were from Oriental Yeast (Tokyo, Japan). Recombinant human M-CSF (Leukoprol) was obtained from Kyowa Hakko (Tokyo, Japan). Recombinant mouse LIF was obtained from Merck Millipore (Darmstadt, Germany). PGE2 was obtained from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals and reagents were of analytical grade.

Immunohistochemistry

To determine the expression of sclerostin, WT, RANKL-Tg, and OPG−/− mice were examined by immunocytochemistry at age 12 weeks. Dissected tibias were fixed in 4% paraformaldehyde (PFA), decalcified with 10% EDTA, and embedded in paraffin. Histological sections were prepared and stained for tartrate-resistant acid phosphatase (TRAP, a marker of osteoclasts). For immunohistochemical staining, histological sections were incubated with an anti-mouse sclerostin antibody (AF1589, R&D Systems, Minneapolis, MN, USA), anti-mouse LIF antibody (AB-449-NA, R&D Systems), anti-osterix antibody (ab22552, Abcam, Cambridge, UK), or anti-β-catenin antibody (ab32572, Abcam) at 4°C overnight. After washing, the sections were incubated with an anti-goat Alexa Fluor488 secondary antibody (A-11055, Thermo Fisher Scientific, Rockford, IL, USA) or anti-goat HRP-conjugated secondary antibody (sc-2056, Santa Cruz Biotechnology, Dallas, TX, USA). The HRP-conjugated antibody was visualized using a DAB kit (DAKO, Carpinteria, CA, USA). Cells were counterstained with propidium iodide (PI) or hematoxylin as described previously.

The sclerostin-positive area and bone area in cortical bone beginning 1 mm from the distal end of growth plate in the anterior tibia were measured with ImageJ software (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij). The number of osteocytes were measured by histomorphometry and expressed as the ratio of sclerostin area/bone area (%), sclerostin-positive area/osteocyte (μm²), and number of osteocytes/bone area (cells/mm²) in fluorescence image. In DAB staining, the number of sclerostin-positive cells were measured by histomorphometry and expressed as the ratio of number of sclerostin-positive osteocytes/bone area (N/mm²). In DAB staining, the number of β-catenin-, LIF-, and osterix-positive cells were measured by histomorphometry and expressed as the ratio of number of positive cells/bone perimeter (N/mm).

To determine the effects of the anti-mouse RANKL antibody on the expression of sclerostin in osteocytes, the antibody (5 mg/kg, sc) was administered once to 8- or 11-week-old OPG−/− mice. For histological analysis, the antibody was administered at age 8 weeks. For analysis of bone histomorphometry, bone mineral density (BMD), serum and bone marrow aspirate markers, and for real-time RT-PCR, the antibody was administered at age 11 weeks.

Measurement of the distance between osteoclasts and osteocytes

The examination areas in cortical bone of 12-week-old OPG−/− mice were in the anterior tibia beginning 1 mm from the distal end of the growth plate. The shortest distance between Howship’s lacunae and lacunae of sclerostin-negative or positive osteocytes was determined at 30 locations in 3 independent sections prepared from each mouse, then the mean value was calculated (Fig. 4A). Measurements were performed blindly by 2 persons.

Bone histomorphometry

For fluorescent labeling of mineralization sites in OPG−/− mice, calcine (Sigma-Aldrich, St. Louis, MO, USA; 10 mg/kg, sc) was injected at 48-hour intervals on days 3 and 5. Five mice of each
group were euthanized on day 7. Their femurs were fixed in 70% ethanol and stained with Villanueva Goldner to identify cellular components. Embedded sections in glycol-methacrylate were subjected to histomorphometric analysis. Images were also visualized by fluorescence microscopy. Bone histomorphometry was evaluated blindly by independent histologists. When the injection of 2nd calcein was not double-labeled in anti-mouse RANKL antibody treatment group, it was excluded from the statistics. In experiments of RANKL-Tg mice, calcein was injected at 72-hour intervals on days 0 and 3. Five mice of each group were euthanized on day 5. When the trabecular bone in RANKL-Tg mice was not in the measurement area, it was excluded from the statistics. Nomenclature and units were used according to the guidelines of the histomorphometry nomenclature committee of the American Society for Bone and Mineral Research.\(^{18}\)

**Measurement of BMD**

BMD was measured in fixed femurs by peripheral quantitative computed tomography (pQCT, XCTResearch SA+, Stratec Medizintechnik, Pforzheim, Germany) using a voxel size of 0.08 × 0.08 × 0.46 mm. Image analysis was carried out using integrated XCT 2000 software version 6.00f. Cortical BMD was defined over 690 mg/cm\(^2\) at 4.5 mm from the end of the epiphysis.

