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Porphyromonas gingivalis-derived Lysine Gingipain Enhances Osteoclast Differentiation Induced by Tumor Necrosis Factor-α and Interleukin-1β but Suppresses That by Interleukin-17A: IMPORTANCE OF PROTEOLYTIC DEGRADATION OF OSTEOPROTTEGRIN BY LYSINE GINGIPAIN

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Porphyromonas gingivalis-derived Lysine Gingipain Enhances Osteoclast Differentiation Induced by Tumor Necrosis Factor-α and Interleukin-1β but Suppresses That by Interleukin-17A

IMPORTANCE OF PROTEOLYTIC DEGRADATION OF OSTEOPROTEGERIN BYLYSINE GINGIPAIN

Periodontitis is a chronic inflammatory disease accompanied by alveolar bone resorption by osteoclasts. Porphyromonas gingivalis, an etiological agent for periodontitis, produces cysteine proteases called gingipains, which are classified based on their cleavage site specificity (i.e. arginine (Rgps) and lysine (Kgps) gingipains). We previously reported that Kgp, a lysine gingipain, degraded osteoprotegerin more efficiently than other cytokines, which might be related to enhancement of osteoclastogenesis by Kgp. These results indicate that the enhancing effect of Kgp on cytokine-induced osteoclastogenesis is dependent on the difference in degradation efficiency between each cytokine and OPG. In addition, elucidation of the N-terminal amino acid sequences of OPG fragments revealed that Kgp primarily cleaved OPG in its death domain homologous region, which might prevent dimer formation of OPG required for inhibition of receptor activator of nuclear factor κB ligand. Collectively, our results suggest that degradation of OPG by Kgp is a crucial event in the development of osteoclastogenesis and bone loss in periodontitis.

Periodontitis is a chronic inflammatory disease caused by infection with various bacteria, including Porphyromonas gingivalis, with alveolar bone resorption by osteoclasts as one of the characteristic symptoms (1). Osteoclasts are multinucleated giant cells that have differentiated from monocyte/macrophage lineage cells by cell-cell interactions with osteoblasts. Osteoblasts express receptor activator of nuclear factor κB (RANK) ligand (RANKL) on the plasma membrane as a result

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2 The abbreviations used are: RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; OPG, osteoprotegerin; F-OPG, FITC-labeled OPG; Rgp, arginine gingipain; Kgp, lysine gingipain; TRAP, tartrate-resistant acid phosphatase; Z, benzoyloxycarbonyl; ck, acyloxyketone; MEM, minimum essential medium.
of stimulation by various pathological as well as physiological bone-resorbing factors (2). RANKL induces osteoclast differentiation by activating intracellular signals mediated by RANK expressed on the plasma membrane of osteoclast precursor cells (3, 4). On the other hand, osteoprotegerin (OPG), a decoy receptor of RANKL secreted by osteoblasts, interferes with the interaction between RANKL and RANK and suppresses osteoclastogenesis (5, 6). Therefore, the relative expression level of RANKL/OPG is a crucial factor in regulation of osteoclastogenesis and bone resorption (1).

Under physiological conditions, calcitriol and parathyroid hormone play central roles in the induction of RANKL expression and suppression of OPG expression in osteoblasts (7), which leads to up-regulation of serum calcium. In pathological situations, it is known that prostaglandin E2, one of the chemical mediators of inflammation; Toll-like receptor ligands, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, IL-6, and IL-17A, are known to stimulate various types of cells, including osteoblasts, to induce RANKL expression and suppress that of OPG (8–11). It is also known that TNF-α directly acts on osteoclast precursor cells to induce their differentiation into osteoclasts in a RANKL-independent manner (12).

In alveolar bone resorption, which is associated with periodontitis, LPS derived from periodontal pathogens, such as P. gingivalis, is one of the major factors contributing to augmentation of osteoclastogenesis directly or indirectly via induction of inflammatory cytokines (13). On the other hand, P. gingivalis produces cysteine proteases called gingipains. Gingipains are the products of three independent genes, namely rgpA, rgpB, and kgp, and the bacterium produces several proteases from these genes, including RgpA(cat), HRgpA, membrane-type RgpA, RgpB, membrane-type RgpB, Kgp, and membrane-type Kgp. These proteases are referred to as arginine gingipains (Rgps) and lysine gingipains (Kgps), depending on the specificity for hydrolysis of either the Arg-Xaa or Lys-Xaa peptide bond, respectively (14, 15). In our previous studies, Kgp but not RgpB augmented osteoclastogenesis induced by calcitriol as well as various TLR ligands in an in vitro co-culture system that utilized mouse osteoblasts and bone marrow cells (16, 17).

Proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-17A, are produced by host cells exposed to pathogen-derived TLR ligands and thought to be involved in inflammatory osteoclastogenesis in periodontitis (1, 18). On the other hand, it has been reported that gingipains and culture supernatants of P. gingivalis degraded and inactivated cytokines, including IL-1β, IL-1 receptor antagonist, IL-6, IL-8, TNF-α, and interferon (IFN)-γ, in vitro (19–21). Therefore, we considered it interesting to explore the effects of gingipains, especially Kgp, on osteoclast differentiation induced by proinflammatory cytokines.

**EXPERIMENTAL PROCEDURES**

**Gingipains**—Two types of gingipains, 105-kDa Kgp and 50-kDa RgpB, were purified from culture supernatants of P. gingivalis HG66, as described previously (22), and reacted immediately before use by incubation for 5 min at 37 °C with 10 mM cysteine in 0.2 M Hepes buffer, pH 8.0, containing 10 mM CaCl2. Activated gingipains were diluted with appropriate medium or buffer containing 0.2 mM cysteine (16, 17). In some experiments, activated Kgp was inactivated by further incubation for 30 min with 0.1 mM benzoyloxy carbonyl-l-phenylalanine-l-lysyl-acyloxy ketone (Z-FK-cck) (Bachem, King of Prussia, PA) (23).

**Cytokines and Antibodies**—Recombinant proteins of human OPG (805-OS), mouse TNF-α (410-MT), mouse IL-1β (4–1-ML), and mouse IL-17A (421-ML) were purchased from R&D Systems (Minneapolis, MN). Human macrophage colony-stimulating factor (M-CSF) (Leukoprol®) was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Goat polyclonal IgG against human OPG (AF805), rat monoclonal IgGs against IL-1β (MAB4011) and IL-17A (MAB421), and biotinylated polyclonal goat IgG (BAF692) for mouse RANK were also obtained from R&D Systems. A rabbit polyclonal antibody against mouse TNF-α (MONOSAN® PS052) was obtained from Sanbio BV (Uden, Netherlands), whereas that for human RANKL (sc-9073) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Horseradish peroxidase (HRP)-linked anti-rat IgG and HRP-linked donkey anti-rabbit IgG were purchased from GE Healthcare. HRP-linked donkey anti-goat IgG and HRP-linked avidin were purchased from Santa Cruz Biotechnology and Invitrogen, respectively.

**Osteoclast Differentiation in Co-cultures of Osteoblasts and Bone Marrow Cells**—Newborn and 6-week-old ddY mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Primary osteoblasts were isolated from the calvaria of newborn mice using a conventional method with collagenase and dispase, as described previously (24). Bone marrow cells were obtained from the femurs and tibiae of 6-week-old mice. Osteoclasts were generated in co-cultures of bone marrow cells and primary osteoblasts, as described previously (24). In brief, osteoblasts (1.25 × 103 cells/well) and bone marrow cells (2.5 × 104 cells/well) were cultured in 50 μl of α-minimal essential medium (α-MEM) (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), antibiotics, and 0.2 mM cysteine in the presence or absence of various cytokines and gingipains in 384-well plates at 37 °C in humidified air containing 5% CO2. The medium was replaced every 3 days with fresh medium containing the same supplemental agents. One day after the second medium change, osteoclast generation was evaluated by counting tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells with three or more nuclei.

In experiments to evaluate the effects of OPG degradation by Kgp on osteoclast differentiation induced by TNF-α, IL-1β, and IL-17A, we used primary osteoblasts and bone marrow cells isolated from OPG-deficient and wild-type C57BL/6 mice (Clea Japan, Inc., Tokyo, Japan). Calvarial osteoblasts and bone marrow cells were isolated from 1-day- and 6-week-old male mice, respectively. The sex of the 1-day-old mice was determined by PCR results of the Y chromosome. Isolation of the cells and induction and evaluation of osteoclast differentiation were performed as described above. Isolation of osteoblasts and bone marrow cells from mice was performed according to a protocol.
approved by the Ethical Board for Animal experiments of Showa University (approval number 13053).

Quantitative Real-time PCR Analysis—Mouse calvarial osteoblasts (2.5 × 10⁴ cells/well) and bone marrow cells (2.5 × 10⁵ cells/well) isolated from ddY mice were cultured for 12 h in 384-well plates in the presence or absence of 50 nm Kgp.

Expression of RANKL (TNfsf11), Opg (TNfsf1b), RANK (TNfsf1a), and Gapdh mRNAs was evaluated using the Taq-Man® gene expression Cell-to-CT™ kit (Invitrogen) with a StepOne Real-Time® PCR system (Invitrogen). The probes and primers for RANKl, Opg, RANK, and Gapdh were supplied by Invitrogen. The assay IDs were Mm00441908_m1 (RANKl), Mm01205928_m1 (Opg), Mm00437135_m1 (Rank), and Mm03302249_g1 (Gapdh). Expression levels of RANKl, Opg, and Rank were normalized to that of Gapdh and expressed as a value relative to that obtained in the control experiments without Kgp.

