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Osteoprotegerin Reduces the Serum Level of Receptor Activator of NF-κB Ligand Derived from Osteoblasts

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Osteoprotegerin (OPG) is a decoy receptor for receptor activator of NF-κB ligand (RANKL). We previously reported that OPG deficiency elevated the circulating level of RANKL in mice. Using OPG−/− mice, we investigated whether OPG is involved in the shedding of RANKL by cells expressing RANKL. Osteoblasts and activated T cells in culture released a large amount of RANKL in the absence of OPG. OPG or a soluble form of receptor activator of NF-κB (the receptor of RANKL) suppressed the release of RANKL from those cells. OPG- and T cell-double-deficient mice showed an elevated serum RANKL level equivalent to that of OPG−/− mice, indicating that circulating RANKL is mainly derived from bone. The serum level of RANKL in OPG−/− mice was increased by ovariectomy or administration of 1α,25-dihydroxyvitamin D3. Expression of RANKL mRNA in bone, but not thymus or spleen, was increased in wild-type and OPG−/− mice by 1α,25-dihydroxyvitamin D3. These results suggest that OPG suppresses the shedding of RANKL from osteoblasts and that the serum RANKL in OPG−/− mice exactly reflects the state of bone resorption. The Journal of Immunology, 2007, 178: 192–200.

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3 Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; MT-MMP, membrane-type matrix metalloproteinase; TACE, TNF-α-converting enzyme; TIMP, tissue inhibitor of metalloproteinases; OPG, osteoprotegerin; CRD, cysteine-rich domain; DcR3, decoy receptor for FasL (18). DcR3 induced osteoclastogenesis of monocytes and macrophages through TNF-α induction (19). DR6 was identified by a homology search of an expressed sequence tag database (20). The ligand for DR6 has not yet been identified.

OPG-deficient (OPG−/−) mice exhibit severe osteoporosis due to enhanced osteoclast differentiation and function (21, 22). OPG−/− mice also showed accelerated bone resorption and formation (21, 23). Juvenile Paget's disease, an autosomal recessive osteopathy, is characterized by rapidly remodeling woven bone. A homozygous deletion of the OPG gene was found in patients with juvenile Paget's disease and the serum level of free RANKL was markedly elevated in one such patient (24). Serum levels of
RANKL were markedly elevated in OPG−/− mice as well. However, it was recently reported that the serum concentration of RANKL-OPG complex is markedly higher than that of free RANKL or OPG in healthy adult females (25). This raises the possibility that the presence of OPG may interfere with the quantification of RANKL level in ELISA.

In the present study, we verified the above possibility using wild-type (WT), RANKL-deficient (RANKL−/−), and OPG−/− mice. We confirmed that the serum level of RANKL in OPG−/− mice is actually 20-fold higher than that of WT mice. We also investigated how OPG and other OPG-related receptors are involved in the shedding of RANKL by cells expressing RANKL.

Our results suggest that OPG plays key roles in the shedding of RANKL in mice. The physiological importance of this new role of OPG is discussed here.

**Materials and Methods**

**Mice**

OPG−/− (C57BL/6) mice (22) and control WT mice were purchased fromCLEA Japan. RANKL−/− (C57BL/6) mice were generated in one of our laboratories (26). C57BL/6 nude (Fox n1nu/nu) mice were purchased from Taconic Farms (27). All procedures for animal care were approved by the animal management committee of Matsumoto Dental University.

**Cell cultures**

Calvarial osteoblasts were prepared as described previously (28). Osteo-

blasts were cultured in α-MEM with 10% FBS in the presence or absence of 10−8 M 1α,25(OH)2D3 (Wako). For T cell activation, CD4+ T cells were isolated from the spleens of 8-week-old WT mice using a MACS CD4+ T cell isolation kit (Miltenyi Biotech). Purified CD4+ T cells were seeded into 96-well anti-mouse CD3 Ab-coated plates or control plates (BD Pharmingen) at 1 × 105 cells/well and were cultured in RPMI 1640 me-
dium with 10% FBS in the presence or absence of 2 μg/ml anti-mouse CD28 Ab (BD Pharmingen).

**Fc-chimeric receptors and protease inhibitors**

The following Fc chimeric proteins of TNFR family members were used: OPG-Fc (mouse OPG [Glu52-Leu56] fused to the Fc region of human IgG1 [Pro85-Lys358] via a polypeptide linker), RANK (mouse RANK [Glu35-Pro146]-Fc), DR6 (human DR6 [Met3-Leu353]-Fc), DcR3 (human DcR3 [Val124-Lys306]-Fc), and control Fc. All recombinant Fc chimeric pro-
tiens were purchased from R&D Systems. The following protease inhibi-
tors were used: aprotinin (Trasylol; Bayer), pepstatin (Peptide Institute), E-64 (Calbiochem), the hydroxamate inhibitor TAPI-2 (Calbiochem) (29), a thiadiazole urea inhibitor (MMP-3 inhibitor III; Calbiochem) (30), a syn-
thetic cyclic peptide inhibitor (H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-
Cys-OH) for gelatinase (MMP29 inhibitor II; Calbiochem) (31), recom-
binant mouse TIMP-1 (R&D Systems), and recombinant mouse TIMP-2, -3 (Calbiochem).