**Measurements of serum and bone marrow aspirate markers**

Mouse serum and bone marrow aspirate samples were collected and subjected to ELISA analysis as described previously.\(^{19}\) Serum activities of TRAP5b and alkaline phosphatase (ALP) were determined using a mouse TRAP assay kit (SBA Sciences, Turku, Finland) and an ALP kit (Wako). Bone marrow aspirate samples were collected from femurs and tibias with 0.5 mL saline. The amounts of sclerostin in bone marrow aspirates were determined by ELISA (Quantikine Mouse/Rat Sost ELISA kit, R&D Systems).

**Real-time RT-PCR**

For preparing total RNA, cultured cells were lysed directly in TRIzol Reagent (Thermo Fisher Scientific). After removing bone marrow cells, the tibias were homogenized in TRIzol using TissueLyser II (Qiagen, Venlo, Netherlands). Total RNA was isolated using RNA isolation kits (PureLink RNA mini kit, Thermo Fisher Scientific) and RNase-free DNase I (Qiagen). First-strand cDNA was synthesized from total RNA using oligo (dT)\(_{12-18}\) primers (Thermo Fisher Scientific) and ReverTra Ace (ToYoBo, Osaka). Real-time RT-PCR for the quantification of cDNA was performed using Fast SYBR Green (Thermo Fisher Scientific) and StepOnePlus system (Thermo Fisher Scientific). The following temperature profile was used: 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. We validated the melting curve and product size in each PCR experiment. Sequences of the primers used for PCR and real-time PCR analyses are described in Supplemental Table S1. Expression levels were calculated using a relative standard curve. Gapdh was used as an internal control for normalization.

**Collection of conditioned medium from osteoclasts or bone marrow–derived macrophages (BMMs)**

Mouse BMMs were prepared as osteoclast precursors as described previously.\(^{19}\) BMMs were cultured in αMEM (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA) in 96-well plates (1 × 10\(^4\) cells/well) with GST-RANKL (200 ng/mL) in the presence of macrophage colony-stimulating factor (M-CSF) (50 ng/mL). Osteoclasts were formed on day 3 in these cultures. The culture medium was changed every 1.5 or 2 days, and conditioned medium from osteoclast cultures (Ocl-CM) was collected from these cultures on days 2, 3.5, and 5. As a control, the BMM culture medium was changed every 1.5 or 2 days, and conditioned medium from BMM cultures (BMM-CM) was collected from BMM cultures treated with only M-CSF on days 2, 3.5, and 5. These conditioned media were filtered (0.45-μm pore size filter; Merck Millipore) and stored at −80°C until further use.

**UMR106 cell cultures**

UMR106 cells (rat osteosarcoma cell line) were obtained from the American Type Culture Collection. Cells were tested for mycoplasma contamination using DAPI staining. The cells were not identified because they showed a typical morphology of UMR 106 cells. UMR106 cells were cultured in medium (αMEM containing 10% FBS) containing 50% Ocl-CM or BMM-CM for 48 hours.

**Osteocyte-like cell cultures**

Primary osteoblasts were prepared from newborn mouse calvariae as described previously.\(^{40}\) Primary osteoblasts (1 × 10\(^4\) cells/well) were cultured in the osteogenic medium (100 μg/mL ascorbic acid and 5 mM β-glycerophosphate from Wako in αMEM containing 10% FBS) in 6-well plates for 5 weeks to differentiate into osteocyte-like cells. The expression of sclerostin was confirmed in the cultured osteocyte-like cells as described previously.\(^{25,41}\) The osteocyte-like cells were cultured in the osteogenic medium containing 50% Ocl-CM or BMM-CM for 48 hours.

**Antibody array analysis**

Soluble factors were detected using an antibody array focusing on cytokines, chemokines, and growth factors (Mouse XL cytokine array kit, R&D Systems) according to the manufacturer's instructions. Briefly, 500 μL of Ocl-CM or BMM-CM was used. After exposure to X-ray film, the intensities of positive signals were measured using ImageJ software (NIH).

