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ABSTRACT

Osteoprotegerin (OPG) is secreted by stromal and osteoblastic lineage cells and inhibits osteoclastogenesis by preventing the interaction of receptor activator of nuclear factor- κ B ligand (RANKL) with receptor activator of nuclear factor- κ B (RANK). In this study, the expression of OPG in osteoclasts themselves and its biological functions during osteoclastogenesis were investigated for the first time. OPG expression *in vivo* in the developing rat maxilla was examined by immunofluorescence analysis. OPG expression in osteoclasts during *in vitro* osteoclastogenesis was determined by reverse-transcription polymerase chain-reaction (RT-PCR), Western blot, and immunofluorescence staining. We determined the function of OPG produced by osteoclasts during osteoclastogenesis by silencing the OPG gene. The effects of OPG on bone-resorbing activity and apoptosis of mature osteoclasts were examined by the assay of resorptive pit formation on calcium-phosphate-coated plate and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, respectively. In the immunofluorescence findings, strong immunoreactivities were unexpectedly seen in multinucleated tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts around the growing and erupting tooth germs in the rat alveolar bone. *In vitro*, OPG expression was significantly increased during the differentiation of osteoclasts from mouse bone-marrow-derived cells treated with a combination of macrophage colony-stimulating factor (M-CSF) and RANKL. Interestingly, it was found that OPG small interfering (si)RNA treatment during osteoclastogenesis enhanced the sizes of osteoclasts, but attenuated their bone-resorbing activity. Also, the increased chromosomal DNA fragmentation and caspase-3 activity in the late phase of osteoclastogenesis were found to be decreased by treatment with OPG siRNA. Furthermore, effects of OPG siRNA treatment on osteoclastogenesis and bone-resorbing activity were recovered by the treatment of exogenous OPG. These results suggest that OPG, expressed by the osteoclasts themselves, may play an auto-regulatory role in the late phase of osteoclastogenesis through the induction of apoptosis.

KEY WORDS: OPG, resorption, M-CSF, apoptosis, RANKL, autoregulation.

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Osteoprotegerin Expressed by Osteoclasts: An Autoregulator of Osteoclastogenesis

INTRODUCTION

Osteoclastogenesis, the development of osteoclasts, is a sequential process that includes progenitor survival, proliferation, and fusion to multinucleated cells, which then activate bone-resorbing osteoclasts. This process is mediated by numerous factors including cytokines, signaling molecules, and transcriptional factors. Among them, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL), which are produced by osteoblasts, are essential for triggering osteoclast differentiation. The binding of RANKL to the receptor activator of nuclear factor- κ B (RANK) induces the formation of multinucleated osteoclasts and maintains the activation and survival of osteoclasts (Arai *et al.*, 1999; Miyamoto *et al.*, 2000, 2001).

Of note, the activities of RANKL during osteoclastogenesis are regulated by osteoprotegerin (OPG), a soluble glycoprotein that is currently known to be secreted by stromal cells and osteoblastic lineage cells (Boyle *et al.*, 2003; Theoleyre *et al.*, 2004; Baud'huin *et al.*, 2007, 2013). OPG as a decoy receptor prevents the binding between RANKL and RANK by binding to RANKL and blocks osteoclast formation *in vitro* (Schoppet *et al.*, 2000; Udagawa *et al.*, 2000). *In vivo* studies have demonstrated that OPG deletion results in enhanced remodeling of bone and osteoporosis (Bucay *et al.*, 1998), whereas OPG overexpression leads to osteopetrosis in mice (Simonet *et al.*, 1997). Several studies have also demonstrated that OPG exerts an anti-apoptotic effect and promotes the survival of immune cells and cancer cells by binding to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (Malyankar *et al.*, 2000; Pritzker *et al.*, 2004; Kobayashi-Sakamoto *et al.*, 2006; Benslimane-Ahmim *et al.*, 2013).

In this study, for the first time, we detected OPG expression in osteoclasts that were involved in alveolar bone resorption to create the eruption pathways and to accommodate growing tooth germs in rats. To the best of the authors' knowledge, although there are reports on the expression and activities of OPG in stromal cells including osteoblasts, the expression and activities of OPG in osteoclasts themselves have been rarely reported. Osteoclasts are formed by the fusion of monocyte lineage cells, but this process should not be limitless. We hypothesized that OPG in the osteoclasts may play the role of a limiting step in an intrinsic mechanism to negatively regulate osteoclastogenesis. To test this hypothesis, we determined OPG expression in osteoclasts *in vivo* and *in vitro*, and assessed its biological roles in regulating the differentiation and survival of osteoclasts *in vitro*.