**In vivo experiments**

To determine the origin of serum RANKL, OPG- and T cell-double-defi-
cient (OPG−/−Fox n1nu/n1nu) mice were created by cross-breeding OPG−/− mice and athymic nude (Fox n1nu/n1nu) mice (27). The double-deficient mice at the age of 8 days were sacrificed and blood was collected. To examine the relationship between age and serum RANKL level, WT and OPG−/− mice at different ages (from 8 days old to 18 wk old) were sacrificed and blood was collected. To examine the effects of estrogen deficiency on the serum level of RANKL, 12 wk-old female WT and OPG−/− mice were ovariectomized (OVX) and sacrificed 4 wk later. To determine the effects of 1α,25(OH)2D3 on the serum level of RANKL, 50 pm 1α,25(OH)2D3 diluted in propylene glycol (0.1 ml/head/day) was injected i.p. into 10-
wk-old WT and OPG−/− mice daily for 2 days. On day 3, the mice were sacrificed and blood was collected. Femora, tibiae, spleen, and thymus were also removed from each mouse. The diaphyses of femora and tibiae were prepared as bone samples. These tissues were used for semiquantitative RT-PCR analysis.

**Measurements of RANKL and OPG**

Serum levels of RANKL and OPG were determined by performing the respective ELISAs (Quantikine M ELISA kit; R&D Systems). The condi-
tioned medium was collected from cultures of osteoblasts and CD4+ T cells and subjected to ELISAs.

**RT-PCR analysis**

For semiquantitative RT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). First-strand cDNA was synthesized from total RNA with oligo(dT) primers and ReverTra Ace (Toyobo) and subjected to PCR amplification with Ex Taq polymerase (Takara). The specific primer pairs and temperature conditions were as follows: for mouse RANKL cDNA amplification, forward: 5′-CGCTC TGTTCCTGTACTTGGAGCCG-3′/reverse: 5′-TCGTTGCTCCCCTTCT GTACAGGT-3′; denaturation at 94°C for 2 min followed by 32 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 1 min. For mouse OPG cDNA amplification, forward: 5′-TGGAGATCAATTCTCAGC-3′/reverse: 5′-TCGATGTTGAGGGCATA-3′; denaturation at 94°C for 2 min followed by 34 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 1 min. For mouse TACE cDNA amplification, forward: 5′-AGCATGGACTCACGTAC-3′/reverse: 5′-TCGACCTGTTAAGGCGCC-3′; denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min. For mouse GAPDH cDNA amplification, forward: 5′-AAGATGATGGTCGCAGGC-3′/reverse: 5′-GCCACCAGCACTGGAATTTT-3′; denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 1 min. For mouse 24-hydroxylase cDNA amplification, forward: 5′-ACCAGTCCTGACATC-3′/reverse: 5′-CCTACACACCTGTCGTA-3′; denaturation at 94°C for 2 min followed by 20 or 22 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 2 min.

**Northern blot analysis**

Total RNA was prepared from bone, intestine, kidney, thymus, and spleen of 12-wk-old WT or OPG−/− mice using TRIzol (Invitrogen Life Tech-
nologies). Total RNA (20 μg) was separated by electrophoresis on a 1.2%-agarose 2% formaldehyde gel and transferred onto a nylon membrane (Roche). The nylon membrane was then hybridized at 68°C in DIG-Easy Hyb buffer (Roche) with a digoxigenin-labeled antisense RNA probe en-
compassing the complete protein-coding sequence of mouse RANKL. Af-
fter hybridization, the membrane was washed at room temperature for 5 min in 2× SSC, 0.1% SDS once, then washed at 68°C for 15 min in 0.1× SSC, 0.1% SDS twice. The bands for RANKL mRNA were immuno-
stained with alkaline phosphatase-conjugated anti-digoxigenin Ab (Roche) and CDP-Star reagent (New England Biolabs).

**OPG knockdown by siRNA and re-expression by OPG expression plasmid in calvarial osteoblasts**

RNA interference-mediated knockdown of OPG protein expression was performed using Silencer pre-designed small interfering RNA (siRNA) (Ambion). The specific RNA oligonucleotides for targeting OPG were as follows: sense, 5′-CGUGGUGUUGCCGAAAACAGATT-3′antisense, 5′-UC UGUUUCGGGAACACCGTT-3′. The scrambled siRNA duplex was used as a negative control (Ambion). WT calvarial osteoblasts were plated in 12-well dishes at 105 cells/well for 24 h before transfection. Transfection of the siRNA was accomplished with the HVJ Envelope Vector kit Geno-
mONE Neo (Ishihara Sango) according to the manufacturer’s instruc-
tions. Forty-eight hours posttransfection, osteoblasts were collected for RNA isolation and the conditioned media were collected for the quantifi-
cation of OPG protein by ELISA. OPG re-expression in OPG osteo-

capsules was accomplished by transient transfection of the pCMV-OPG expression plasmid using the HVJ Envelope Vector kit GenomONE-Neo. Seventy-two hours posttransfection, osteoblasts were collected for RNA isolation and the conditioned media were collected for the quantification of OPG protein by ELISA.