**Immunoblot Analysis of Degradation of OPG, TNF-α, IL-1β, and IL-17A by Kgp—**Degradation of OPG, TNF-α, IL-1β, and IL-17A by Kgp in cell-free systems was evaluated by quantitative detection of the intact cytokines using Western blotting. Kgp and the cytokines were incubated in α-MEM containing 10% FBS and 0.2 mm cysteine at 37 °C. To examine the concentration-dependent degradation of OPG, TNF-α, IL-1β, and IL-17A, Kgp at 0, 0.5, 5, or 50 nm was incubated for 15 h with a 25 ng/ml concentration of each cytokine. To examine the time-dependent degradation of the cytokines, Kgp (50 nm) was incubated with the cytokines (25 ng/ml) for 0, 1, 3, or 15 h under the same conditions as described above.

The reaction mixtures (20 μl) were denatured by boiling in buffer containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% sucrose, 0.1% bromphenol blue, and 10% 2-mercaptoethanol at their final concentrations and then separated by SDS-8% polyacrylamide gel electrophoresis (for TNF-α, IL-1β, and IL-17A) or SDS-10% polyacrylamide gel electrophoresis (for OPG) and electrotransferred onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blocked with 1% nonfat dried skimmed milk powder in 20 mm Tris-HCl (pH 7.6) containing 150 mm NaCl and 0.1% Tween 20 and subjected to immunoblotting using the primary antibodies described above, namely those against TNF-α, IL-1β, IL-17A, and OPG, diluted in the blocking buffer described above at a dilution ratio of 1:10 for detection of TNF-α and 1:1000 for IL-1β, IL-17A, and OPG. Next, the membranes were further incubated with the appropriate secondary antibodies conjugated with HRP (1:5000). Secondary antibodies were quantitatively detected using Versa Doc 5000 MP (Bio-Rad) after incubation with ECL™ Prime Western Blotting Detection Reagent (GE Healthcare).

Degradation of TNF-α, IL-1β, and IL-17A by Kgp in the coculture system was also examined. Osteoblasts (1.25 × 10³ cells/well) and bone marrow cells (2.5 × 10⁴ cells/well) isolated from OPG-deficient male mice were co-cultured in the presence or absence of TNF-α, IL-1β, or IL-17A (10 ng/ml) and Kgp (50 nm) as described above. At 0, 1, 3, 6, and 15 h after changing media on day 6, the culture supernatants (20 μl) were denatured and applied to SDS-8% polyacrylamide gel electrophoresis. Cytokines remaining intact were detected by Western blot analyses as described above.

**Immunoblot Analysis of Degradation of RANKL and RANK by Kgp in Cell Culture Systems—**Degradation of RANKL by Kgp was evaluated using cultured osteoblasts. Primary mouse osteoblasts were cultured in 6-well plates for 3 days in α-MEM supplemented with 10% FBS in the presence of 10 nm calcitriol (Sigma-Aldrich) to induce the expression of RANKL. The cells were further cultured for 0, 0.5, 1, 3, or 18 h in the presence or absence of Kgp (50 nm). Degradation of RANK by Kgp was evaluated in cultured macrophages. Mouse bone marrow cells were cultured in α-MEM supplemented with 10% FBS for 3 days in the presence of M-CSF (50 ng/ml). Attached cells were treated with Kgp (50 nm) for 0, 1, 3, or 18 h in the same medium. Cells were washed with PBS and lysed in 10 mm Tris-HCl (pH 7.8) containing 1% Nonidet P-40, 0.15 m NaCl, and a protease inhibitor mixture containing EDTA (Roche Applied Science). The cell lysates (5 μg of protein) were subjected to SDS-PAGE (10% polyacrylamide gel) under a reducing condition and electrotransferred onto the membranes. The membranes were blocked and subjected to immunoblotting using antibodies against RANKL, RANK, and β-actin, as described above.

**Determination of N-terminal Amino Acid Sequence—**OPG (25 μg, 0.5 nmol) and Kgp (2 pmol) were incubated for 0, 15, 30, or 60 min at 37 °C in 20 μl of Hanks’ balanced salt solution (Wako Pure Chemicals) containing 0.2 mM cysteine. The reaction products were separated using SDS-12% polyacrylamide gel electrophoresis under reducing conditions, as described above. Electrophoresis was performed in the presence of 1 mm sodium thioglycolate. After electrotransfer to a PVDF membrane (Millipore), staining was performed with 0.1% Coomassie Brilliant Blue R-250 (Sigma-Aldrich) in 45% methanol and 10% acetic acid for 5 min, followed by decolorization in a solution containing 45% methanol and 7% acetic acid for 15 min and in 90% methanol for 40 s and then washing in pure water. Bands of 37- and 19-kDa fragments of OPG were cut, and the N-terminal amino acid sequences of the fragments were analyzed using ABI Procise® 491HT (Invitrogen) at the Nippon Research Institute of Biomatrix (Tokyo, Japan).