**Western blot analysis**

Cells were lysed in 0.1% NP-40 lysis buffer (20 mM Tris [pH 7.5], 50 mM β-glycerophosphate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM Na\(_3\)VO\(_4\), and 1 × protease inhibitors cocktail [Sigma-Aldrich]). Whole cell extracts were electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (Clear blot P membrane, Atto, Tokyo). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), the membrane was incubated with an anti-mouse sclerostin antibody (R&D Systems) or an anti-β-actin antibody (A5441, Sigma-Aldrich) in TBS-T containing 5% skim milk. After washing, the membrane was incubated with HRP-conjugated secondary antibody, and the bound antibodies were visualized using ECL (GE Healthcare Life Sciences, Little Chalfont, UK), followed by exposure to X-ray film.

**Statistical analysis**

The results are expressed as the mean ± SD for 4 or more animals. The significance of differences between two groups was determined by unpaired 2-tailed Welch's t test and that among 3 groups was determined by ANOVA with Scheffe's test.
Any p values less than 0.05 were considered to be statistically significant. Each in vitro experiment was performed at least three times with similar results.

**Results**

Enhancement of Wnt/β-catenin signaling in OPG−/− mice

We first measured serum TRAP5b activity, a bone resorption marker, and ALP activity, a bone formation marker, in WT (OPG+/+) and OPG−/− mice (Supplemental Fig. S1A). Both markers were significantly higher in OPG−/− mice than in WT mice. Real-time PCR analysis also confirmed that the expression of Ctsk (encoding cathepsin K, a marker of osteoclasts), Alpl (encoding ALP), Sp7 (encoding osteonexin), and Col1a1 (encoding collagen, type I, alpha 1) mRNA was significantly higher in OPG−/− mice (Supplemental Fig. S1B). Histomorphometric analysis also demonstrated that mineral apposition rate (MAR) was significantly higher in OPG−/− mice (Supplemental Fig. S1C). These results suggested that both bone resorption and formation were markedly enhanced in OPG−/− mice.

Wnt/β-catenin signaling promotes osteoblast differentiation and bone formation. Therefore, we examined whether Wnt/β-catenin signaling increases in OPG−/− mice. The expression of Axin2 mRNA, a target gene of Wnt/β-catenin signaling, was significantly higher in OPG−/− mice (Fig. 1A). Furthermore, an immunohistochemical study showed that β-catenin-positive signals were stronger in cells on the bone surface, including osteoblasts, from the tibias of 12-week-old OPG−/− mice (Fig. 1B, C). These results suggested that Wnt/β-catenin signaling was markedly enhanced in OPG−/− mice. Therefore, we examined the expression of Wnt ligands in tibias from WT and OPG−/− mice (Fig. 1D). Real-time PCR analysis revealed that the expression levels of Wnt ligand mRNAs in OPG−/− mice were similar to those in WT mice, suggesting that the enhancement of Wnt/β-catenin signals in OPG−/− mice did not depend on the expression of Wnt ligands.

Downregulation of sclerostin expression in OPG−/− mice

Osteocytes are known to account for a large fraction of bone cells, including osteoblasts and osteoclasts. Furthermore, osteocytes specifically express sclerostin to inhibit Wnt/β-catenin signals. Therefore, we examined the expression of sclerostin in tibias of 12-week-old OPG−/− and WT mice using immunohistochemical techniques (Fig. 2A). The expression of sclerostin in the tibias of OPG−/− mice was lower than that in WT mice, as reported previously. The quantification analysis confirmed that the ratio of sclerostin area per bone area in OPG−/− mice was significantly decreased compared with that in WT mice (Fig. 2A, left bar graph). Furthermore, the amount of sclerostin in each osteocyte was determined from sclerostin-positive areas in osteocytes. The sclerostin-positive areas in OPG−/− mice were significantly fewer than those in WT mice. However, the number of osteocytes in OPG−/− mice was similar to that in WT mice (Fig. 2B). Real-time PCR analysis of osteocyte markers in tibias from WT and OPG−/− mice confirmed that the expression levels of Sost, but not Mepe or Phex, were significantly lower in OPG−/− mice (Fig. 2C). In our pilot studies, the amounts of sclerostin protein in bone marrow aspirates from WT mice were approximately 90 times higher than that in serum (bone marrow 5.4 ng/mL, serum 0.06 ng/mL). Therefore, we evaluated the amounts of sclerostin in bone marrow aspirates from WT and OPG−/− mice (Fig. 2D). The amounts of sclerostin in bone marrow aspirates were significantly lower in OPG−/− mice, indicating that sclerostin secreted from osteocytes was retained in bone tissues including bone marrow. These results suggested that the enhanced bone resorption may suppress the expression of sclerostin, which in turn promotes bone formation in OPG−/− mice.