**Biotinylation of RANKL and analysis for RANKL disappearance in the culture medium of osteoblasts**

One microgram of mouse RANKL (R&D Systems) was dissolved in 0.5 ml of 40 mM NaHCO3 (pH 8.0) containing 14 μl of 5 mg/ml sulfosuccinimi-
dyl-biotin (Biotinylation kit; Dojin Laboratories), and incubated at 25°C for 2 h. Free sulfosuccinimidyI-biotin was removed from the reaction mixture using a gel-filtration column according to the manufacturer’s in-
structions (Dojin Laboratories). WT and OPG−/− osteoblasts were plated in a 96-well plate at 104 cells/well. After culture for 24 h, the medium was changed to the fresh medium containing 50 ng/ml biotinylated RANKL (100 μl/well). After incubation for 48 h, the culture medium was collected
and subjected to SDS-PAGE, followed by blotting to a clear blot membrane-P (Atto). The biotinylated RANKL was detected with streptavidin-HRP (Roche) using the ECL Plus detection system (GE Healthcare).

Results

**OPG deficiency results in markedly elevated circulating levels of RANKL in mice**

We previously reported that OPG−/− mice showed marked elevated serum levels of RANKL compared with WT mice (23). However, it was reported that the serum concentration of the RANKL-OPG complex is extremely high in healthy human adults (25). This raises the possibility that the presence of OPG may interfere with the quantification of RANKL in ELISA. We first measured the concentrations of RANKL and OPG in serum from WT, OPG−/−, and RANKL−/− mice in the respective ELISA kits (Fig. 1A). The serum level of RANKL in OPG−/− mice was 20-fold higher than that in WT mice. In contrast, the serum level of OPG in RANKL−/− mice was equivalent to that in WT mice. This indicated that the presence of RANKL in serum did not interfere with OPG measurement. To examine whether OPG interferes with the quantification of RANKL in this ELISA, the serum obtained from OPG−/− mice was incubated with increasing concentrations of mouse rOPG-Fc for 2 days and subjected to RANKL measurement (Fig. 1B). RANKL measurements were barely affected by the presence of physiological concentrations of OPG (2-5 ng/ml), but 70% interference was observed at 10 ng/ml OPG. When OPG−/− mouse serum was incubated with a high concentration of OPG-Fc (200 ng/ml), the observed RANKL level was almost equivalent to that in WT mouse serum (Fig. 1B). RANKL−/− mouse serum was also incubated with increasing concentrations of mouse rRANKL for 2 days and subjected to OPG measurements. The quantification of serum OPG was not affected even at a high concentration of rRANKL (50 ng/ml) (Fig. 1C). These results suggest that the serum concentrations of OPG and RANKL in WT mice shown in Fig. 1A closely reflect the true values.

**Effects of OPG gene inactivation on expression levels and patterns of RANKL transcripts in vivo**

OPG withdrawal may affect the expression level of RANKL mRNA or induce a short RANKL mRNA transcript encoding a soluble form of RANKL. The expression of RANKL mRNA in various tissues from WT and OPG−/− mice was analyzed by Northern blotting using an antisense RNA probe encompassing the complete protein-coding sequence of mouse RANKL (Fig. 2). Four RANKL mRNA species were observed in thymus and spleen: a major transcript of 2.4 kb and three minor transcripts of 10.4, 6.0, and 0.5 kb (Fig. 2). In agreement with the previous finding (32), kidney and intestine scarcely expressed RANKL mRNA. Bone exclusively expressed the 2.4-kb transcript. The expression levels and patterns of RANKL transcripts in OPG−/− mice were quite similar to those in WT mice (see Fig. 5A).

**Regulation of RANKL mRNA expression in osteoblasts**

RANKL is highly expressed in osteoblasts and lymphoid cells (14). We examined whether OPG is involved in the shedding of RANKL from osteoblasts. Osteoblasts obtained from OPG−/− mice spontaneously released RANKL even in the absence of any osteotropic factors. The concentration of RANKL was significantly increased following the addition of 1α,25(OH)2D3 (Fig. 3A). RT-PCR analysis showed that the mRNA expression of RANKL in osteoblasts was also up-regulated by the addition of 1α,25(OH)2D3, concomitantly with the up-regulation of 1α,25(OH)2D3-24-hydroxylase mRNA expression (Fig. 3B). The expression of TACE, which has been proposed to be an enzyme responsible for the shedding of RANKL, was not regulated by 1α,25(OH)2D3. Northern blot analysis confirmed that WT and OPG−/− osteoblasts exclusively expressed a 2.4-kb transcript of 28S and 18S rRNA. The lower panel of ethidium bromide (EtBr) staining shows the amount of RNA in each lane.