MATERIALS & METHODS

Animals

Eight-week-old C57BL/6 male mice and Sprague-Dawley rats were used. This study was conducted in accordance with the guidelines of the Chonnam National University Institutional Animal Care and Use Committee.

Isolation of Bone-marrow-derived Cells and Osteoclast Formation

Bone-marrow-derived cells (BMDCs) were harvested by flushing femurs and tibias with α -minimum essential medium (α -MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (GIBCO BRL) and filtered through a 70- μ m nylon cell strainer (BD Biosciences, Durham, NC, USA). Following red blood cell (RBC) lysis, cells were re-suspended in α -MEM supplemented with 25 ng/mL M-CSF (R&D Systems, Minneapolis, MN, USA) for 16 hr. Non-adherent cells were harvested and cultured with M-CSF (50 ng/mL) and RANKL (R&D Systems, 100 ng/mL) for 4, 7, and 11 days. Cells were treated with OPG (R&D Systems) on day 7 after the induction of osteoclastogenesis. The culture medium was exchanged with fresh medium every 3 days, and osteoclast formation was evaluated.

RT-PCR and Quantitative Real-time PCR

The RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was conducted with an RT system containing Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA). Real-time amplification of complementary (c)DNA was conducted in a Rotor-Gene 3000 System (Corbett Research, Mortlake, Australia) with the SYBR Green PCR Master Mix Reagent Kit (Qiagen, Valencia, CA, USA). The primers used were as follows: OPG, 5'-ACAGTTTGCCTGGGACCAAA-3' and 5'-TCACAGAGGTCAATGTCTTGA-3'; NFATc1, 5'-TCATCCTGTCCAACACCAAA-3' and 5'-TCACCCTGGTGTCTTCTTC-3'; OSCAR, 5'-ACCTGGCACCTACTGTGCT-3' and 5'-TCCATCTCCAGGCAGTCTCT-3'; TRAP, 5'-CAGCAGCC AAGGAGGACTAC-3' and 5'-ACATAGCCACACCGTTC TC-3'; ATP6vOd2, 5'-CATTCTTGAGTTTGAGGCCGAC-3' and 5'-AGCTTGAGCTAACAACCGCA-3'; DC-STAMP, 5'-T TGCCGCTGTGGACTATCTG-3', 5'-TTGGGTTCTTGCTT CTCTCC-3'; α -Integrin, 5'-TCGTTTCTATCCACCGCAG-3', 5'-GAAGACCAGCGAGCAGTTGA-3'; β_3 -Integrin, 5'-GTGG GACACAGCAAACAACC-3' and 5'-TTAAGTCCCCCGGTAG GTGA-3'; Cathepsin K, 5'-CCATATGTGGGCCAGGATGAA-3' and 5'-CGTTCCCCACAGGAATCTCT-3'; Bcl-2, 5'-GGGTGT GAGTGGATTCTGGG-3' and 5'-CGGGCAGGAGTTAACACA GT-3'; Bax, 5'-CCAAGAAGCTGAGCGAGTGT-3' and 5'-CA CGTCAGCAATCATCTCTG-3'; and β -actin, 5'-GATCTGGCA CCACACCTTCT-3' and 5'-GGGGTGTGAAGGTCTCAAA-3'. The amplified cDNA products were resolved on a 1.5% agarose gel by electrophoresis. The mean cycle threshold values were used for assessment of the relative level of gene expression.

Immunofluorescence Staining

The samples of alveolar bone containing the maxillary molar germs at postnatal day 9 were isolated, fixed in 4% paraformaldehyde solution, and then decalcified. Sections from paraffin-embedded tissues were cut at 5- μ m thickness. Cultured osteoclasts *in vitro* were fixed in 3.7% paraformaldehyde. Rabbit polyclonal antibodies for OPG (ABBIOTEC, San Diego, CA, USA) and TRAP (Santa Cruz Biotech, Santa Cruz, CA, USA) were used as primary antibodies. After the endogenous peroxidase activity was blocked, immunofluorescence staining was performed with a TSA™ kit (Invitrogen). The samples were counterstained with DAPI, and examined with an LSM confocal microscope (Carl Zeiss, Göttingen, Germany).

Western Blot Analysis

Protein extracts were prepared with a CytoBuster Protein Extraction Reagent (Novagen, Madison, WI, USA) and transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was incubated with purified rabbit polyclonal primary antibody raised against OPG (ABBIOTEC), TRAP (Santa Cruz Biotech), Bax (Cell Signaling Technology, Danvers, MA, USA), or Bcl-2 (Santa Cruz Biotech) overnight. The purified mouse monoclonal primary antibody to β -actin (Sigma-Aldrich) was used as the reference. The blots were incubated with the horseradish-peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody (Cell Signaling Technology), subsequently developed with the HRP Substrate Luminol Reagent (Millipore Corporation, Bedford, MA, USA) and photographed.