Downregulation of sclerostin expression in RANKL-Tg mice

To clarify whether the enhancement of bone resorption diminished the expression of sclerostin, we examined the expression of sclerostin in RANKL-Tg mice, which exhibited increased bone resorption. The expression level of sclerostin in RANKL-Tg mice was lower than that in WT mice (Fig. 3A). Quantification analysis showed that the ratio of sclerostin area per bone area in RANKL-Tg mice was significantly decreased compared with WT mice (Fig. 3A, left bar graph). In addition, the sclerostin-positive area per osteocyte was significantly decreased in RANKL-Tg mice than WT mice (Fig. 3A, right bar graph). However, the number of osteocytes in RANKL-Tg mice was similar to that in WT mice (Fig. 3B). The expression levels of Sost mRNA, but not Mepe or Phex, were significantly lower in tibias of RANKL-Tg mice (Fig. 3C). The amounts of sclerostin protein were also significantly lower in bone marrow aspirates from RANKL-Tg mice (Fig. 3D). We then examined bone resorption and formation markers in RANKL-Tg mice. TRAP5b activity in serum was higher in RANKL-Tg mice than WT mice as expected (Fig. 3E). ALP activity in serum was also higher in RANKL-Tg mice. The expression of Col1a1 mRNA of bone formation marker in tibias was higher in RANKL-Tg mice (Fig. 3F). Histomorphometric analysis also demonstrated that MAR of trabecular bone was significantly higher in RANKL-Tg mice (Fig. 3G). Thus, both bone resorption and formation were accelerated in RANKL-Tg mice. However, the expression of Alpl mRNA was not significantly higher in RANKL-Tg than that in WT mice (Fig. 3F). MAR of endosteal in RANKL-Tg mice was comparable to that in WT mice (data not shown). The coupling of bone formation and resorption was milder in RANKL-Tg mice than that in OPG−/− mice. These results strongly suggested that the state of bone resorption regulated the expression of sclerostin.

Osteoclast-derived factors suppress the expression of sclerostin

To clarify whether osteoclasts affect the expression of sclerostin in osteocytes in vivo, we examined the two-dimensional spatial relationship between osteoclasts and sclerostin-positive osteocytes using immunohistochemical techniques (Fig. 4A). We measured the distance between osteoclasts and sclerostin-positive or -negative osteocytes in the cortical area of tibias from OPG−/− mice because a large number of osteoclasts as well as osteocytes were observed in the bone area in these mice. The distance between osteoclasts and sclerostin-positive osteocytes was approximately 3 times greater than that between osteoclasts and sclerostin-negative osteocytes (Fig. 4B). Osteocytes expressing sclerostin maintained a certain distance from osteoclasts. These results suggested that the osteoclast-derived factors inhibited the expression of sclerostin in osteocytes.

We next examined whether osteoclast-derived factors suppress the expression of sclerostin using UMR106 cells, an osteosarcoma cell line (Fig. 4C). These cells are known to constitutively express sclerostin and are widely used to examine
the regulation of sclerostin expression.\textsuperscript{(23,25,41)} Ocl-CM collected from osteoclast cultures was added into UMR106 cell cultures. The expression of sclerostin in UMR106 cells was markedly decreased within 48 hours by the addition of Ocl-CM treated with RANKL and M-CSF, but not BMM-CM with M-CSF alone (Fig. 4C). The addition of neither recombinant RANKL nor M-CSF decreased the expression of sclerostin in UMR106 cells.

We further confirmed that osteoclasts secreted some factors that suppress the expression of sclerostin (Fig. 4D). The expression of sclerostin in UMR106 cells was decreased by treatment with PGE\textsubscript{2}, as reported previously.\textsuperscript{(24)} Treatment with Ocl-CM cultured for 5 days markedly decreased the expression level of sclerostin. Ocl-CM cultured for 2 and 3.5 days exhibited weaker effects on the expression of sclerostin. Osteoclasts appeared from day 3 in the BMM cultures treated with M-CSF and RANKL, and the number of osteoclasts reached maximum on day 5. BMM-CMs collected at each time point failed to suppress the expression of sclerostin (Fig. 4E). The preliminary experiments showed that when Ocl-CM was boiled for 15 minutes, the suppressive effects of Ocl-CM completely disappeared (data not shown). This result suggested that the osteoclast-derived factors had a character as proteins. As reported previously,\textsuperscript{(25,41)} sclerostin was expressed in long-term cultures of primary osteoblasts. Treatment of these cultures with Ocl-CM for 48 hours suppressed the sclerostin expression (Fig. 4F).

