Knockdown of sphingomyelin synthase 2 inhibits osteoclastogenesis by decreasing RANKL expression in mouse primary osteoblasts

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ABSTRACT

Sphingomyelin is a major lipid of the plasma membrane and is enriched in microdomains of the plasma membrane that are critical for signal transduction. However, the function of sphingomyelin in the cell membrane of osteoblasts has not been clarified. Therefore, we examined how sphingomyelin synthase 2 (SMS2) affects osteoclast differentiation by osteoblasts. We knocked down the expression of SMS2 with siRNA targeting the *Sgms2* gene in mouse primary osteoblasts. The effects of SMS2 knockdown in osteoblasts were examined using polymerase chain reaction and western blotting. The knockdown of SMS2 suppressed the formation of TRAP-positive multinucleated cells by co-culture of osteoblasts and bone marrow cells compared to the control. We found that receptor activator of nuclear factor κ B ligand (RANKL) mRNA expression was significantly reduced by 1,25(OH)₂D₃ stimulation in SMS2 siRNA osteoblasts. The knockdown of SMS2 repressed the expression of retinoid-X-receptor- α (RXR α) regardless of 1,25(OH)₂D₃ stimulation. TRAP-positive multinucleated cell formation was significantly reduced by RXR α siRNA in osteoblasts in a co-culture system. These results suggest that SMS2 regulates osteoclast differentiation by inducing RANKL expression via RXR α .

Bone is a dynamic tissue that undergoes continuous remodeling throughout the lifespan of humans. Bone mass is maintained through the balance between the functions of osteoblasts and osteoclasts. An imbalance between bone formation and resorption, therefore, results in various metabolic bone diseases (11). Bone metabolism is regulated by multiple hormones, growth factors, and cytokines.

Osteoclasts are multinucleated cells with a unique membrane known as the ruffled border membrane

with bone resorption function (26). They are formed by differentiation and fusion of monocyte-macrophage cells under the control of osteoblast-lineage cells (21). Differentiation from monocytes to osteoclasts is regulated by the receptor activator of nuclear factor κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Bone resorption factors, including 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), parathyroid hormone, interleukin (IL)-6, and IL-11, regulate the expression of RANKL and osteoprotegerin (OPG) genes in osteoblasts. In addition, they regulate calcium homeostasis, bone remodeling, proliferation, differentiation, and physiological processes (10, 19, 28). 1,25(OH)₂D₃ binds to the vitamin D receptor (VDR) and promotes heterodimerization of VDR with a retinoid-X-receptor (RXR) to genomically regulate diverse cellular processes (9).

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Sphingolipids such as sphingomyelin, ceramide, and glycosphingosides are components of lipid bilayers. Sphingomyelin is the most abundant sphingolipid in the cell membrane and plays a role in maintaining its function and integrity. In recent years, sphingomyelin has been shown to be enriched in microdomains of cell membranes that are important for signal transduction, together with cholesterol (18). On the other hand, multiple stimuli cause the hydrolysis of sphingomyelin in the cell membrane to form ceramide, which is a second messenger (6). Thus, these sphingolipids participate in signaling associated with cell differentiation and proliferation (5). Sphingomyelin synthase (SMS) synthesizes sphingomyelin from ceramide, and of the two SMS (SMS1 and SMS2), SMS2 is present in the cell membrane (7, 29). SMS2 has been confirmed to be enriched in specific lipid microdomains, where it may regulate the function of these microdomains through sphingomyelin synthesis (27). Sphingomyelin turnover is induced by the activation of neutral sphingomyelinase (nSMase) during the induction of differentiation of the human leukemia HL-60 cell line by the addition of $1,25(\text{OH})_2\text{D}_3$ and the enhancement of ceramide production (16).

It remains unclear how SMS2 might be involved in osteoclast differentiation. Therefore, in this study, we investigated whether SMS2 affected osteoclast differentiation induced by $1,25(\text{OH})_2\text{D}_3$ in mouse primary osteoblasts.

MATERIALS AND METHODS

Animals. Slc:ddY mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). Animal handling and experimental protocols were approved by the Animal Research Committee of Osaka Dental University.