**Fragmentation of Fluorinated OPG by Kgp—**Recombinant human OPG (1 nmol) was incubated with 10 nmol of fluorescein isothiocyanate (FITC) isomer I (Sigma-Aldrich) for 4 h at room temperature in 0.1 m sodium borate buffer, pH 8.0. It has been reported that preferential labeling of the N-terminal amino group of proteins with FITC could be achieved at pH 8.0 for differentiating the dissociation constants of the α-amino and ε-amino groups (i.e., 8–9 and around 10, respectively) (25). Unreacted FITC was removed by gel filtration (Sephadex G-25, GE Healthcare) using PBS as an eluate. FITC-labeled OPG (F-OPG) (0.15 mg/ml) was incubated for 0.25–18 h with Kgp (25 nm) at 37 °C in Hanks’ balanced salt solution containing 0.2 mM cysteine. The reaction mixtures (30 μl) were separated by SDS-10% polyacrylamide gel electrophoresis under reducing conditions, as described above, and fluorescence derived from F-OPG and its fragments was detected using a fluorescence detection system (Printgraph type DX, ATTO Co., Tokyo, Japan).

**Binding of F-OPG to Osteoblasts—**F-OPG (1.5 μg/ml) was incubated for 15 min in the presence or absence of Kgp or Z-FK-ck-inactivated Kgp (30 nm) in α-MEM containing 10%...
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FBS. The reaction of F-OPG and Kgp was terminated by the addition of Z-FK-ck (0.1 mM). Mouse calvarial osteoblasts (2 × 10⁵ cells) were cultured in a 6-well plate for 3 days in α-MEM containing 10% FBS in the presence of 10 nM calcitriol and then washed three times with PBS and treated for 30 min at 37 °C with α-MEM plus 10% FBS containing 1.5 μg/mL F-OPG, F-OPG pretreated with Kgp, or F-OPG pretreated with Z-FK-ck-inactivated Kgp. The cells were washed three times with PBS and observed under a fluorescence microscope.

Degradation of OPG Mutants by Kgp—Full-length human OPG cDNA was cloned into pCAGGS mammalian expression vector (26) kindly provided by Dr. J. Miyazaki (Osaka University). The resulting pCAGGS-OPG, an expression plasmid for wild-type OPG, was used as a template for preparation of expression plasmids for OPG mutants by PCR using KOD DNA polymerase (KOD-Plus-Mutagenesis Kit, TOYOBO Co. Ltd., Osaka, Japan). Primer pairs used for preparation of pCAGGS-OPG(K258A), pCAGGS-OPG(K262A), and pCAGGS-OPG(K258A/K262A), the expression plasmids of mutant OPGs of which Lys-258, Lys-262, and both of them were substituted by Ala, were 5’-GCCATCAAAAAAGACCAAGATA-3’/5’-CCATAACTTCAGCAGCTGAAGTC-3’; 5’-GCAGACC-AAGATATAGTCAGAGAGAAGTGTTGCCATAACTTCAGC-3’; and 5’-GCCATCAAAAAAGCCAGAC-CAAGATA-3’/5’-CCATAACTTCAGCAGCTGAAGTC-3’, respectively. Wild type and the mutant OPG proteins were expressed in CHO-K1 cells. Culture supernatants were treated with 50 nM Kgp for various periods up to 3 h and analyzed by Western blotting using anti-human OPG antibody.

Statistical Analysis—A Mann-Whitney U test and a Bonferroni post hoc test were used for statistical analyses, with p values less than 0.05 considered to be significant.

RESULTS

Kgp Enhanced Osteoclast Differentiation Induced by IL-1β and TNF-α but Suppressed That Induced by IL-17A—We examined the effects of Kgp on osteoclast differentiation induced by IL-1β, TNF-α, and IL-17A using a co-culture system of mouse bone marrow cells and osteoblasts. Kgp (50 nM) induced formation of TRAP-positive multinucleated osteoclasts in culture without the addition of any cytokine. Each of the three cytokines (10 ng/mL) induced osteoclast differentiation, whereas Kgp significantly enhanced the number of osteoclasts formed in the presence of either IL-1β or TNF-α (Fig. 1A). Enhancement of IL-1β- and TNF-α-induced osteoclast formation by Kgp was dependent on the concentration of Kgp (Fig. 1C). In contrast, Kgp suppressed osteoclast differentiation induced by IL-17A.