Because TGF-\beta and Cthrc1 are proposed to be coupling factors between bone resorption and formation,\textsuperscript{(9,13)} we examined whether these cytokines suppress the expression of sclerostin. Treatment with recombinant TGF-\beta1 or Cthrc1 failed to suppress the expression of sclerostin in UMR106 cell cultures (Supplemental Fig. S2A, B). As previously reported,\textsuperscript{(25)} recombinant OSM effectively suppressed the expression of sclerostin in UMR106 cells (Supplemental Fig. S2C). However, the anti-OSM neutralizing antibody could not block the inhibitory effect of the Ocl-CM on sclerostin expression in UMR106 cells (Supplemental Fig. S2D). The expression of Osm mRNA in cultured osteoclasts was comparable to that in BMM (Supplemental Fig. S2E). The expression of Osm mRNA in tibias from OPG\textsuperscript{−/−} mice was comparable to that from WT mice (Supplemental Fig. S2F). As previously reported,\textsuperscript{(25)} recombinant CT-1 effectively suppressed the expression of sclerostin in UMR106 cells in culture (Supplemental Fig. S2G). However, the anti-CT-1 neutralizing antibody failed to block the inhibitory effect of the
Ocl-CM on sclerostin expression in UMR106 cells (Supplemental Fig. S2H). The expression of Ctf1 (encoding CT-1) mRNA in cultured osteoclasts was comparable to that in BMM (Supplemental Fig. S2I). These results indicate that cultured osteoclasts may not express enough amounts of CT-1 to suppress the sclerostin expression under our experimental conditions. We also measured pH in the conditioned medium. The pH value in the medium was between 7.2 and 7.6. These results suggested that factors secreted from osteoclasts, but not RANKL, M-CSF, TGF-β1, Cthrc1, OSM, or acidic conditions, suppressed the expression of sclerostin.

Osteoclast-derived LIF inhibits the expression of sclerostin

Using an antibody array, we profiled the cytokines, chemokines, and growth factors in Ocl-CM (Fig. 5A). Four factors among the evaluated 111 proteins showed a 4-fold increase in Ocl-CM compared with BMM-CM. The expression of CCL22, receptor for advanced glycation end products (RAGE), LIF, and matrix metalloproteinase (MMP)-9 was reportedly increased in osteoclast differentiation.(34,44–46) Consistent with the previous report,(33) we confirmed that osteoclasts strongly expressed Lif mRNA as well as Ctsk mRNA (Fig. 5B). Recombinant LIF markedly suppressed the expression of sclerostin in UMR106 cell cultures as shown previously(25) (Fig. 5C). The suppressive effects of Ocl-CM and LIF on the sclerostin expression in UMR106 cell cultures were abrogated by the treatments with an anti-LIF antibody (Fig. 5D). These results suggested that the osteoclast-derived LIF inhibited the expression of sclerostin in osteocytes.

The effects of anti-RANKL antibody treatment on the expression of sclerostin

We next examined whether the inhibition of osteoclast formation by administration of an anti-RANKL antibody increases the expression of sclerostin in vivo. The antibody was injected once into 8-week-old OPG−/− mice (Fig. 6). An injection of the anti-RANKL antibody to these mice markedly increased cortical BMD with a reduction of cortical porosity and TRAP activity at age 12 weeks (Fig. 6A, B). The expression of Ctsk mRNA and the level of serum TRAP5b activity also decreased in OPG−/− mice treated with the anti-RANKL antibody (Fig. 6C, D). The expression level of Lif mRNA was higher in tibias of OPG−/− mice (Fig. 6E). Treatment of OPG−/− mice with anti-RANKL antibody significantly decreased the expression of Lif mRNA. Immunohistochemical analysis showed that LIF-positive

Fig. 2. Expression of sclerostin in OPG−/− mice. (A) The expression of sclerostin in cortical areas of tibias in 12-week-old WT and OPG−/− mice. Sclerostin-positive signals (green), nuclei (red). Left bar graph, the ratio of sclerostin area/bone area (%). Right bar graph, sclerostin-positive area/osteocyte (μm²) (n = 6). (B) Number of osteocytes/bone area (cells/mm²) (n = 6). (C) Real-time RT-PCR analysis of the expression of Sost, Mepe, Phex, and Opg mRNAs in tibias of 12-week-old WT and OPG−/− mice (n = 4). (D) ELISA analysis of sclerostin in bone marrow aspirates from 12-week-old WT and OPG−/− mice (n = 6). Data are expressed as the mean ± SD in A–D. For statistical analysis, unpaired 2-tailed Welch’s t tests were performed. **p < 0.01. Scale bar = 50 μm.
Signals were strongly observed in large cells (indicated by arrows; probably osteoclasts) on the bone surface from the tibias of OPG−/− mice (Fig. 6F, G). Administration of anti-RANKL antibody decreased LIF-positive signals in tibias from OPG−/− mice (Fig. 6F, G). These results suggested that treatment with the anti-RANKL antibody markedly suppressed the expression of LIF.