OPG Production Assay

The respective levels of OPG in the culture medium and BMDC lysates, which were prepared with CytoBuster (Novagen, Madison, WI, USA), were determined with a mouse OPG Immunoassay kit (R&D Systems). OPG quantity was measured as absorbance at 400 nm in a Microplate Reader (Thermo Fisher Scientific, Rockford, IL, USA).

TRAP Staining

TRAP staining was performed with the TRAP assay kit (Sigma Aldrich). BMDCs were fixed with 3.7% formaldehyde for 30 sec and incubated for 1 hr at 37°C, with protection from light in a mixture of fast Garnet GBC, sodium nitrite, naphthol AS-BI phosphoric acid, acetate, and the tartrate of the leukocyte acid phosphatase.

Transfection

BMDCs were transfected with specific siRNA against OPG or scrambled control RNA with Lipofectamine 2000 (Invitrogen). Both siRNA treatments were performed at osteoclastogenesis induction days 4 and 7. The level of OPG expression was determined by real-time RT-PCR and Western blot analysis.

Figure 1. Osteoprotegerin (OPG) protein in tissue sections was detected by immunofluorescence. **(a)** OPG was localized in multinucleated cells (arrows) around the rapidly growing cap stage 3rd molar germ at postnatal day 9. **(b)** OPG-immunoreactive cells (arrows) were also found in multinucleated cells located occlusal to the erupting 2nd molar germ at postnatal day 9. **(c, d)** High magnifications from 2 adjacent sections show OPG **(c)**- and tartrate-resistant acid phosphatase (TRAP) **(d)**-immunopositive osteoclasts, respectively. **(e)** A negative control omitting primary antibody did not show any reaction. DF, dental follicle; yellow dotted line, osteoclasts; blue dotted line, blood vessels; 2nd, 2nd molar tooth germ; and 3rd, 3rd molar tooth germ.

Bone Resorption Assay

BMDCs were seeded into a fluoresceinamine-labeled sodium chondroitin sulfate-labeled calcium-phosphate-coated 48-well plate from the Bone Resorption Assay Kit (COSMO Bio, Tokyo, Japan) at a density of 2×10^4 cells *per* well. Fluorescence intensity in the conditioned medium was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm, with FLx800, Microplate Fluorescence Reader (BIO-TEK, Winooski, VT, USA). To visualize resorption pits, we filled the wells with 5% sodium hypochlorite (Sigma-Aldrich) and dried them.

Cathepsin K and Caspase-3 Activity Assay

Cathepsin K and caspase-3 activity was measured in osteoclast extracts with the Cathepsin K Activity Assay Kit (BioVision, Milpitas, CA, USA) and the Caspase-3/CPP32 Colorimetric

Assay Kit (BioVision), respectively. Cathepsin K intensity and caspase-3 activities were measured with the FLx800, Microplate Fluorescence Reader (BIO-TEK) and Microplate Reader (Thermo Fisher Scientific), respectively.

TUNEL Assay

BMDCs were fixed with 3.7% formaldehyde, and TUNEL assays were performed with the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA). Apoptotic cell death was visualized by microscopy at 100x magnification.

Statistical Analysis

Data (mean $\pm$ SD) were acquired from 3 independent experiments and assessed by analysis of variance (ANOVA). The *p* values $< .05$ were considered statistically significant.

RESULTS

OPG is Expressed in Osteoclasts *in vivo* and *in vitro*

In the immunofluorescence analysis, OPG was unexpectedly localized in multinucleated cells of the alveolar bone around the developing 3rd molar tooth germ that was undergoing rapid growth (Fig. 1a). OPG was also found in the multinucleated cells located occlusal to the developing 2nd molar germ that was undergoing eruptive movement (Fig. 1b). For further identification of these cells, 2 adjacent sections were stained with anti-sera against tartrate-resistant acid phosphatase (TRAP) and OPG, respectively. The OPG-expressing multinucleated cells were found to be TRAP-immunopositive osteoclasts (Figs. 1c, 1d).