On the other hand, RgpB (50 nM) did not have any effect on osteoclast differentiation induced by IL-1β, TNF-α, or IL-17A in the same co-culture system (Fig. 1B). These results are consistent with our previous observation that RgpB did not affect osteoclast differentiation induced by calcitriol or LPS (16).

As shown in Fig. 1C, Kgp induced osteoclast differentiation in a co-culture system of osteoblasts and bone marrow cells in a concentration-dependent manner, even without the addition of any cytokine. Although Kgp was prone to induce the expression of Rankl mRNA in co-cultures of mouse osteoblasts and bone marrow cells, we did not observe a significant difference between the expression level of Rankl mRNA in a control culture and that in a culture containing Kgp. In addition, Kgp did not affect the expression of Opg mRNA or that of Rank mRNA in the co-cultures (Fig. 2). It cannot be denied that the knock down-regulation of Rankl expression by Kgp was involved in osteoclast differentiation induced by Kgp, whereas it is possible that other mechanisms were also involved, such as degradation of factors that inhibit osteoclast differentiation, including interferons (27).

TNF-α and IL-1β More Stable Than OPG toward Degradation by Kgp—We previously reported that Kgp degraded OPG, a protein that inhibits RANKL-RANK interaction, and augmented osteoclast differentiation induced by calcitriol and various TLR ligands, including LPS (16). On the other hand, it was
shown that Kgp degrades several cytokines, including TNF-α (19). Therefore, we examined the degradation of TNF-α, IL-1β, and IL-17A by Kgp in complete medium containing 10% FBS, which was used for the experiments of osteoclast differentiation described above, in comparison with that of OPG. After incubation of each cytokine (25 ng/ml) with Kgp, cytokines that remained intact were quantitatively detected by Western blotting. Although all of the tested cytokines were degraded by Kgp in a concentration-dependent manner, TNF-α and IL-1β were more stable than IL-17A and OPG. After 15-h reactions with 50 nM Kgp, 50% of TNF-α and IL-1β remained non-degraded, whereas neither IL-17A nor OPG was detected (Fig. 3A). Time course findings also revealed that 3-h reactions with Kgp (50 nM) were adequate for clearance of IL-17A and OPG (Fig. 3B). These results clearly showed that TNF-α and IL-1β were more stable than OPG in regard to degradation by Kgp, whereas IL-17A and OPG were equally susceptible to Kgp. In addition, they suggest that the enhancing effects of Kgp on TNF-α and IL-1β-induced osteoclast differentiation are due to the relative stability of these cytokines toward Kgp in comparison with OPG. On the other hand, the susceptibility of IL-17A to Kgp might cause suppression of IL-17A-induced osteoclast differentiation by Kgp (Fig. 1).

**Cell-associated RANKL and RANK Stable in Cultures Containing Kgp**—To examine the degradation of RANKL expressed in osteoblasts by Kgp, mouse osteoblasts pretreated with calcitriol to induce the expression of RANKL were cultured for various periods in the presence of Kgp. The expression level of RANKL did not decrease but rather increased after an 18-h culture in the presence of Kgp (Fig. 3C). There is a possibility that a tendency to increase the expression of Rankl mRNA by Kgp (Fig. 2) might reflect the increased amount of RANKL protein observed after an 18-h incubation with Kgp (Fig. 3C). Furthermore, Kgp did not affect the amount of RANK protein expressed in mouse bone marrow macrophages (Fig. 3D).
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FIGURE 4. Effects of Kgp on osteoclast differentiation induced by TNF-α, IL-1β, and IL-17A in co-cultures of bone marrow cells and osteoblasts obtained from wild-type and OPG-deficient mice. A, calvarial osteoblasts and bone marrow cells were isolated from 1-day- and 6-week-old wild-type male C57BL/6 mice, respectively, and then co-cultured for 6 days with 10 ng/ml TNF-α, IL-1β, or IL-17A in the presence or absence of 50 nM Kgp. The numbers of TRAP-positive multinucleated cells formed in wells of 384-well plates were counted under a microscope. Results are expressed as the mean ± S.D. of four cultures. *, p < 0.05 and **, p < 0.01, respectively. B, OPG-deficient osteoblasts and bone marrow cells isolated from 1-day- and 6-week-old OPG-deficient male C57BL/6 mice were co-cultured for 6 days in 384-well plates with 10 ng/ml TNF-α, IL-1β, or IL-17A in the presence or absence of 50 nM Kgp. The numbers of TRAP-positive multinucleated cells formed in wells of 384-well plates were counted under a microscope. Results are expressed as the mean ± S.D. of seven cultures. **, p < 0.01, NS, not significant. C and D, degradation of TNF-α, IL-1β, and IL-17A by Kgp in OPG-deficient co-culture systems. Osteoblasts and bone marrow cells isolated from male OPG-deficient and wild-type C57BL/6 mice were co-cultured in 96-well plates containing 10% FBS in the presence or absence of 50 nM Kgp. The numbers of TRAP-positive multinucleated cells formed in wells of 384-well plates were counted under a microscope. Results are expressed as the mean ± S.D. of four cultures. *, p < 0.05 and **, p < 0.01, respectively.