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**FIGURE 1.** Evidence that OPG deficiency markedly elevated the circulating level of RANKL in mice. A. Serum concentrations of RANKL and OPG in WT, OPG−/−, and RANKL−/− mice at the age of 4 wk. Serum obtained from WT, OPG−/−, and RANKL−/− mice was subjected to RANKL and OPG ELISAs. Data are expressed as the mean ± SD of four mice. *p < 0.01. n.d., Not detectable. B. Effects of OPG-Fc on the measurement of serum RANKL levels of OPG−/− mice. Serum obtained from OPG−/− mice was incubated with increasing concentrations of OPG-Fc for 2 days. Concentrations of RANKL in the serum were determined by ELISA. C. Effects of rRANKL on the measurement of serum OPG levels of RANKL−/− mice. Serum obtained from RANKL−/− mice was incubated with increasing concentrations of RANKL for 2 days. Concentrations of OPG in the serum were determined by ELISA.

**FIGURE 2.** Effects of OPG gene inactivation on expression levels and patterns of RANKL transcripts. Total RNA was isolated from bone, intestine, kidney, thymus, and spleen of 12-wk-old WT and OPG−/− mice, and analyzed by Northern blot hybridization to detect RANKL transcripts. The positions of 28S and 18S rRNA are noted. The lower panel of ethidium bromide (EtBr) staining shows the amount of RNA in each lane.
FIGURE 3. Regulation of RANKL mRNA expression in osteoblasts. A, Spontaneous release of RANKL into culture medium from OPG−/− osteoblasts. Calvarial osteoblasts from OPG−/− mice were cultured for 16 h in 6-cm diameter dishes (3 × 105 cells) in the presence or absence of 1α,25(OH)2D3 (10−8 M). Concentrations of RANKL in the culture medium were determined by ELISA. Data are expressed as the mean ± SD of four cultures.* Significantly different between the two groups; p < 0.01. B, Expression of RANKL, TACE, 24-hydroxylase, and GAPDH mRNAs in OPG−/− osteoblasts treated with or without 1α,25(OH)2D3. OPG−/− osteoblasts were cultured for 16 h in 6-cm diameter dishes (3 × 105 cells) in the presence or absence of 1α,25(OH)2D3 (10−8 M). Total RNA was isolated from the osteoblasts, and analyzed for the expression of RANKL, TACE, 24-hydroxylase, and GAPDH mRNAs by semiquantitative RT-PCR. ID no., The identification number of the mice examined. C, Northern blot analysis of RANKL mRNA expression in WT and OPG−/− osteoblasts. WT and OPG−/− osteoblasts were cultured for 16 h in a 6-cm dish (3 × 105 cells) in the presence or absence of 1α,25(OH)2D3 (10−8 M). Total RNA was isolated from the osteoblasts and analyzed by Northern blot hybridization. The positions of 28S and 18S rRNA are noted. The lower panel of ethidium bromide (EtBr) staining shows the amount of RNA in each lane. D, Effects of OPG knockdown by siRNA on RANKL mRNA expression in WT and OPG−/− osteoblasts. WT osteoblasts cultured in a 12-well plate were transfected with various amounts of siRNA targeting OPG (50 – 500 pmol/well) or a scrambled control siRNA. After 48 h posttransfection, cells were harvested and analyzed by semiquantitative RT-PCR for OPG and RANKL mRNA levels (upper panel). Concentrations of OPG in the culture medium were determined by ELISA (lower panel). E, Effects of OPG re-expression by an OPG expression plasmid on RANKL mRNA expression in OPG−/− osteoblasts. OPG−/− osteoblasts cultured in a 12-well plate were transfected with various amounts of an OPG expression plasmid (0 – 60 μg/well) or a GFP expression plasmid as a negative control. Seventy-two hours posttransfection, cells were harvested and analyzed by semiquantitative RT-PCR for OPG and RANKL mRNA levels (upper panel). Concentrations of OPG in the culture medium were determined by ELISA (lower panel). F, Effects of OPG on RANKL loss in culture medium of osteoblasts. WT and OPG−/− osteoblasts were plated in a 96-well plate at 1 × 104 cells/well and cultured in α-MEM containing 10% FBS (0.1 ml/well). After incubation for 24 h, the medium was changed to the fresh medium containing biotinylated recombinant soluble RANKL (50 ng/ml). After incubation for 48 h, the culture media were subjected to SDS-PAGE, followed by Western blotting. Biotinylated RANKL was detected with streptavidin-HRP.

RANKL mRNA, in which expression was up-regulated by the addition of 1α,25(OH)2D3 (Fig. 3C). Treatment with 1α,25(OH)2D3 did not induce other sizes of RANKL transcripts in osteoblasts.