To confirm OPG expression in osteoclasts, we induced *in vitro* osteoclast differentiation of mouse BMDCs. BMDCs were cultured with a combination of M-CSF and RANKL, classic inducers of osteoclast differentiation. As shown in Figs. 2A and 2B, osteoclasts expressed OPG and TRAP. The level of OPG transcription and translation reached a peak at day 7 after RANKL treatment and declined thereafter. The TRAP level increased in a time-dependent manner. Immunofluorescence staining (Fig. 2D) clearly demonstrated that OPG was expressed in the TRAP-positive osteoclasts. As shown in Fig. 2C, the OPG level in cell lysates and culture medium was analyzed by ELISA at day 11 after treatment with M-CSF only or M-CSF/RANKL or M-CSF/RANKL/OPG siRNA. Mouse BMDCs were incubated with OPG siRNA or control siRNA for 6 hr before treatment with M-CSF or M-CSF/RANKL. Compared with M-CSF-only treatment, M-CSF/RANKL treatment significantly elevated the OPG level in both cell lysates and culture medium, and furthermore, the OPG expression was successfully decreased by treatment with OPG siRNA.

OPG siRNA Enhances Multinucleation of Osteoclasts but Attenuates Their Bone-resorbing Activity

To investigate the function of OPG produced by osteoclasts during osteoclastogenesis, we silenced the OPG gene by treatment with specific OPG siRNA. Mouse BMDCs were incubated with OPG siRNA or control siRNA for 6 hr before treatment with

M-CSF or M-CSF/RANKL, and osteoclast formation was evaluated by TRAP staining at day 11. Multinucleated TRAP-positive osteoclasts were formed after treatment with a combination of M-CSF and RANKL. Surprisingly, OPG siRNA treatment, but not control siRNA, in the presence of M-CSF and RANKL significantly increased the osteoclast size. The cell planar surface area of osteoclasts and the number of nuclei after OPG silencing on day 11 were approximately seven- and six-fold greater, respectively, than those in osteoclasts treated with control siRNA (Fig. 3A).

Next, we examined the role of OPG in bone resorption. We performed a pit formation assay to directly assess the resorption activity of OPG-silenced osteoclasts by comparing the resorption pits on dentin surfaces. As shown in Fig. 3B, BMDCs that were cultured in the presence of M-CSF and RANKL for 11 days produced a number of irregularly shaped pits of various sizes, whereas cells treated only with M-CSF did not form any pits. Unexpectedly, OPG siRNA-treated BMDCs cultured with a combination of M-CSF and RANKL showed less pit formation. Control siRNA did not cause a discernible difference in the formation of resorption pits after the cells were cultured with a combination of M-CSF and RANKL. We also confirmed these results by measuring the level of fluoresceinamine released from the resorption pits. Moreover, the level of fluoresceinamine was recovered to that in the M-CSF/RANKL-treated group when the cells pre-treated with OPG siRNA for 7 days were treated with exogenous OPG.

OPG is Involved in the Regulation of Organic Matrix Resorption Activity

For further investigation of the effect of OPG gene silencing on osteoclast activity, the levels of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), and osteoclast-associated, immunoglobulin-like receptor (OSCAR) for differentiation, dendritic cell-specific transmembrane protein (DC-STAMP) and ATP6vOd2 (an essential component of the osteoclast-specific proton pump that mediates extracellular acidification in bone resorption) for cell fusion, α_v and β_3 integrin for cell attachment, and TRAP and cathepsin K for organic matrix dissolution were determined at

Figure 2. Expression of OPG during osteoclastogenesis of BMDCs. For osteoclastogenesis, mouse BMDCs were cultured in the presence of M-CSF or M-CSF/RANKL. OPG and TRAP expression was analyzed by real-time RT-PCR (A) or Western blotting (B) at the indicated times. The relative expression of OPG and TRAP protein was normalized to β -actin expression and quantified by Scion Image software. Also, the OPG protein level in cell lysates and culture medium was analyzed by ELISA at day 11 after treatment with M-CSF and RANKL. Cells were treated with OPG siRNA and control siRNA at days 4 and 7 (C). Immunofluorescence analyses of OPG expression (D-a, b) and TRAP staining (D-c, d) were performed in the osteoclasts from BMDCs at day 11 after treatment with M-CSF or M-CSF/RANKL. The gel image (A, B) is representative of 3 independent experiments.

the transcriptional level. The increase in OPG expression by M-CSF and RANKL treatment was confirmed, and it was successfully silenced by siRNA treatment (Fig. 4A). Apparently, OPG expression was not related to gene regulation for cell