Kgp Did Not Affect Osteoclast Differentiation Induced by Inflammatory Cytokines in Co-cultures of OPG-deficient Cells—The results described above indicated that both the observed enhancement of TNF-α- and IL-1β-induced osteoclast differentiation and suppression of IL-17A-induced osteoclast differentiation by Kgp were highly dependent on the presence of OPG. Therefore, we examined the effects of Kgp on osteoclast differentiation induced by TNF-α, IL-1β, and IL-17A in a coculture system with OPG-deficient osteoblasts and bone marrow cells. Whereas TNF-α, IL-1β, and IL-17A at 10 ng/ml significantly increased the number of multinucleated osteoclasts in wild-type co-cultures (Fig. 4A) and OPG-deficient co-cultures (Fig. 4B), Kgp at 50 nM did not change the number of osteoclasts formed by these cytokines in OPG-deficient co-cultures (Fig. 4B).

Because there could be a possibility that insufficient degradation of the cytokines by Kgp produced the faint effects of Kgp on osteoclastogenesis in OPG-deficient co-cultures, we performed Western blot analyses of culture supernatants for TNF-α, IL-1β, and IL-17A (Fig. 4, C and D). However, the half-lives of these cytokines in the co-cultures were similar to those obtained in the cell-free systems (Fig. 3B). Hence, it would appear that the activities of TNF-α and IL-1β to induce osteoclast formation waned partially, and that of IL-17A decreased greatly in the presence of Kgp.

On the other hand, osteoclast differentiation was induced by Kgp (50 nM) without the addition of any cytokine, suggesting that osteoclasts formed in the presence of each cytokine with Kgp included osteoclasts of which differentiation was induced by Kgp itself.

Kgp Primarily Cleaved OPG at Its Death Domain Homologous Region—Results of this as well as our previous study (16) suggest the importance of degradation and inactivation of OPG by Kgp in osteoclast differentiation at pathogenic foci of periodontitis. Therefore, we attempted to determine the sites of OPG cleavage by Kgp. Time course findings indicated that human OPG (56 kDa) was primarily cleaved by Kgp, resulting in a 37-kDa fragment (Fig. 5A, Fr. A). Analysis of the N-terminal amino acid sequence of the 37-kDa fragment revealed that it had the same N-terminal sequence as that of the original OPG (Fig. 5B), indicating that hydrolysis by Kgp primarily occurred at a Lys residue residing in its death domain homologous region (Fig. 5C). Next, we analyzed the N-terminal sequence of the fragment(s) in another band with an approximate size of 19 kDa (Fig. 5A, Fr. B). Although we could not identify the specific cleavage site, sequence analysis indicated that the band contained a mixture of fragments, including those having N termini indicated by arrows in Fig. 5B.

On the other hand, several fragments of OPG other than fragments A and B were detected after treatment with Kgp (Fig. 5A), indicating that Kgp cleaved multiple sites in the OPG molecule. Incubation of F-OPG with Kgp for 15 min resulted in the preferential appearance of a fluorescent fragment with a molecular mass of around 37 kDa. Because the reaction condition of FITC labeling of OPG was thought to preferentially label the N-terminal α-amino group by fluorescein, the 37-kDa fluorescent fragment was considered to correspond to fragment A. The 37-kDa fragment decreased with increased incubation time with Kgp, and bands with fluorescence at 25 kDa or lower molecular masses were produced (Fig. 5D), indicating that fragment A was further degraded by Kgp.

Analysis of the N-terminal amino acid sequence of fragment B suggested that Kgp primarily cleaves OPG at Lys-258 and/or Lys-262. We then prepared amino acid substitution mutants of OPG, in which Lys-258, Lys-262, or both were substituted by alanine. Contrary to our expectation, Kgp degraded these mutants with a similar efficiency as seen in its degradation of wild-type OPG (Fig. 5E). One possible explanation for these results is that Kgp cleaved OPG at Lys residues other than Lys-258 and Lys-262, due to their substitution with alanine. Another explanation is that Kgp preferably cleaves OPG at a lysine residue different from these. In that case, the molecular weight of fragment A indicates that the primary cleavage site of OPG by Kgp resides in the vicinity of Lys-258 and Lys-262.