Changes in OPG production in osteoblasts may alter the expression of RANKL. We performed both OPG knockdown by siRNA in WT osteoblasts and OPG re-expression by an OPG expression plasmid in OPG−/− osteoblasts. Transfection of 250 pM siRNA for the OPG sequence into WT osteoblasts led to an ∼70% reduction in the OPG protein (Fig. 3D). However, RANKL mRNA expression was not affected by the OPG knockdown. Transfection of an OPG expression plasmid in OPG−/− osteoblasts dose-dependently increased OPG secretion into the culture medium, but had no effect on RANKL mRNA expression (Fig. 3E). Thus, RANKL mRNA expression was not affected by the changes in OPG production. OPG has been shown to bind to cell surface heparan sulfates and to be internalized (33). Tat et al. (34) also reported that OPG accelerated internalization of RANKL. We examined the possibility that RANKL internalization is impaired in OPG−/− osteoblasts and soluble RANKL accumulates in the culture medium. Biotinylated soluble RANKL was added for 48 h to WT and OPG−/− osteoblast cultures, and the loss of biotinylated RANKL in the culture medium was determined by SDS-PAGE, followed by Western blotting (Fig. 3F). Similar levels of biotinylated RANKL were detected in cultures of WT and OPG−/− osteoblasts (Fig. 3F). These results suggest that the elevated RANKL level in culture medium of OPG−/− osteoblasts is due to the shedding from the osteoblasts.

Inhibition of the shedding of RANKL from osteoblasts by OPG and RANK

The amino acid sequence of the CRDs in OPG shows high homology to that of the CRDs in three TNFR members: RANK, DcR3, and DcR6 (Fig. 4A) (16). We next examined the effects of OPG and these OPG-related receptors on the shedding of RANKL.
by OPG−/− osteoblasts. Before measuring the effects of these molecules on the shedding of RANKL, the interference by OPG, RANK, DcR3, and DR6 in the RANKL assay was evaluated in the presence of RANKL at 50 pg/ml, because the concentration of RANKL in the culture medium of OPG−/− osteoblasts was 50–100 pg/ml (Fig. 3A). It was reported that OPG exists and acts as a homodimeric form (35). OPG-Fc also exists as a homodimer (35), suggesting that OPG-Fc similarly behaves like a native protein. OPG-Fc at concentrations higher than 2 ng/ml and RANK-Fc at 50 ng/ml strongly interfered with the assay of RANKL at 50 pg/ml (Fig. 4B). In contrast, DR6-Fc and DcR3-Fc even at 1,000 ng/ml barely interfered with the RANKL assay (data not shown). We therefore examined the effects of OPG-Fc at 0.5–2 ng/ml and RANK-Fc at 1–10 ng/ml on the shedding of RANKL from OPG−/− osteoblasts (Fig. 4C). The concentrations of RANKL in

**FIGURE 4.** Effects of OPG and OPG-related receptors on the shedding of RANKL by OPG−/− osteoblasts. A. The modular structures of the CRDs of OPG and OPG-related receptors. The amino acid sequence of the CRDs of mouse OPG (mOPG) shows high homology to that of the CRDs of mouse RANK (mRANK), human DcR6 (hDcR6), and human DcR3 (hDcR3). The crystallized modules A1, A2, B1, and B2 in the CRDs are pattern-coded as shown in the inset. B. Evaluation of interference by OPG and RANK in the RANKL assay. RANKL at 50 pg/ml was measured in an ELISA system in the presence of increasing concentrations of OPG-Fc and RANK-Fc. The results are expressed as the ratio of the observed values to the expected value (50 pg/ml). C. Effects of OPG-Fc, RANK-Fc, DcR3-Fc, and DR6-Fc on the shedding of RANKL from OPG−/− osteoblasts. OPG−/− osteoblasts were cultured for 96 h in 24-well plates (3 × 10^4 cells/well) in the presence of various concentrations of OPG-Fc and OPG-related receptor-Fcs. Concentrations of RANKL in the culture medium were determined by ELISA, and corrected according to the interference curves shown in B. Data are expressed as the mean ± SD of four cultures. *p < 0.001, significantly different from the control cultures treated with IgG-Fc.