We next examined the expression of sclerostin in tibias from OPG−/− mice treated with the anti-RANKL antibody or vehicle. Administration of the antibody significantly increased the expression of Sost mRNA in tibias from OPG−/− mice (Fig. 6H). Immunohistochemical analysis also confirmed that sclerostin-positive osteocytes significantly increased in tibias from OPG−/− mice treated with the antibody (Fig. 6I, J). Along with the increased expression of sclerostin, the expression of Axin2 and β-catenin-positive signals was significantly decreased in those mice (Fig. 6K–M). These findings suggested that treatment with the anti-RANKL antibody suppressed LIF expression, which in turn upregulated sclerostin expression, thereby decreasing Wnt/β-catenin signals.
The effects of anti-RANKL antibody treatment on bone formation

We next examined whether the increased expression of sclerostin by anti-RANKL antibody administration impacts bone formation (Fig. 7). The injection of the anti-RANKL antibody significantly decreased the expression of Sp7, Col1a1, and Alpl mRNAs in tibias and serum ALP activity from OPG−/− mice (Fig. 7A, B). An immunohistochemical analysis showed that osterix-positive signals were weaker in OPG−/− mice treated with the antibody (Fig. 7C, D). A histomorphometric analysis also demonstrated that administration of anti-RANKL antibody significantly decreased MAR in femurs from OPG−/− mice (Fig. 7E).

We further confirmed the effects of the anti-RANKL antibody on the expression of sclerostin in RANKL-Tg mice (Supplemental Fig. S3). Administration of the antibody reduced the serum TRAP5b activity in these mice (Supplemental Fig. S3A). In contrast, the expressions of sclerostin mRNA and protein were increased in RANKL-Tg mice treated with the anti-RANKL antibody (Supplemental Fig. S3B–D). In contrast, ALP activity in serum was significantly decreased in RANKL-Tg mice treated with the antibody (Supplemental Fig. S3E). These results suggested that treatment with the anti-RANKL antibody suppressed bone formation probably because of the increased expression of sclerostin. Thus, the osteoclast-derived LIF suppresses the expression of sclerostin in osteocytes, thereby facilitating bone formation (Fig. 7F).

Discussion

Here, we demonstrated that the suppression of sclerostin expression associated with bone resorption was critical for coupling bone resorption to formation using OPG−/− and RANKL-Tg mice. These mice exhibited a high bone turnover state with a decreased expression of sclerostin in osteocytes. Injection of an anti-RANKL antibody into these mice inhibited osteoclastogenesis and markedly increased the expression of sclerostin, thereby suppressing bone formation with the decreased Wnt/β-catenin signals. Osteoclasts secreted LIF to suppress the sclerostin expression in UMR106 cell and osteocyte cultures. Thus, osteoclastic suppression of sclerostin in osteocytes promotes bone formation through the activation of Wnt/β-catenin signals (Fig. 7F).
Clinical studies reported that bone resorption contributes to bone turnover state \(^{(27,50)}\) and is associated with the serum level of sclerostin \(^{(30,31,51,52)}\). Denosumab, an anti-RANKL antibody, suppressed bone turnover and increased serum sclerostin in postmenopausal women \(^{(52)}\). Bisphosphonate, a widely used anti-bone-resorbing agent, has also been shown to suppress bone turnover in osteoporosis patients and OPGL \(^{-/-}\) mice \(^{(47,48,51)}\). These previous clinical findings and our results strongly indicate that factors secreted from osteoclasts or factors released from bone matrices suppress sclerostin expression to enhance bone formation.

We have found that LIF is a candidate factor that is secreted from osteoclasts to suppress the sclerostin expression. An antibody array analysis revealed that LIF was contained in Ocl-CM (Fig. 5A). RT-PCR and immunohistochemical analyses also confirmed that osteoclasts strongly expressed LIF in cultures and in OPGL \(^{-/-}\) mice. Consistent with the previous report \(^{(25)}\), LIF suppressed the sclerostin expression in UMR106 cells in culture. Furthermore, anti-LIF antibody inhibited the suppressive effects of Ocl-CM (Fig. 5D). Roles of LIF in bone turnover were highlighted by bone phenotypes of LIF \(^{-/-}\) mice \(^{(27)}\). These mice exhibited a low trabecular bone mass associated with decreased osteogenesis and enhanced adipogenesis. Based on previous and the present studies, it is likely that Wnt/β-catenin signals may be suppressed because of the increased expression of sclerostin in LIF \(^{-/-}\) mice.