Cell culture. Primary osteoblastic cells were isolated from the calvaria of 1-day-old male Slc:ddY mice and cultured in α -MEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 unit/mL penicillin G sodium, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 292 mg/mL L-glutamine (Invitrogen, Carlsbad, CA, USA) at 37°C in an atmosphere containing 5% CO_2 . Bone-marrow cells obtained from the tibias of 6- to 8-week-old male Slc:ddY mice were cultured in α -MEM containing 10% FBS.

Antibodies and reagents. Anti-VDR (sc-13133), anti-RXR α (sc-553), and anti- β -actin antibodies (sc-

4778) were obtained from Santa Cruz Biotechnology (Paso Robles, CA, USA). Horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodies were obtained from Cell Signaling (Danvers, MA, USA). A Chemi-Lumi One Markers Kit was purchased from Nacalai Tesque (Kyoto, Japan).

Tartrate-resistant acid phosphatase (TRAP) buffer solution was prepared by mixing 0.1 M sodium acetate (Wako Pure Chemicals, Osaka, Japan) and 50 mM tartaric acid (Wako Pure Chemicals) in a 1 : 1 ratio, followed by adjustment with TRAP staining solution, which was prepared by mixing 50 mL of TRAP buffer solution, 5 mg of naphthol AS-MX phosphate (Sigma-Aldrich), 0.5 mL of N,N-dimethylformamide (Wako Pure Chemicals), and 25 mg of Fast Red Violet LB salt (Sigma-Aldrich).

Liquid chromatography tandem mass spectrometry (LC-MS/MS). Primary osteoblastic cells were homogenized in PBS. Protein was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA), and cell lysate was processed for lipid extraction. Measurements of sphingomyelin species in lipid extracts were performed using LC-MS/MS as described previously (14, 15). The amounts of sphingomyelin species with various carbon chains (d18:1/16:0, d18:0/16:0, d18:1/18:1, d18:1/18:0, d18:1/20:1, d18:1/20:0, d18:1/22:1, d18:1/22:0, d18:1/24:1, d18:1/24:0, d18:1/26:1, d18:1/26:0, d16:1/18:1, d16:1/20:0, and d18:2/16:0 h) were determined. Each sphingomyelin was normalized to the total protein amount.

Osteoclast differentiation assay. Primary osteoblasts at a density of $2.5 \times 10^3/\text{well}$ and freshly prepared bone marrow cells at a density of $2.5 \times 10^5/\text{well}$ were cocultured in α -MEM containing 10% FBS and 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ (Sigma-Aldrich) in 96-well plates. Osteoclasts formed within 7 days in cocultures. All cultures were incubated in quadruplicate, and medium was replenished on day 3. Cells were fixed in 4% paraformaldehyde in PBS (–) and an acetone/ethanol mixture (1 : 1). Fixed cells were treated with TRAP staining solution for 10 min, washed with water, and observed by light microscopy. TRAP-positive multinucleated cells containing at least three nuclei were counted as osteoclasts. Representative results from at least three independent experiments are expressed as the mean $\pm$ SEM of three cultures.

Western blotting. Cells were plated in 24-well tissue culture plates ($3.0 \times 10^4/\text{well}$) and grown to subcon-

fluency in α -MEM supplemented with 10% FBS. Confluent monolayers were cultured in a medium containing 0.1% bovine serum albumin with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ for 24 h. Cells were washed twice with PBS and dissolved directly on the plate using sodium dodecyl sulfate (SDS) sample buffer and were briefly sonicated to shear DNA with a Branson digital sonifier 450 (Branson Ultrasonics, Danbury, CT, USA). Samples were boiled at 99°C for 3 min and proteins were separated by SDS-polyacrylamide gel electrophoresis. After transfer of the proteins to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Texas City, TX, USA), membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing Tween-20 (TBS-T). Membranes were incubated overnight with primary antibody in 5% skim milk/TBS-T, followed by washing with TBS-T. Membranes were then incubated with peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) at a 1 : 5000 dilution for 1 h, and immunoreactive bands were visualized using the Chemi-Lumi One Markers Kit (Nacalai Tesque). Signals were analyzed using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The same membranes were stripped and reprobed with antibodies recognizing β -actin for normalization of results.

