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Lysine-specific gingipain promotes lipopolysaccharide- and active vitamin D_3-induced osteoclast differentiation by degrading osteoprotegerin

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SYNOPSIS

Porphyromonas gingivalis is one of the major pathogens of periodontitis, a condition characterized by excessive alveolar bone resorption by osteoclasts. The bacterium produces cysteine proteases called gingipains, which are classified according to their cleavage-site specificity into lysine-specific (Kgp) and arginine-specific gingipains (Rgps). In this study, we examined the effects of gingipains on osteoclast differentiation. In co-cultures of mouse bone marrow cells and osteoblasts, formation of multinucleated osteoclasts induced by 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] was augmented by Kgp but not by RgpB. A physiological concentration (0.1 nM) of 1α,25$(OH)_2D_3$ induced the osteoclast formation in the presence of 100 nM Kgp to the extent comparable to that induced by 10 nM 1α,25$(OH)_2D_3$. Kgp also enhanced osteoclastogenesis induced by various microbial components including lipopolysaccharide. Combined use of Kgp and 1α,25$(OH)_2D_3$ or lipopolysaccharide also increased the number of resorption pits developed on dentin slices, indicating the osteoclasts formed in the presence of Kgp possess bone-resorbing activity. The enhanced osteoclastogenesis by Kgp was correlated with a depletion of osteoprotegerin in co-culture media and proteolytic activity-dependent, since benzyloxycarbonyl-phenylalanyl-lysyl-acycloxyketone, an inhibitor of Kgp, completely abolished osteoclastogenesis induced by Kgp. Kgp digested osteoprotegerin, since its recombinant protein was susceptible to degradation by Kgp in the presence of serum. As a result, Kgp did not augment osteoclastogenesis in co-cultures of osteoprotegerin-deficient osteoblasts and bone marrow cells. In addition, enhanced osteoclastogenesis by Kgp was abolished by excess amount of recombinant osteoprotegerin. These findings suggest that degradation of osteoprotegerin is one of the mechanisms underlying promotion of osteoclastogenesis by Kgp.

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Keywords

periodontitis; bacterial protease; osteoclastogenesis; bone resorption

INTRODUCTION

Periodontitis is a chronic inflammatory disease accompanied by a decrease in alveolar bone volume due to excessive bone resorption by osteoclasts. Osteoclasts are multinucleated giant cells differentiated from monocyte/macrophage lineage cells by cell-cell interactions with osteoblasts. Osteoblasts produce key factors, such as receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), which regulate osteoclast differentiation [1, 2]. RANKL is a membrane-associated factor that induces osteoclast differentiation by activating intracellular signals mediated via RANK (receptor activator of NF-κB) expressed on the plasma membrane of osteoclast precursors [3, 4]. In contrast, OPG is a soluble-form decoy receptor for RANKL that inhibits osteoclast differentiation by interrupting RANKL-RANK binding [5, 6]. Thus, the balance between the expression level of RANKL and that of OPG in osteoblasts is critical for regulation of osteoclastogenesis and control of bone mineral density.

RANKL expression in osteoblasts is induced by bone-resorbing factors such as 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] and parathyroid hormone under physiological conditions. However, in pathological situations, including periodontitis, compounds derived from bacteria, such as LPS, a cell-wall constituent of Gram-negative bacteria, as well as lipoteichoic acid and peptidoglycan (PGN) have been reported to be the major factors that facilitate osteoclastogenesis [7–9]. LPS and other bacterial components, including PGN and CpG-DNA, are recognized by Toll-like receptors (TLRs), which are widely expressed in various kinds of cells [10, 11]. Among the bacteria found in subgingival microflora, *Porphyromonas gingivalis* has been proposed to be a potent etiological agent of periodontitis.

P. gingivalis secretes cysteine proteases called gingipains and it is known that more than 85% of the proteolytic activity derived from this bacterium is attributed to them [12]. Gingipains are the products of 3 independent genes, namely, *rgpA*, *rgpB*, and *kgp*, and the bacterium produces several proteins from these genes, including $RgpA_{(cat)}$, HRgpA, membrane type (mt)-RgpA, RgpB, mt-RgpB, Kgp, and mt-Kgp, which are bacterial cell surface associated variants of proteases. These proteases are referred to as arginine-specific (Rgps) and lysine-specific (Kgp) gingipains, depending on the specificity for hydrolysis of either the Arg-Xaa or Lys-Xaa peptide bond, respectively [12]. Rgps are known to activate the kinin-kallikrein system, blood coagulation system, and protease-activated receptors on platelets [13], while Kgp reportedly degrades fibrinogen/fibrin [14]. Gingipains destroy matrix components directly and indirectly by stimulating the expression and secretion of matrix metalloproteases (MMPs) [15], and also degrades several cytokines, components of complement systems, and receptors on immune cells [16–19]. All of these activities of gingipains are considered to be involved in the progression of periodontitis.