OPG Cleaved by Kgp Did Not Bind to Osteoblasts—To ascertain whether Kgp-cleaved OPG loses its ability to associate with RANKL, we compared the binding of F-OPG to calcitriol-treated osteoblasts with that of F-OPG after treatment with active Kgp or Z-FK-ck-inactivated Kgp. It is well known that
calcitriol induces the expression of RANKL in osteoblasts (28). Incubation of F-OPG with active Kgp clearly lowered the binding of F-OPG to calcitriol-treated osteoblasts. On the other hand, Kgp inactivated by Z-FK-ck did not affect the binding of F-OPG to the cells (Fig. 5F). These results indicate that OPG loses its capability to bind to RANKL expressed on the cell surface of osteoblasts after fragmentation by Kgp.

**DISCUSSION**

Several reports have described the involvement of gingipains in periodontal bone destruction. Induced expression of RANKL in mouse osteoblasts was observed in vitro after infection with wild-type *P. gingivalis* but not after infection with *P. gingivalis* deficient in gingipain genes (29). Immunization with an *rgpA* DNA vaccine in mice (30) and that with an RgpA-Kgp complex in rats (31) protected the animals from alveolar bone loss after oral infection of *P. gingivalis*. Involvement of gingipains in alveolar bone loss was also suggested in a mouse periodontitis model by using *P. gingivalis* mutants deficient in gingipain genes, in which the relative contribution of each gingipain in bone resorption was indicated to be Kgp > RgpB > RgpA (32). In this study, we found that osteoclast differentiation induced by IL-1β and TNF-α was augmented by Kgp in vitro but not by RgpB. Our observations are consistent with the previous in vivo study (32).

Bacterial components, such as LPS, not only directly induce osteoclastogenesis but also stimulate production of proinflammatory cytokines from host cells, including macrophages and periodontal fibroblasts (13). Among the various proinflammatory
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Inflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-17A are especially considered to be major causative factors of inflammatory bone destruction in periodontitis (33, 34). Bone loss after infection with *P. gingivalis* was found to be reduced in TNF-α receptor-deficient mice (35). Also, administration of antagonists against TNF and IL-1 reduced the number of osteoclasts as well as bone loss in a non-human primate periodontitis model (36), indicating that these cytokines are major inducers of osteoclastogenesis in periodontitis. It was also reported that alveolar bone loss induced by *P. gingivalis* infection in IL-6-deficient mice was milder than that in wild-type mice (37). Also, in addition to its role as a chemotactic factor for neutrophils, IL-17A induces periodontal bone destruction (38). These cytokines stimulate osteoblasts to express RANKL, which in turn induces differentiation and activation of osteoclasts via RANKL-RANK interaction (8–11). Along with induction of RANKL expression in osteoblasts, mouse TNF-α directly acts on osteoclast progenitor cells to induce differentiation into osteoclasts (12), whereas it is also known that IL-1 stimulates osteoclasts to enhance their osteolytic activity in a RANKL-independent manner (39).

In the present study, Kgp enhanced osteoclast differentiation induced by TNF-α and that by IL-1β, whereas it instead suppressed that induced by IL-17A (Fig. 1A). On the other hand, RgpB did not have an effect on osteoclast differentiation induced by these cytokines (Fig. 1B). It is known that serum α₂-macroglobulin inhibits Rgps but not Kgp because of a lack of lysine residues in the bait region of the inhibitor (40); thus, it is thought that RgpB does not maintain its proteolytic activity in culture medium. We also found that TNF-α and IL-1β were more stable than OPG toward degradation by Kgp, whereas IL-17A and OPG were equally susceptible to it (Fig. 3). Therefore, the relative stability/susceptibility of a cytokine and OPG (Fig. 4). One possible explanation is that the cleavage site(s) in OPG cleaved by Kgp is considered to be an important factor to determine the enhancing/suppressive effects of Kgp on cytokine-induced osteoclast differentiation. The importance of OPG degradation by Kgp was confirmed in the present experiments using OPG-deficient osteoclasts because no enhancing effect of Kgp on osteoclast differentiation induced by TNF-α and IL-1β was observed in the absence of OPG (Fig. 4). On the other hand, RANKL and RANK expressed on the cell surface of osteoblasts and macrophages were stable against degradation by Kgp (Fig. 3, C and D), which might be one of the causes of enhanced osteoclast differentiation in the presence of Kgp.

Our findings of degradation of TNF-α, IL-1β, and IL-17A by Kgp (Fig. 3, A and B) led us to suspect that suppression by Kgp of osteoclast differentiation induced by these cytokines, especially that induced by IL-17A, would occur in co-cultures with OPG-deficient cells. However, the numbers of osteoclasts formed by treatment with TNF-α and IL-1β in the presence of Kgp were nearly the same as in its absence. Although the number of osteoclasts formed by the treatment with IL-17A in the presence of Kgp showed a tendency to be lower than the number formed by IL-17A without Kgp, we could not find a significant difference between these groups (Fig. 4B). That may have been due, at least in part, to osteoclastogenesis caused by Kgp. Kgp (50 nm) induced osteoclast formation even in the absence of proinflammatory cytokines (Figs. 1 A and C and 4A), which was also observed in co-cultures with OPG-deficient cells (Fig. 4B). IL-17A with Kgp induced the formation of almost the same number of osteoclasts as did Kgp alone (Figs. 1 A and C and 4 A and B), indicating that the contribution of IL-17A to induction of osteoclast differentiation was minimal as a consequence of its rapid degradation by Kgp.