Figure 3. Involvement of OPG in osteoclastogenesis and bone resorption. **(A)** Effect of OPG siRNA on osteoclastogenesis. BMDCs were treated with M-CSF or M-CSF/RANKL or M-CSF/RANKL/OPG siRNA for 11 days and TRAP-stained. Cells were treated with OPG siRNA and control siRNA at days 4 and 7 after induction. M-CSF-only treatment failed to induce osteoclastogenesis, and the osteoclasts after the OPG siRNA treatment were apparently increased in size. The size of the TRAP-positive cells was analyzed at days 7 and 11, and the number of nuclei was counted at day 11 after the treatment. **(B)** Effect of OPG siRNA on bone-resorbing activity of osteoclasts. BMDCs were incubated in a culture medium overnight at a density of 1×10^6 cells/mL. The next day, non-adherent cells were harvested in a phenol-free α -MEM supplemented with 10% FBS and 1% alveolar-arterial gradient (A-A), M-CSF (50 ng/mL), and RANKL (100 ng/mL) and immediately seeded into a fluoresceinamine-labeled sodium chondroitin sulfate-labeled calcium-phosphate-coated plate at a density of 2×10^4 cells per well. Resorption pits were visualized by light microscopy at day 11 after treatment of M-CSF or M-CSF/RANKL or M-CSF/RANKL/OPG siRNA. Exogenous OPG (200 ng/mL) was also treated to the cells pre-treated with M-CSF/RANKL/OPG siRNA for 7 days. Apparently, resorption pit size and the level of the released fluoresceinamine measured at day 11 were reduced by OPG siRNA treatment, but recovered by exogenous OPG to the levels in the M-CSF/RANKL or M-CSF/RANKL/OPG control siRNA treated group. Data are from 3 independent experiments. * $p < .05$.

differentiation, cell fusion, and cell attachment except for the increase in NFATc1 at day 11. In contrast, OPG siRNA, but not control siRNA, in combination with M-CSF and RANKL significantly down-regulated the expression of TRAP and cathepsin K, compared with that after the treatment with M-CSF and RANKL combination (Fig. 4B). Inhibition of cathepsin K activities was also confirmed by spectrophotometry (Fig. 4C).

OPG siRNA Inhibits Apoptosis of Mature Osteoclasts

Based on the present findings that OPG silencing induced a larger cell size, OPG involvement in cell death was further investigated. This was supported by studies which showed that

OPG regulates apoptosis in other cells (Emery *et al.*, 1998; Shipman and Croucher, 2003; Zinonos *et al.*, 2011). To examine the intracellular effect of OPG expressed during osteoclastogenesis on apoptosis of mature osteoclasts, we evaluated the effect of OPG siRNA on the appearance of apoptotic nuclear condensation, the activation of caspase cascades, and the expression of the Bax pro-apoptotic factor and Bcl-2 anti-apoptotic factor. In the late stages of osteoclastogenesis induced by M-CSF and RANKL in BMDCs, the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive mature osteoclasts was clearly increased. Interestingly, the increment of TUNEL-positive cells was found to be decreased by treatment with OPG siRNA on day 11 (Fig. 5A). Moreover, the increase of Bcl-2 mRNA and the inhibition of activation of caspase-3, an apoptosis final effector molecule, were induced by treatment with OPG siRNA. Control siRNA did not show any effects on the apoptosis of mature osteoclasts in the late stage of osteoclastogenesis (Figs. 5B, 5C). However, the size increase and anti-apoptotic figures of osteoclasts by OPG down-regulation were obviously recovered in a dose-dependent manner by the exogenous OPG treatment (Fig. 5D).

DISCUSSION

This study clearly demonstrated the expression of OPG in osteoclasts *in vivo*, as well as in primary cultured osteoclasts from bone marrow and RAW 264.7 cells *in vitro* (Appendix). Sequencing analysis revealed that the OPG mRNA expressed in osteoclasts was identical to the previously reported OPG mRNA (Genbank NM_008764). Moreover, OPG expression was significantly increased in the late phase of osteoclastogenesis. Of note, down-regulation of OPG in osteoclasts induced an increase in their size and multinucleation, as well as longer survival and reduced bone-resorbing activity, despite the significantly increased size; furthermore, exogenous OPG treatment to OPG siRNA-treated cells recovered the bone-resorbing activity, and inhibited multinucleation and anti-apoptosis. This study also suggested that OPG in osteoclasts may play a negative role in osteoclastogenesis. A negative feedback in osteoclastogenesis was previously reported in the inhibition of NFATc1 by DYRK1A, a serine/threonine kinase (Lee *et al.*, 2009).