Quantitative real-time reverse transcription (RT)-PCR. Total RNA was isolated using an Illustra RNeasy Mini RNA Isolation Kit (GE Healthcare, Amersham, UK) from cultured cells at 24 h after the addition of 10^{-8} M $1,25(\text{OH})_2\text{D}_3$. The quantity and purity of extracted total RNA were determined spectrophotometrically at 260 and 280 nm. One microgram of total RNA was reverse-transcribed into cDNA using the RNA to cDNA EcoDry Premix (Clontech Laboratories, Inc., Palo Alto, CA, USA). Relative levels of mRNA expression were measured by quantitative real-time PCR using predesigned and preformulated gene-specific primer and probe sets for osteoclast differentiation factor molecules (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA). Analysis was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's protocol. Gene expression in multiplex reactions was quantified using the comparative Ct method with normalization of the amount of the target (FAM^{TM}) to endogenous GAPDH (VIC^{TM}) expression. Probes for detecting cDNAs encoding RANKL, OPG, VDR, RXR α , and GAPDH were designed and synthesized by Applied Biosystems, based on gene

sequences in the Celera database. Assay IDs of TaqMan Gene Expression Assay probes were: RANKL, Mm00441908_m1; OPG, Mm00435452_m1; RXR α , Mm0044182_m1; VDR, Mm00437297_m1; and SMS2, Mm00512327_m1. The commercial number of TaqMan GAPDH control reagents was 4352932E.

Small interfering RNA (siRNA). Validated SMS2 (gene *Sgms2*) and RXR α (gene *Rxra*) siRNA and negative-control siRNA were purchased from Ambion (Grand Island, NY, USA) and transfected into cells using RNAi MAX reagent (Invitrogen) according to the manufacturer's protocol, without removal of medium for the duration of 1-day experiments. Knockdown of the target protein and gene was confirmed by immunoblotting and real-time PCR, respectively.

Statistical analysis. Results are presented as means $\pm$ SDs of five experiments and were analyzed by unpaired Student's *t*-tests. Differences with *P* values of less than 0.05 were considered significant.

RESULTS

SMS2 knockdown decreases sphingomyelin in primary osteoblasts

To determine whether regulation of osteoclast differentiation is dependent on SMS2, SMS2 expression was downregulated using *Sgms2*-targeted small interfering RNA (SMS2 siRNA) in primary osteoblasts. Successful knockdown was confirmed by RT-PCR (Fig. 1A). The levels of cellular sphingomyelin were measured by LC-MS/MS. As expected from the reduction of SMS2 expression, cellular sphingomyelin content was also reduced in SMS2 siRNA primary osteoblasts (Fig. 1B). These observations demonstrate that SMS2 and cellular sphingomyelin content was down-regulated by transient treatment with siRNA in primary osteoblasts.

SMS2 in primary osteoblasts potentiated osteoclast differentiation in cocultures with bone marrow cells

Osteoclasts are generated when bone marrow cells and osteoblasts are cocultured in the presence of $1,25(\text{OH})_2\text{D}_3$. To determine whether osteoclast differentiation in response to a $1,25(\text{OH})_2\text{D}_3$ stimulus requires SMS2, we used an osteoclast coculture system in which primary osteoblasts were treated with siRNA negative control and SMS2 siRNA. SMS2 siRNA reduced the number of osteoclasts formed (Fig. 2). Taken together, these coculture data demon-

Fig. 1 Sphingomyelin expression is reduced by SMS2 siRNA in primary osteoblasts. Mouse calvarial osteoblasts were transfected with SMS2 siRNA or control siRNA for 24 h. **A**. Gene expression of SMS2 mRNA was compared between control and SMS2 siRNA. **B**. The level of each molecular species of sphingomyelin was compared between control and SMS2 siRNA. Symbols represent mean values of four wells and vertical bars indicate SEMs. *Significant difference between the control and SMS2 siRNA groups ($P < 0.05$).

Fig. 2 SMS2 siRNA in primary osteoblasts blocked osteoclast formation. Mouse calvarial osteoblasts were transfected with SMS2 siRNA or control siRNA for 24 h, and the osteoblast and bone marrow cell cocultures were then treated with $1,25(\text{OH})_2\text{D}_3$ for 7 days. **A**. Cells were fixed and stained for TRAP. **B**. The number of TRAP-positive multinucleated cells contain three or more nuclei were scored. Symbols represent mean values of four wells and vertical bars indicate SEM. *Significant difference between the control and SMS2 siRNA groups ($P < 0.05$). Scale bar = 250 μm .