**FIGURE 5.** Effects of OPG and RANK on the shedding of RANKL by activated T cells. A. Release of RANKL from activated T cells into the culture medium. CD4 T cells were isolated from spleens of WT mice, and cultured in the presence or absence of soluble anti-CD28 Ab (αCD28) in 96-well plates (1 × 10^5 cells/well) that had been precoated with anti-CD3 Ab (αCD3). After incubation for 96 h, the culture medium was collected, and concentrations of RANKL were determined by ELISA, n.d., not detectable. B. Expression of RANKL, OPG, TACE, and GAPDH mRNAs in T cells cultured with or without an anti-CD28 Ab and/or anti-CD3 Ab. CD4 T cells (1 × 10^5 cells/well) were cultured for 16 h in 96-well plates precoated with anti-CD3 Ab (αCD3). Some cultures were treated with anti-CD28 Ab (αCD28). Total RNA was isolated from T cells and analyzed for the expression of RANKL, OPG, TACE, and GAPDH mRNAs by semiquantitative RT-PCR. C. Evaluation of interference by OPG and RANK in the RANKL assay. RANKL at 500 pg/ml was measured in an ELISA system in the presence of increasing concentrations of OPG-Fc and RANK-Fc. The results are expressed as the ratio of the observed values to the expected value (500 pg/ml). D. Effects of OPG-Fc and RANK-Fc on the shedding of RANKL by activated T cells. CD4 T cells were cultured in an anti-CD3 Ab-coated dish and costimulated with anti-CD28 Ab for 96 h. Various concentrations of Fc-chimeric proteins were added to the T cell cultures. Concentrations of RANKL in the culture medium were determined by ELISA, and corrected according to the interference curves shown in C. Data are expressed as the mean ± SD from four cultures. *p < 0.001, significantly different from the control cultures treated with IgG-Fc.
Rankl from osteoblasts, OPG. To identify the proteases responsible for the shedding of RANKL, we measured the release of OPG, which inhibits the shedding of RANKL, by osteoblasts. Involvement of MT-MMPs and TACE in the shedding of RANKL was strongly inhibited by OPG-Fc on the shedding of RANKL was stronger than DR6-Fc and DcR3-Fc did not (data not shown). The inhibitory effect of OPG-Fc on the shedding of RANKL was stronger than 20 ng/ml and RANK-Fc at higher than 50 ng/ml strongly interfered with the shedding of RANKL, whereas MMP29 inhibitor III (31) and MMP3 inhibitor III (30) did not (Fig. 6B). The biological activity of MMPs is suppressed by TIMPs, which are also expressed by osteoblasts. TIMP-2 and TIMP-3 inhibited the release of RANKL, whereas MMP2/9 inhibitor III (31) and MMP3 inhibitor III (30) did not (Fig. 6B). The release of RANKL from OPG-/- osteoblasts was not inhibited by aprotinin (a serine protease inhibitor), pepstatin A (an aspartate protease inhibitor), or E-64 (a cysteine protease inhibitor), when these agents were added at 10 times higher concentrations than those used for the ordinary preparation of cell lysates (Fig. 6A). Mouse osteoblasts are known to express MMP-2, MMP-3, MMP-13, and MT1-MMP (9, 10). TNF-α protease inhibitor-2 (TAPI-2), a broad spectrum inhibitor of MMPs, MT-MMPs, and TACE (29), strongly inhibited the shedding of RANKL, whereas MMP29 inhibitor III (31) and MMP3 inhibitor III (30) did not (Fig. 6B). The biological activity of MMPs is suppressed by TIMPs, which are also expressed by osteoblasts. TIMP-2 and TIMP-3 inhibited the release of RANKL, but TIMP-1 did not (Fig. 6C). MT-MMPs are selectively inhibited by TIMP-2 and TIMP-3 but not by TIMP-1 (36–38). TACE is selectively inhibited by TIMP-3 but not TIMP-1 or TIMP-2 (39). These findings suggest that MT-MMPs and TACE, but not serine proteases, aspartate proteases, or cysteine proteases are involved in the shedding of RANKL in OPG-/- osteoblasts.

Effects of various types of protease inhibitors on the shedding of RANKL by OPG-/- osteoblasts. Effects of (A) inhibitors of serine, aspartate, and cysteine proteases (aprotinin, pepstatin A, and E-64), (B) inhibitors of metalloproteinases (TAPI-2, MMP29 inhibitor III, and MMP3 inhibitor III), and (C) tissue inhibitor of metalloproteinases (TIMP-1, TIMP-2 and TIMP-3) on the shedding of RANKL were examined in cultures of OPG-/- osteoblasts. OPG-/- osteoblasts were cultured for 96 h in 24-well plates (3 x 10^5 cells/well) in the presence or absence of several types of protease inhibitors at the concentrations indicated. Concentrations of RANKL in the culture medium were determined by ELISA. Data are expressed as the mean ± SD from four cultures. * Significantly different from the control cultures; **p < 0.01.

Inhibition of the shedding of RANKL from activated T cells by OPG and RANK

Activated CD4+ T cells have been shown to release excess amounts of RANKL (13). Therefore, we hypothesized that activated T cells release RANKL, because hemopoietic cells, including T cells, barely produce OPG. When T cells obtained from WT mice were activated by plate-bound anti-CD3 Ab together with soluble anti-CD28 Ab, the concentration of RANKL in the culture medium was markedly elevated (Fig. 5A). RT-PCR analysis confirmed that activated T cells strongly expressed RANKL mRNA and weakly expressed OPG mRNA (Fig. 5B). The expression of TACE mRNA was also up-regulated by T cell activation (Fig. 5B). We then examined whether OPG-Fc and RANK-Fc could suppress the release of RANKL by activated T cells. The OPG and RANK in the RANKL assay was evaluated in the presence of RANKL at 500 pg/ml, because the conditioned medium of activated T cells contained nearly 500 pg/ml RANKL (Fig. 5A). OPG-Fc at higher than 20 ng/ml and RANK-Fc at higher than 50 ng/ml strongly interfered with the RANKL assay at 500 pg/ml RANKL (Fig. 5C). Accordingly, OPG-Fc and RANK-Fc at concentrations up to 20 and 50 ng/ml, respectively, were used in the experiments with T cell cultures. OPG-Fc and RANK-Fc dose-dependently suppressed the shedding of RANKL by activated T cells (Fig. 5D), but DR6-Fc and DcR3-Fc did not (data not shown). The inhibitory effect of OPG-Fc on the shedding of RANKL was stronger than that of RANK-Fc in cultures of activated T cells.