In addition to these host-degenerative actions, it was reported that immunization with gingipains protected *P. gingivalis*-induced periodontal bone loss in rats [20]. In addition, RANKL expression in mouse osteoblasts was observed *in vitro* following infection with wild-type *P. gingivalis*, but not after infection with a gingipains-deficient mutant [21]. Furthermore, involvement of gingipains in alveolar bone loss was suggested in a murine periodontitis model by using *P. gingivalis* mutants deficient of gingipains, in which the relative contribution of each gingipain in bone destruction was shown to be Kgp > RgpB > HRgpA [22]. These observations indicate that gingipains are involved not only in soft tissue destruction and inflammation, but also in osteoclastogenesis and osteoclast-dependent bone resorption associated with periodontitis.

Despite what is known about gingipains, the precise mechanisms underlying their promotion of osteoclastic bone resorption have not been elucidated. In this study, we found that Kgp degrades osteoprotegerin, and thereby augments osteoclast differentiation in the co-culture of bone marrow cells and osteoblasts induced by physiological and pathological bone-resorbing factors, namely, $1\alpha,25(OH)_2D_3$ and various microbial components. These results provide new insights into the treatment and prevention of bone loss associated with periodontitis.

EXPERIMENTAL

Reagents

LPS from *Escherichia coli* (O55:B5) and peptidoglycan from *Staphylococcus aureus* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly(I:C) RNA was from GE Healthcare Bio-Science Co. (Piscataway, NJ, USA). LPS from *P. gingivalis* W83 was prepared in our laboratories. Phosphothioate-stabilized CpG DNA (TCCATGACGTTCCTGATGCT) was kindly donated by Dr. Y. Choi, University of Pennsylvania School of Medicine. $1\alpha,25(OH)_2D_3$ and recombinant human M-CSF (Leucoprol) were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively. Recombinant human OPG, the monoclonal antibody for human OPG were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Benzyloxycarbonyl-L-phenylalanyl-L-lysyl-acyloxyketone (Z-FK-ck) was purchased from Bachem (King of Prussia, PA, USA) [23]. CellTiter 96 Aqueous One Solution Cell Proliferation Assay was from Promega (Madison, WI, USA).

Gingipains

Two types of gingipains, 105-kDa Kgp and 50-kDa RgpB, were purified from the culture supernatant of *P. gingivalis* HG66, as described by Pike *et al*. [24], then incubated for 5 minutes at 37°C with 10 mM cysteine in 0.2 M HEPES buffer (pH 8.0) containing 10 mM $CaCl_2$ before use to reactivate the proteases by reducing the active center thiol partially oxidized during purification process. To block the enzyme activity of Kgp, reactivated Kgp was further incubated for 30 minutes with 0.1 mM Z-FK-ck that titrates the active center of Kgp. The activated and inactivated gingipains were then diluted with the appropriate medium or buffer [19].

Cell cultures

Newborn and 6-week-old ddY mice were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). Primary osteoblasts were isolated from the calvaria of newborn mice using a conventional method with collagenase and dispase, as previously described [25]. 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 earlier [25]. In brief, cells were cultured in α-MEM supplemented with 10% FBS and various concentrations of $1\alpha,25(OH)_2D_3$ or LPS in culture plates at 37°C under 5% CO_2/95% air. The medium was replaced every 3 days with fresh containing the same concentrations of $1\alpha,25(OH)_2D_3$ or LPS. Osteoclast generation was evaluated by determining the enzymatic activity of tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts, or by counting TRAP-positive multinucleated cells with 3 or more nuclei.

Pit formation assay

Osteoblasts (2×10^4 cells) and bone marrow cells (2×10^5 cells) were co-cultured for 1 h to allow attachment on dentin slices (4 mm in diameter, 0.2 mm in thickness) in 96-well culture plates, as described above. The dentin slices with osteoblasts and bone marrow cells were then transferred to 48-well culture plates, and further incubated for 6 days in the presence of various concentrations of $1\alpha,25(OH)_2D_3$ or LPS. After wiping off the cells with cotton, the dentin slices were immersed in toluidine blue O (Sigma-Aldrich) to stain resorption pits formed by mature osteoclasts. The numbers of resorption pits on the dentin slices were counted under a microscope.