Fig. 3 SMS2 siRNA in primary osteoblasts blocked induction of RANKL gene expression in primary osteoblasts. Cells were transfected with SMS2 siRNA or control siRNA for 24 h and then treated with $1,25(\text{OH})_2\text{D}_3$ for 24 h. RANKL and OPG mRNA expression was then detected by quantitative real-time PCR. Symbols represent mean values of four wells and vertical bars indicate SEMs. *Significant difference between the control and SMS2 siRNA groups ($P < 0.05$).

strate a functional role for SMS2 in osteoclast differentiation.

SMS2 knockdown inhibited RANKL mRNA expression in primary osteoblasts

To clarify the mechanisms involved in suppression of osteoclast differentiation by SMS2 siRNA, we investigated the effects of SMS2 siRNA on RANKL and OPG mRNA expression in primary osteoblasts. We observed a decrease in $1,25(\text{OH})_2\text{D}_3$ stimulated RANKL mRNA expression with SMS2 siRNA (Fig. 3A). Transfection with SMS2 siRNA did not change the induction of OPG mRNA expression (Fig. 3B). SMS2 siRNA affected RANKL, but not OPG, mRNA expression with $1,25(\text{OH})_2\text{D}_3$ in primary osteoblasts.

SMS2 siRNA inhibited RXR α expression in primary osteoblasts

Although qPCR data showed that SMS2 is necessary for $1,25(\text{OH})_2\text{D}_3$ stimulated RANKL expression in osteoblasts, we sought to determine whether the requirement for SMS2 in $1,25(\text{OH})_2\text{D}_3$ signaling was limited to RANKL induction. We demonstrated that SMS2 siRNA in primary osteoblasts significantly decreased RXR α mRNA and protein expression (Fig. 4). However, neither VDR mRNA nor protein expression was changed (Fig. 4). These results indicate that SMS2 may be required for RXR α expression and regulate osteoclast differentiation.

RXR α in primary osteoblasts potentiated osteoclast differentiation in cocultures of bone marrow cells

To determine whether primary osteoblasts are dependent on RXR α for their ability to induce osteoclast differentiation in response to a $1,25(\text{OH})_2\text{D}_3$

stimulus, we used an osteoclast coculture system in which primary osteoblasts were treated with siRNA negative control and RXR α siRNA. RXR α siRNA-treated cocultures had a significant reduction in the number of osteoclasts formed (Fig. 5). Taken together, these coculture data demonstrate a functional role for RXR α in osteoclast differentiation.

DISCUSSION

In this study, we examined the effects of SMS2 on osteoclast differentiation induced by $1,25(\text{OH})_2\text{D}_3$ in mouse primary osteoblasts. Our results showed that SMS2 siRNA downregulated the expression of RXR α , which heterodimerizes with VDR to influence RANKL mRNA expression in the presence of $1,25(\text{OH})_2\text{D}_3$. These effects ultimately resulted in enhanced osteoclast differentiation.

Previous studies have shown that SMS2 knockout mice causes type 2 diabetes by impairing glucose metabolism (12, 20). We have clarified that osteoblasts regulate glucose metabolism by ablation of osteoblasts that express osteocalcin (30), suggesting that SMS2 may influence osteoblastic function expression. SMS and nSMase contribute interconversion between ceramide and sphingomyelin (4). It supposed that activation of nSMase contributes increase of ceramide in this cycle, but it is pointed out significance of activation control of SMS recently (6, 24). In the present experiment, SMS2 siRNA led to a decrease in sphingomyelin levels in mouse primary osteoblasts.

Hydrolysis of sphingomyelin produces the intracellular signaling molecule ceramide. This triggers responses including cell death (13), differentiation (17), and inflammation (8). SMS2 is ubiquitous in

Fig. 4 SMS2 siRNA in primary osteoblasts inhibits RXR α expression. Cells were transfected with SMS2 siRNA or control siRNA for 24 h and then treated with 1,25(OH) $_2$ D $_3$ for 24 h. **A**. VDR and RXR α mRNA expression detected by quantitative real-time PCR. Symbols represent mean values of four wells, and vertical bars indicate SEMs. *Significant difference between the control and SMS2 siRNA groups ($P < 0.05$). **B**. Protein expression levels of VDR and RXR α were detected by western blot.

the plasma membrane and the Golgi apparatus, where it is responsible for sphingomyelin and diacylglycerol formation and ceramide consumption (29). This control of activation is important in the regulation of cell death and cell proliferation via these lipids. A previous report has suggested that such SMS2-dependent lipid regulation regulates inflammatory responses (3).