LIF is reported to be expressed in osteoblasts and chondrocytes \(^{(27,53,54)}\). Grimand and colleagues \(^{(53)}\) reported that LIF is not expressed in osteoclasts under a physiological condition. In contrast, it has been shown that TGF-β induced the expression of LIF in osteoclasts \(^{(34)}\). We also showed that osteoclasts strongly expressed LIF in OPGL \(^{-/-}\) mice. Furthermore, when osteoclast differentiation was inhibited by the administration of anti-RANKL antibody, most LIF-positive signals disappeared in OPGL \(^{-/-}\) mice. These results suggest that TGF-β released from bone matrix associated with bone resorption induces LIF expression in OPGL \(^{-/-}\) mice. Thus, osteoclasts dominantly expressed LIF under the high bone turnover state observed in OPGL \(^{-/-}\) mice.

LIF binds to the receptor complex of glycoprotein 130 (gp130)/LIF receptor (LIFR) \(^{(27)}\). Osteoblast and osteocyte-specific gp130 conditional knockout mice exhibited the increased expression of sclerostin in tibias \(^{(55)}\). These findings suggest that LIF suppresses the expression of sclerostin through gp130 signaling in osteocytes. OSM binds to receptor complexes of OSM receptor/gp130 and those of LIFR/gp130 \(^{(25)}\) suggesting that OSM also suppresses sclerostin expression during bone resorption. Walker and colleagues \(^{(25)}\) reported that an administration of OSM into mice inhibited the expression of sclerostin in osteocytes. CT-1 also binds to receptor complexes of LIFR/gp130. \(^{(11)}\) Recombinant CT-1 inhibited the expression of sclerostin in UMR106 cells \(^{(25)}\). We confirmed that recombinant OSM at 50 ng/mL effectively suppressed the expression of sclerostin in UMR106 cells in culture (Supplemental Fig. S2C). However, the anti-OSM or -CT-1 neutralizing antibody could not block the inhibitory effect of the Ocl-CM on sclerostin expression in UMR106 cells (Supplemental Fig. S2D, H). The concentration of OSM in the medium from osteoclast cultures treated with RANKL and M-CSF was less than 10 pg/mL. Furthermore, the expression of

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### Fig. 5. Effects of osteoclast-derived LIF on the expression of sclerostin.

A) An antibody array in Ocl-CM and BMM-CM. BMMs were cultured with or without GST-RANKL (200 ng/mL) in the presence of M-CSF (50 ng/mL). Ocl-CM and BMM-CM were collected from these cultures on day 3.5 (D3.5) and 5 (D5). The conditioned media were examined by an antibody array kit. The colored bar shows the fold upregulation of intensities of cytokines and chemokines. (B) Real-time PCR analysis of the expression of Lif and Ctsk mRNAs in the cultured osteoclasts. BMMs were cultured with or without GST-RANKL (200 ng/mL) in the presence of M-CSF (50 ng/mL) at each time point \((n = 4)\). (C, D) Western blot analysis of sclerostin in UMR106 cells. (C) UMR106 cells were cultured in the presence or absence of LIF \((10 \text{ng}/\text{mL}, 5 \times 10 \text{ng}/\text{mL}, \text{or } 10 \text{ng}/\text{mL})\) or PGE2 \((10 \text{ng}/\text{mL})\). (D) UMR106 cells were cultured in the presence or absence of LIF \((10 \text{ng}/\text{mL})\) or Ocl-CM or with or without anti-LIF antibody \((2 \text{ μg}/\text{mL})\). Data are expressed as the mean ± SD in B. For statistical analysis, ANOVA with Scheffe’s test were performed. **\(p<0.01\). Western blots are representative of 3 independent experiments performed.
OSM mRNA in tibias from OPG−/− mice was similar to that in WT mice (Supplemental Fig. S2). Based on these results, it is unlikely that OSM is highly involved in the suppression of sclerostin expression in high bone turnover states.