Involvement of MT-MMPs and TACE in the shedding of RANKL by osteoblasts

It is difficult to characterize the enzymes involved in the shedding of RANKL from osteoblasts because WT osteoblasts spontaneously release a large amount of OPG, which inhibits the shedding of RANKL. To identify the proteases responsible for the shedding of RANKL from osteoblasts, OPG-/- osteoblasts were cultured in the presence or absence of several kinds of protease inhibitors (Fig. 6). The release of RANKL from OPG-/- osteoblasts was not inhibited by aprotinin (a serine protease inhibitor), pepstatin A (an aspartate protease inhibitor), or E-64 (a cysteine protease inhibitor), when these agents were added at 10 times higher concentrations than those used for the ordinary preparation of cell lysates (Fig. 6A). Mouse osteoblasts are known to express MMP-2, MMP-3, MMP-13, and MT1-MMP (9, 10). TNF-α protease inhibitor-2 (TAPI-2), a broad spectrum inhibitor of MMPs, MT-MMPs, and TACE (29), strongly inhibited the shedding of RANKL, whereas MMP29 inhibitor III (31) and MMP3 inhibitor III (30) did not (Fig. 6B). The biological activity of MMPs is suppressed by TIMPs, which are also expressed by osteoblasts. TIMP-2 and TIMP-3 inhibited the release of RANKL, but TIMP-1 did not (Fig. 6C). MT-MMPs are selectively inhibited by TIMP-2 and TIMP-3 but not by TIMP-1 (36–38). TACE is selectively inhibited by TIMP-3 but not TIMP-1 or TIMP-2 (39). These findings suggest that MT-MMPs and TACE, but not serine proteases, aspartate proteases, or cysteine proteases are involved in the shedding of RANKL in OPG-/- osteoblasts.

Effects of T cell deficiency on serum RANKL levels in OPG-/ mice.

Serial concentrations of RANKL in OPG- and T cell double-deficient mice. OPG- and T cell double-deficient mice were produced by crossing OPG-/- mice and athymic nude mice (Fox n1nu/nu mice). Blood was collected from WT (OPG+/-/Fox n1nu/nu) mice, athymic nude (OPG+/-/Fox n1nu/nu) mice, athymic nude (OPG+/-/Fox n1nu/nu) mice, OPG+/-/Fox n1nu/nu mice, and OPG- and T cell double-deficient (OPG+/-/Fox n1nu/nu) mice at the age of 8 days. Serum concentrations of RANKL were measured by ELISA. Data are expressed as the mean ± SD from four mice.
Both OPG−/− osteoblasts and activated T cells released a large amount of RANKL in the culture medium and OPG-Fc effectively suppressed the release of RANKL from both types of cells. We examined which tissues, bone cells or T cells, were responsible for the elevated RANKL in OPG−/− mice. OPG- and T cell-double-deficient (OPG−/−) mice were created by crossing OPG−/− mice with Foxn1−/− mice. Serum levels of RANKL in OPG−/− mice were significantly increased by OVX (Fig. 8B). Circulating levels of RANKL in OPG−/− mice were significantly increased by OVX (Fig. 8A). Serum levels of RANKL in OPG−/− mice were gradually decreased with age (Fig. 8B). These results suggest that the high levels of serum RANKL detected in OPG−/− mice were derived not from T cells but from bone tissues. When 1α,25(OH)2D3 (50 pM/head/day) was administered i.p. to WT and OPG−/− mice for 2 days, serum concentrations of RANKL in WT mice were significantly increased in response to 1α,25(OH)2D3 administration but were still lower than those in untreated OPG−/− mice. Administration of 1α,25(OH)2D3 to OPG−/− mice further elevated the increased concentration of serum RANKL (Fig. 8C). Concomitantly, 1α,25(OH)2D3 induced hypercalcemia in mice with both genotypes (data not shown), suggesting that serum levels of RANKL reflected bone resorption. In contrast to RANKL, serum concentrations of OPG in WT mice were not affected by 1α,25(OH)2D3 administration (data not shown). Semi-quantitative RT-PCR showed that the thymus and spleen of WT and OPG−/− mice expressed RANKL mRNA, but the expression levels were much lower than those in bone (Fig. 8D, upper). The expression of RANKL mRNA in bone but not thymus or spleen was up-regulated by 1α,25(OH)2D3 administration. OPG mRNA was highly expressed in bone but not in thymus or spleen in WT mice (Fig. 8D, lower). 1α,25(OH)2D3 administration failed to down-regulate the OPG mRNA expression in bone (Fig. 8D, lower).