Several studies have reported that the absence of cell-cell fusion in osteoclasts resulted in severely impaired bone-resorbing activity. Bone-resorbing activity of mononuclear osteoclasts is lower than that of multinucleated osteoclasts (Yagi *et al.*, 2005; Miyamoto, 2011; Chiu *et al.*, 2012). In addition, TRAP activities in multinucleated osteoclastic giant cells of medium size are size-dependent, and the large cells reveal, in part, low activities (Metze *et al.*, 1987). In this study, the size and bone-resorbing activity of osteoclasts induced by RANKL were increased throughout the differentiation processes. In particular, osteoclasts treated with RANKL and OPG siRNA showed about seven-fold increase in size, but unexpectedly a two-fold decrease in bone-resorbing activity during the late phase of osteoclastogenesis, compared with osteoclasts treated with RANKL and scrambled control siRNA. Moreover, reduction in OPG by siRNA treatment did not affect the expression of OSCAR, an osteoclast differentiation marker gene (Merck *et al.*, 2004), *Atp6vOd2* and DC-STAMP, fusion-related molecules (Yagi *et al.*, 2005; Lee *et al.*, 2006), and α v and β 3 integrin, adhesion molecules (Lacey *et al.*, 1998; Nakamura *et al.*, 1999), but of note, it negatively affected the activity and expression of TRAP and cathepsin K, bone lytic enzymes (Lacey *et al.*, 1998; Boyle *et al.*, 2003). These results are consistent with the findings of a previous report, which suggested that large osteoclasts had low TRAP activities (Metze *et al.*, 1987). Regarding the cell size, it is certain that the increase in size was accomplished by multinucleation (Fig. 3). However, the mRNA level of DC-STAMP and *ATP6vOd2* was not significantly altered. We cannot provide logical data for this discrepancy, but it is conceivable that other fusion-related genes may exist in addition to these 2 genes and/or that co-regulatory factors may function in controlling the size. Otherwise, OPG siRNA could cause increased survival of precursors in addition to osteoclasts, so the greater availability of precursor fusion partners could permit greater multinucleation.

In this study, the increased sizes of osteoclasts by OPG siRNA treatment were further investigated with respect to apoptosis. Recently, Masuda *et al.* (2013) demonstrated that anti-apoptotic proteins such as Bcl-xL and Mcl-1 positively regulated cell viability and negatively regulated the bone-resorbing activity of osteoclasts both *in vitro* and *in vivo*. In this study, Bax expression was not altered, but expression of Bcl-2, an anti-apoptotic factor, was increased by OPG siRNA treatment. Thus, this indicates that silencing of OPG by a specific siRNA in osteoclasts during the late phase of osteoclastogenesis inhibited apoptosis, followed by excessive cell fusion and reduction in bone-resorbing activity.

In contrast to the studies that demonstrated the positive effect of OPG on the survival of cells including vascular endothelial cells and smooth-muscle cells (Chikatsu *et al.*, 2002; Collin-Osdoby, 2004; Chollet *et al.*, 2010), in this study, OPG siRNA strongly enhanced the survival of mature osteoclasts. The difference in OPG function may depend on the cell types. Osteoclasts are terminally differentiated cells, and they undergo rapid apoptosis in the absence of trophic factors such as M-CSF and RANKL. Thus, it is suggested that extracellularly secreted OPG from osteoclasts, which was demonstrated in this study, may

Figure 4. Effects of OPG on the expression of osteoclastogenesis-related genes. **(A)** The increased OPG expression by M-CSF and RANKL treatment for 7 and 11 days was silenced by siRNA treatment. **(B)** Effects of OPG siRNA on mRNA expression of differentiation marker genes (NFATc1 and OSCAR), fusion-related genes (DC-STAMP and *ATP6vOd2*), adhesion molecules (α_v and β_3 integrin), and lytic enzymes (TRAP and cathepsin K) during osteoclastogenesis. Cells were treated with OPG siRNA and control siRNA on days 4 and 7. The mRNA levels were analyzed by real-time RT-PCR at the indicated times after treatment with M-CSF or M-CSF/RANKL. Expression of TRAP and cathepsin K enzymes was significantly affected by OPG silencing. **(C)** Cathepsin K activities were measured at the indicated times after treatment with M-CSF and RANKL. Data were acquired from 3 independent experiments. * $p < .05$.