LIF, a secreted protein, can act on osteocytes,(28) since LIFRs are expressed in osteocytes as well as osteoblasts.(25) As shown in Fig. 4A, B, osteocytes away from osteoclasts had strong signals positive for sclerostin, whereas osteocytes near osteoclasts did not. LIF was expressed in osteoclasts in our experimental conditions and suppressed expression of sclerostin. These results suggest that LIF may act on osteocytes through the lacunar-canalicular system.

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**Fig. 6.** Effects of the anti-RANKL antibody on the expression of sclerostin in OPG−/− mice. (A) Cortical bone mineral densities in femurs of 12-week-old OPG−/− mice treated with vehicle or the anti-RANKL antibody (n = 6). (B) Histological analysis of TRAP in the cortical area of the femur in OPG−/− mice treated with vehicle (left panel) or the anti-RANKL antibody (right panel). TRAP (red). (C) Real-time RT-PCR analysis of the expression of Ctsk mRNA in tibias of OPG−/− mice treated with vehicle or the anti-RANKL antibody (n = 4). (D) TRAP5b activity in serum from OPG−/− mice treated with vehicle or the anti-RANKL antibody (n = 6). (E) Real-time PCR analysis of the expression of Lif mRNA in WT and OPG−/− mice (left) and in OPG−/− mice treated with vehicle or the anti-RANKL antibody (right) (n = 6). (F) Immunohistochemical analysis of LIF in the cortical areas of tibias in WT (outer left panel), OPG−/− (inner left panel) mice, and OPG−/− mice treated with vehicle (inner right panel) or the anti-RANKL antibody (outer right panel). (G) LIF-positive cells/bone perimeter (N/mm) (n = 5). (H) Real-time RT-PCR analysis of the expression of Sost mRNA in OPG−/− mice treated with vehicle or the anti-RANKL antibody (n = 4). (I) Immunohistochemical analysis of sclerostin in OPG−/− mice treated with vehicle (left panel) or the anti-RANKL antibody (right panel). (J) Sclerostin-positive cells/bone area (N/mm²) (n = 5). (K) Real-time RT-PCR analysis of the expression of Axin2 mRNA in OPG−/− mice treated with vehicle or the anti-RANKL antibody (n = 4). (L) Immunohistochemical analysis of β-catenin in OPG−/− mice treated with vehicle (left panel) or the anti-RANKL antibody (right panel). (M) β-Catenin-positive cells/bone perimeter (N/mm) (n = 5). In A, C–E, G, H, I, K, and M, data are expressed as the mean ± SD. For statistical analysis, unpaired 2-tailed Welch’s t tests were performed. *p < 0.05, **p < 0.01. Scale bar = 50 μm.
Mechanical stimulation inhibited the expression of sclerostin in bone. This finding indicates that changes in mechanical properties with low bone mass might impact the expression of sclerostin in OPG−/− mice. It was reported that mechanical stimulation affects not only the expression of sclerostin but also other osteocyte markers such as Mepe and Phex. The expression of Sost as well as that of Mepe and Phex was decreased by fluid shear stress in the three-dimensional culture model. However, the expression of Mepe and Phex in OPG−/− and RANKL-Tg mice remained unchanged compared with control mice (Figs. 2B and 3B). Therefore, it is unlikely that the changes in mechanical properties dominantly contribute to the suppression of sclerostin expression in those mice. Although mechanical loading was reported to stimulate the expression of dentin matrix protein 1 (DMP-1) in alveolar bone in young mice, DMP-1-positive osteocytes were evenly distributed in the cortical bone of aged OPG−/− mice as well as in that of WT mice. Furthermore, the osteocytic lacunar-canalicul system was reportedly well arranged in the cortical bone of OPG−/− mice similar to that in WT mice. Based on the previous and our present studies, the expression of sclerostin is not greatly affected by changes in the mechanical properties of bone with enhanced bone resorption in OPG−/− and RANKL-Tg mice.

In the present study, we demonstrated that osteoclasts produce inhibitory factors that suppress the expression of sclerostin. Inhibition of osteoclast formation using an anti-RANKL antibody inhibited bone formation through increased expression of sclerostin. The administration of bisphosphonates or anti-RANKL neutralizing antibody to postmenopausal osteoporosis was reported to increase serum sclerostin and decrease serum bone-specific ALP. These findings suggest that anti-resorbing agents suppress bone formation due to increasing sclerostin expression. Based on previous and our present studies, administration of anti-resorbing agents with an anti-sclerostin antibody may effectively increase bone formation in osteoporosis.

Disclosures

HY is an employee of Oriental Yeast Co. All other authors state that they have no conflicts of interest.

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