The human promyelocytic leukemia cell line HL60 has been widely used to study cell differentiation along the monocyte-macrophage pathway. 1,25(OH) $_2$ D $_3$, tumor necrosis factor- α (TNF- α), and γ -interferon induce differentiation of HL60 cells into monocytes (1, 23, 25). Each of these differentiations is associated with changes in sphingolipid metabolism. Differentiation of HL60 cells into monocytes is regulated by a pathway involving sphingomyelin and its derivatives. *In vitro*, it has been confirmed that 1,25(OH) $_2$ D $_3$ degrades sphingomyelin by activating nSMase. In this case, ceramide functions as a second messenger (6). Furthermore, exogenous sphingomyelinase promoted differentiation to monocytes induced by 1,25(OH) $_2$ D $_3$. Then we examined whether SMS2 affects osteoclast differentiation induced by 1,25(OH) $_2$ D $_3$. We found that promotion of osteoclast differentiation was suppressed when SMS2 was knocked down in mouse primary osteoblasts. Thus,

these data suggest that osteoclast differentiation is mediated by SMS2. Further analysis revealed that SMS2 knockdown inhibited the increase in RANKL mRNA expression caused by 1,25(OH) $_2$ D $_3$ in mouse primary osteoblasts. These results indicated that induction of RANKL by 1,25(OH) $_2$ D $_3$ was mediated by SMS2 signaling.

VDR bound to 1,25(OH) $_2$ D $_3$ forms a heterodimer with the vitamin A receptor RXR α , which then regulates transcription of target genes in the nuclei of osteoblasts (31). RXR α is a nuclear receptor that regulates the action of multiple endocrine processes through heterodimerization with a variety of hormone receptors, including thyroid receptor, peroxisome proliferator-activated receptor, VDR, and other retinoic-acid receptors (2). In addition, it has been reported that ceramide is involved in S1P phosphatase SPP in MC3T3-E1 cell line to control the transcriptional activity of AP-1 and increase retinoic acid receptor alpha and RXR α (22). Therefore, it is important to investigate whether SMS2 affects VDR and RXR α protein expression. We observed no change in VDR protein expression with SMS2 knockdown, but RXR α protein expression was decreased in mouse primary osteoblasts. RXR belongs to the nuclear receptor superfamily and has three isoforms: alpha, beta and gamma. Therefore, we examined the

Fig. 5 RXR α siRNA in primary osteoblasts blocks osteoclast formation. Mouse calvarial osteoblasts were transfected with RXR α siRNA or control siRNA for 24 h, and mouse calvarial osteoblast and bone marrow cell cocultures were then treated with 1,25(OH) $_2$ D $_3$ for 7 days. **A**. Cells were fixed and stained for TRAP. **B**. The number of TRAP-positive multinucleated cells contain three or more nuclei were scored. Symbols represent mean values of four wells, and the vertical bars indicate SEM. *Significant difference between the control and RXR α siRNA groups ($P < 0.05$). Scale bar = 250 μ m.

expression of all three, but no differences were found for the β and γ isoforms (data not shown). In summary, knockdown of SMS2 suppressed RXR α protein expression, but not VDR to which 1,25(OH) $_2$ D $_3$ directly binds. Similarly, after SMS2 knockdown in mouse primary osteoblasts, RXR α mRNA expression was decreased. Therefore, when siRNA knockdown of RXR α was performed to confirm osteoclast differentiation, as expected, it was suppressed.

In conclusion, we have shown that knockdown of SMS2 reduces RXR α expression and decreases RANKL expression after 1,25(OH) $_2$ D $_3$ stimulation in osteoblasts, which may suppress osteoclast differentiation. Further studies are needed to clarify the significance of SMS2 in the mechanism of RXR α expression regulation.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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