Although it has been reported that HRgpA, RgpB, and Kgp (10 nm each) did not induce osteoclast differentiation in a mouse co-culture system (41), another report noted that Rgp and/or Kgp is required for induction of RANKL mRNA expression in mouse primary osteoblasts after infection with *P. gingivalis in vitro* (29). In our experimental settings, Kgp was apt to induce the expression of Rankl mRNA in a co-culture of osteoblasts and bone marrow cells (Fig. 2). Therefore, it is possible that Kgp stimulated osteoblasts to produce RANKL or some components of bone marrow cells to produce bone-resorbing factors, such as inflammatory cytokines. Further studies are required for clarification of the mechanism of osteoclast differentiation induced by Kgp.

Another interesting finding of our study is the primary cleavage site(s) in OPG cleaved by Kgp. A major fragment with a molecular mass of ~37 kDa emerged immediately after treatment with Kgp and had the same N-terminal amino acid sequence as that of the original OPG (Fig. 5), indicating that Kgp primarily cleaved OPG at lysine residue(s) residing in the death domain homologous region, but not at those in the RANKL-binding region of the OPG molecule (42). It is known that OPG molecules form a homodimer with a disulfide bridge at the C-terminal cysteine residue of each subunit, which is required for association with and inhibition of RANKL (43). Whereas OPG bound to osteoblasts, the 37-kDa fragment of OPG obtained after Kgp treatment did not (Fig. 5F). Therefore, it is highly plausible that cleavage of OPG at the death domain homologous region by Kgp results in loss of RANKL inhibition activity in OPG (Fig. 5G).

To confirm that one of the primary cleavage sites of OPG by Kgp is at Lys-258 or Lys-262, we prepared recombinant OPG mutants, in which Lys-258 and/or Lys-262 was substituted by alanine, and then compared their degradation by Kgp with that of wild-type OPG. Contrary to our expectations, the half-life of OPG in the presence of Kgp was not elongated by these mutations (Fig. 5E). One possible explanation is that the cleavage site specificity of Kgp is low enough to choose lysine residues other than Lys-258 and Lys-262 when they are substituted by other amino acids.

Although we focused on OPG degradation, there remains a possibility that Kgp degrades other proteinaceous or peptide factors that inhibit osteoclast differentiation in the co-culture system used in this study. It was previously reported that gingipains degrade cytokines that suppress osteoclastogenesis, such as IL-4 and IFN-γ (15). Further studies are required to elucidate the contribution of degradation of inhibitory factors other than OPG to osteoclastogenesis.

In addition to the degradation by Kgp of proteinaceous factors that directly stimulate or inhibit osteoclastogenesis, gingipains potentially modulate osteoclast differentiation through alteration of bacterial colonization and recruitment of leukocytes. It is known that gingipains have cell adhesion domains...
(15), which may facilitate colonization of *P. gingivalis*. Arginine residues that appear in matrix proteins after their cleavage by Rgps reportedly increase the affinity of the matrices to bacterial bodies through their pilis (44). Furthermore, gingipains degrade not only cytokines and chemokines but also complement component 5a, a chemotactic factor for neutrophils, and its receptor (14), which may inhibit neutrophil recruitment. On the other hand, Rgps activate prekallikrein and facilitate bradykinin production (14), which may induce accumulation of inflammatory cells.

To elucidate the involvement of Kgp in osteoclastogenesis after infection with *P. gingivalis*, we subcutaneously inoculated wild-type, *kg*,-deficient, and *rgpA/rgpB*-deficient strains of *P. gingivalis* to the heads of mice. We could not find the colonization of *kg*,-deficient bacteria, whereas the other two strains colonized and induced inflammation and bone destruction at the infected foci (data not shown). Although these observations reinforce the idea that Kgp is crucial for osteolysis in periodontitis, we could not demonstrate the specific role of Kgp in degradation of OPG and enhancement of osteoclastogenesis in vivo.

Along with other pathogenic and physiological situations, the RANKL/OPG ratio is considered to be important for determination of the condition of alveolar bone in patients with periodontitis (1). Although the expression level ratio of RANKL and OPG is important, we think that the influence of proteolytic degradation of these factors is another factor for determination of the RANKL/OPG ratio. In conclusion, we propose that degradation of OPG by Kgp is one of the crucial events in the development of bone loss in periodontitis, where proinflammatory cytokines play important roles in osteoclastogenesis.

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**REFERENCES**

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