Figure 5. Effect of OPG siRNA on apoptosis during osteoclastogenesis. Cells were treated with OPG siRNA and control siRNA on 4 and 7 days. **(A)** TUNEL assay was performed at day 11 after treatment with M-CSF or M-CSF/RANKL. **(B)** To determine the effect of OPG siRNA on apoptosis-related genes during osteoclastogenesis, we analyzed the expression of Bax and Bcl-2 mRNA by real-time RT-PCR at the indicated times after treatment with M-CSF or M-CSF/RANKL. **(C)** Caspase-3 activities were measured after treatment with M-CSF and RANKL. Data were acquired from 3 independent experiments. **(D)** Exogenous OPG was treated to the cells pre-treated with M-CSF/RANKL/OPG siRNA for 7 days, and afterward the osteoclast sizes by multinucleation and apoptotic figures were observed at day 11. By the OPG treatment, multinucleation was decreased, whereas apoptotic cells were increased. * $p < .05$.

indirectly induce apoptosis of osteoclasts themselves by limiting RANKL availability for binding to RANK. Conversely, secreted OPG during osteoclastogenesis may bind to TRAIL and inhibit apoptotic cell death, which is opposite the effects of OPG binding to RANKL. However, the anti-apoptotic effects due to binding of OPG to TRAIL should be negligible because the affinity of OPG for RANKL is greater than that for TRAIL (Truneh *et al.*, 2000). Also, we cannot rule out binding of OPG to the osteoclast surface and subsequent direct intracellular regulation of pro- or anti-apoptotic machinery. This proposed mechanism is supported by both the OPG structure that has a C-terminal heparin-binding domain (Yamaguchi *et al.*, 1989) and by the reports that OPG activates the integrin signaling pathway (Lane *et al.*, 2013) and OPG can be internalized (Standal *et al.*, 2002). A further study is needed to address this mechanism in osteoclasts.

In conclusion, it was found that OPG expression was detected in mature osteoclasts during osteoclastogenesis, and silencing of OPG expression resulted in not only an increase in the cell size but also in reduction of apoptosis and bone-

resorption activity of mature osteoclasts. Although it is well-known that osteoclasts and osteoblast lineage cells communicate with each other bidirectionally through paracrine factors (Matsuo and Irie, 2008), these results clearly demonstrate that OPG from osteoclasts plays an autoregulatory role during the late phase of osteoclastogenesis through the induction of apoptosis. This study provides clues regarding the serious question of how the OPG/RANKL/RANK or OPG/TRAIL/death receptor system interacts during osteoclastogenesis. Also, this study engenders the hypothesis that induction of apoptosis of mature osteoclasts by OPG could be an effective therapy to treat diseases associated with bone loss. To test this hypothesis, the theory that intracellular action and direct binding of OPG onto the cell membrane causes release of factors of OPG-induced apoptosis and OPG expression under non-RANKL-induced osteoclastogenesis should be investigated further. Moreover, it should be carefully noted that the *in vitro* circumstances are different from those *in vivo*, in which the osteoclasts can be affected by bone matrix components and proximal cells such as osteoblasts and bone-lining cells.

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REFERENCES

- Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, *et al.* (1999). Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. *J Exp Med* 190:1741-1754.
- Baud'huin M, Lamoureux F, Duplomb L, Rédini F, Heymann D (2007). RANKL, RANK, osteoprotegerin: key partners of osteoimmunology and vascular diseases. *Cell Mol Life Sci* 64:2334-2350.
- Baud'huin M, Duplomb L, Teletchea S, Lamoureux F, Ruiz-Velasco C, Maillason M, *et al.* (2013). Osteoprotegerin: multiple partners for multiple functions. *Cytokine Growth Factor Rev* 24:401-409.
- Benslimane-Ahmim Z, Poirier F, Delomenie C, Lokajczyk A, Grelac F, Galy-Fauroux I, *et al.* (2013). Mechanistic study of the proangiogenic effect of osteoprotegerin. *Angiogenesis* 16:575-593.
- Boyle WJ, Simonet WS, Lacey DL (2003). Osteoclast differentiation and activation. *Nature* 423: 337-342.

- Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, *et al.* (1998). Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 12:1260-1268.
- Chikatsu N, Takeuchi Y, Fukumoto S, Yano K, Fujita N, Tsuruo T, *et al.* (2002). Clonal endothelial cells produce humoral factors that inhibit osteoclast-like cell formation in vitro. *Endocr J* 49:439-447.
- Chiu YH, Mensah KA, Schwarz EM, Ju Y, Takahata M, Feng C, *et al.* (2012). Regulation of human osteoclast development by dendritic cell-specific transmembrane protein (DC-STAMP). *J Bone Miner Res* 27:79-92.
- Chollet ME, Brouland JP, Bal dit Sollier C, Bauduer F, Drouet L, Bellucci S (2010). Evidence of a colocalisation of osteoprotegerin (OPG) with von Willebrand factor (VWF) in platelets and megakaryocytes alpha granules. Studies from normal and grey platelets. *Br J Haematol* 148:805-807.
- Collin-Osdoby P (2004). Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res* 95:1046-1057.
- Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, *et al.* (1998). Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* 273:14363-14367.
- Kobayashi-Sakamoto M, Hirose K, Nishikata M, Isogai E, Chiba I (2006). Osteoprotegerin protects endothelial cells against apoptotic cell death induced by *Porphyromonas gingivalis* cysteine proteinases. *FEMS Microbiol Lett* 264:238-245.
- Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, *et al.* (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93:165-176.
- Lane D, Matte I, Laplante C, Garde-Granger P, Rancourt C, Piche A (2013). Osteoprotegerin (OPG) activates integrin, focal adhesion kinases (FAK), and Akt signaling in ovarian cancer cells to attenuate TRAIL-induced apoptosis. *J Ovarian Res* 6:82-91.
- Lee SH, Rho J, Jeong D, Sul JY, Kim T, Kim N, *et al.* (2006). v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. *Nat Med* 12:1403-1409.
- Lee Y, Ha J, Kim HJ, Kim YS, Chang EJ, Song WJ, *et al.* (2009). Negative feedback inhibition of NFATc1 by DYRK1A regulates bone homeostasis. *J Biol Chem* 284:33343-33351.
- Malyankar UM, Scatena M, Suchland KL, Yun TJ, Clark EA, Giachelli CM (2000). Osteoprotegerin is an alpha vbeta 3-induced, NF-kappa B-dependent survival factor for endothelial cells. *J Biol Chem* 275:20959-20962.
- Masuda H, Hirose J, Omata Y, Tokuyama N, Yasui T, Kadono Y, *et al.* (2013). Anti-apoptotic Bcl-2 family member Mcl-1 regulates cell viability and bone-resorbing activity of osteoclasts. *Bone* 58:1-10.
- Matsuo K, Irie N (2008). Osteoclast-osteoblast communication. *Arch Biochem Biophys* 473:201-209.
- Merck E, Gaillard C, Gorman DM, Montero-Julian F, Durand I, Zurawski SM, *et al.* (2004). OSCAR is an FcRgamma-associated receptor that is expressed by myeloid cells and is involved in antigen presentation and activation of human dendritic cells. *Blood* 104:1386-1395.
- Metzke K, Ciplea AG, Hettwer H, Barckhaus RH (1987). Size dependent enzyme activities of multinucleated (osteoclastic) giant cells in bone tumors. *Pathol Res Pract* 182:214-221.
- Miyamoto T (2011). Regulators of osteoclast differentiation and cell-cell fusion. *Keio J Med* 60:101-105.
- Miyamoto T, Arai F, Ohneda O, Takagi K, Anderson DM, Suda T (2000). An adherent condition is required for formation of multinuclear osteoclasts in the presence of macrophage colony-stimulating factor and receptor activator of nuclear factor kappa B ligand. *Blood* 96:4335-4343.
- Miyamoto T, Ohneda O, Arai F, Iwamoto K, Okada S, Takagi K, *et al.* (2001). Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood* 98:2544-2554.
- Nakamura I, Pilkington MF, Lakkakorpi PT, Lipfert L, Sims SM, Dixon SJ (1999). Role of alpha(v)beta(3) integrin in osteoclast migration and formation of the sealing zone. *J Cell Sci* 112(Pt 22):3985-3993.
- Pritzker LB, Scatena M, Giachelli CM (2004). The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Bio Cell* 15:2834-2841.
- Schoppert M, Preissner KT, Hofbauer LC (2000). RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. *Arterioscler Thromb Vasc Biol* 22:549-553.
- Shipman CM, Croucher PI (2003). Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. *Cancer Res* 63:912-916.
- Simonet WS, Lacey DL, Dunstan CR, Kelly M, Chang MS, Luthy R, *et al.* (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 89:309-319.
- Standal T, Seidel C, Hjertner O, Plesner T, Sanderson RD, Waage A, *et al.* (2002). Osteoprotegerin is bound, internalized, and degraded by multiple myeloma cells. *Blood* 100:3002-3007.
- Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D (2004). The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine Growth Factor Rev* 15:457-475.
- Truneh A, Sharma S, Silverman C, Khandekar S, Reddy MP, Deen KC, *et al.* (2000). Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem* 275:23319-23325.
- Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, *et al.* (2000). Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. *Endocrinology* 141:3478-3484.
- Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, *et al.* (2005). DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. *J Exp Med* 202:345-351.
- Yamaguchi K, Kinoshita M, Goto M, Kobayashi F, Tsuda E, Morinaga T, *et al.* (1998). Characterization of structural domains of human osteoclastogenesis inhibitory factor. *J Biol Chem* 273:5117-5123.
- Zinonos I, Labrinidis A, Lee M, Liapis V, Hay S, Ponomarev V, *et al.* (2011). Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin in vivo. *J Bone Miner Res* 26:630-643.