**FIGURE 8.** Origin of serum RANKL in OPG−/− mice. A, Effects of OVX on serum RANKL levels in WT and OPG−/− mice. Female WT and OPG−/− mice at the age of 12 wk were OVX or sham operated. Blood was collected from those mice 4 wk after surgery. Serum concentrations of RANKL were measured by ELISA. Data are expressed as the mean ± SD from four mice. *, Significantly different from the sham-operated controls; p < 0.01. B, Age-dependent changes in serum RANKL levels in OPG−/− mice. Serum concentrations of RANKL were measured by ELISA. Data are expressed as the mean ± SD of four mice. *, Significantly different from the sham-operated controls; p < 0.001. C, Effects of 1α,25(OH)2D3 administration on serum RANKL levels in WT and OPG−/− mice. WT and OPG−/− mice at the age of 10 wk were injected i.p. with 1α,25(OH)2D3 (50 pM/head/day) for 2 days. On day 3, blood was collected from the mice by heart puncture. *, Significantly different from the sham-operated controls; p < 0.001. D, Effects of 1α,25(OH)2D3 on RANKL and OPG mRNA expression in bone, thymus, and spleen were determined by semi-quantitative RT-PCR. Total RNA was analyzed for the expression of RANKL and OPG mRNAs by semi-quantitative RT-PCR. Data represent typical RANKL mRNA expression in four individual WT and OPG−/− mice (upper) and OPG mRNA expression in six individual WT mice (lower). ID no., The identification number of mice examined.

**Discussion**

We previously reported that OPG−/− mice showed marked elevated serum levels of RANKL compared with WT mice (23). However, the recent study demonstrated the possibility that the presence of OPG interferes with the quantification of the serum level of RANKL in humans (25). Using serum obtained from WT, RANKL−/−, and OPG−/− mice, we examined this possibility. 1) The serum level of OPG in RANKL−/− mice was equivalent to that in WT mice. 2) RANKL measurements were barely affected by the presence of physiological concentrations of rOPG (2–5 ng/ml). 3) Experiments of mixture of serum from OPG−/− mice and WT mice showed that circulating OPG in WT mice did not interfere with the RANKL assay (data not shown). These results suggest that the circulating level of RANKL is actually elevated in OPG−/− mice.

Northern blot analysis showed that the expression levels and patterns of RANKL transcripts in several tissues in OPG−/− mice were similar to those in WT mice. Neither OPG knockdown by siRNA in WT osteoblasts nor OPG re-expression by an OPG expression plasmid in OPG−/− osteoblasts alters RANKL mRNA expression. Treatment of osteoblasts with 1α,25(OH)2D3 up-regulated the expression of the 2.4-kb RANKL transcript but did not induce the other sizes of RANKL transcripts. In addition, disappearance of biotinylated RANKL in culture medium of OPG−/− osteoblasts was similar to that of WT osteoblasts. These results suggest that neither the transcriptional events nor the decrease in RANKL degradation is involved in the elevated RANKL level in serum in OPG−/− mice and in culture medium of OPG−/− osteoblasts. Thus, it was proposed that OPG may prevent the shedding of RANKL-expressing cells.

Using OPG−/− mice, we investigated 1) whether OPG inhibits the shedding of RANKL, 2) which proteases are involved in the shedding of RANKL, 3) which organ is responsible for the elevated serum level of RANKL, and 4) whether serum RANKL levels reflect the state of bone resorption. OPG−/− osteoblasts spontaneously released RANKL into the culture medium. Activated T cells obtained from WT mice also released a large amount of...
RANKL, WT osteoblasts but not activated T cells secreted OPG in the culture medium. Soluble RANK also inhibited the shedding of RANKL in OPG−/− osteoblasts. Similarly, the release of RANKL from activated T cells was inhibited by the addition OPG or soluble RANK. These results suggest that activated T cells release RANKL, because they do not produce OPG, and that the binding of OPG and soluble RANK to RANKL is important for the suppression of the shedding of RANKL. MT-MMPs and TACE were suggested to be involved in the shedding of RANKL in OPG−/− osteoblasts. The expression of TACE mRNA as well as RANKL mRNA was up-regulated in activated T cells, indicating that TACE is especially important for the shedding of RANKL in activated T cells. Neither DR6 nor DcR3 showed any effect on the shedding of RANKL by osteoblasts or activated T cells. These results suggest that the binding of OPG or soluble RANK to the membrane-associated RANKL in osteoblasts and T cells protects RANKL against attack by some proteases.

In agreement with the previous study (32), the RANKL transcripts were found in bone, thymus, and spleen but not in intestine and kidney in WT and OPG−/− mice. Activated T cells released a large amount of RANKL. These findings raised the possibility that not all but some of the soluble RANKL found in OPG−/− mice originated from T cells. However, our experiments using OPG−/− and T cell-deficient mice suggested that T cells are not involved in the elevated levels of RANKL in serum in OPG−/− mice. The serum level of RANKL in OPG−/− mice was closely correlated with the state of bone resorption: OVX and 1α,25(OH)2D3, was increased up to 30 ng/ml (43). Serum osteoclastogenesis by osteoprotegerin ligand serum levels in healthy young women. J. Bone Miner. Res. 12: 328–337.


Disclosures

The authors have no financial conflict of interest.

References