Immunoblot analysis

For detection of proteolysis of OPG by Kgp or RgpB in a cell-free system, the recombinant human OPG (5 μg/ml) were separately incubated with 0, 5, or 100 nM Kgp or RgpB at 37°C in Hanks' balanced salt solution or in α-MEM containing 10% FBS. Products of the cell-free reactions were separated using SDS-polyacrylamide (10%) gel electrophoresis in a reducing condition, and electro-transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 3% nonfat dry milk in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.1% Tween 20, and subjected to immunoblotting with the antibody for OPG, followed by incubation with the secondary antibody conjugated with horseradish peroxidase. Secondary antibody was detected using an ECL detection kit (GE Healthcare Bio-Science, Piscataway, NJ, USA).

Quantification of OPG

The amount of OPG in the co-culture medium was quantified using a mouse OPG/TNFRSF11B Immunoassay kit (R&D Systems) according to the manufacturer's instructions.

Osteoclast differentiation in co-cultured cells from OPG deficient mice

Osteoblasts and bone marrow cells were isolated as described above from 6-week-old OPG-deficient C57BL/6 mice obtained from Clea Japan, Inc., Tokyo, Japan. The OPG-deficient

osteoblasts, and bone marrow cells were co-cultured in the presence or absence of 1 ng/ml of LPS from *E. coli* or *P. gingivalis* and 100 nM Kgp for 5 days, after which TRAP-positive cells with 3 or more nuclei were counted as osteoclasts.

Animal experiments

Isolation of osteoblasts and bone marrow cells from mice was performed according to the protocol approved by the Ethical Board for Animal Experiments at Showa University.

Statistical analysis

Data are expressed as the mean ± SD. Student's t test was used for statistical analyses, with p values < 0.05 considered significant.

RESULTS

Kgp enhanced osteoclast formation induced by 1α,25(OH)$_2$D$_3$

We examined the effects of Kgp on osteoclast formation using a classical culture system, in which bone marrow cells and osteoblasts were co-cultured in the presence of 1α,25(OH)$_2$D$_3$. In the absence of Kgp, 1α,25(OH)$_2$D$_3$ at 0.1 nM did not induce the formation of TRAP-positive multinucleated osteoclasts, whereas the presence of Kgp with 1α,25(OH)$_2$D$_3$ enhanced formation in a concentration-dependent manner (Fig. 1A). Furthermore, a small number of osteoclasts appeared in the culture with 100 nM Kgp in the absence of 1α,25(OH)$_2$D$_3$. Also, the number of osteoclasts was increased by the addition of 1α,25(OH)$_2$D$_3$ as compared to the control in a dose-dependent manner, which was reflected in TRAP activity (Fig. 1B). We also examined whether the effects of Kgp on bone resorption coincided with osteoclast formation. As in the case of osteoclast formation, the number of resorption pits formed in the presence of 100 nM Kgp was significantly greater than that formed in the absence of Kgp (Fig. 1C and D).

Kgp enhanced osteoclast formation in the presence of microbial constituents

Since Kgp is produced by *P. gingivalis*, a Gram-negative bacterium, we examined the effects of Kgp and microbial constituents, such as LPS, PGN, unmethylated CpG DNA, and poly(I:C) RNA, on osteoclast formation in co-cultures of osteoblasts and bone marrow cells. In the absence of Kgp, LPS from *E. coli* at a concentration of 10 ng/ml or lower did not induce the formation of multinucleated osteoclasts, as only mononuclear osteoclasts or osteoclast precursors appeared (Fig. 2A, B). In contrast, *E. coli* LPS induced osteoclast formation in a dose-dependent manner in the presence of 100 nM Kgp (Fig. 2A, B), as was observed in the culture with Kgp plus 1α,25(OH)$_2$D$_3$ (Fig. 1B). Resorption pits were formed in the presence of both *E. coli* LPS and Kgp, but not in the presence of LPS alone (Fig. 2C, D). While LPS from *P. gingivalis* at 1 ng/ml significantly fostered the osteoclast differentiation in the presence of 100 nM Kgp, its activity to promote osteoclastogenesis seemed weaker than that from *E. coli* (Fig. 2E). The higher concentration of *P. gingivalis* LPS up to 100 ng/ml rather suppressed the osteoclast differentiation. In addition to LPS from *E. coli* and *P. gingivalis*, other microbial constituents, such as unmethylated CpG DNA, PGN, and poly(I:C) RNA, also induced osteoclast formation exclusively in the presence of Kgp (Fig. 2F).

Enzymatic activity of Kgp was involved in osteoclast formation

To examine if the specific enzymatic activity of Kgp is necessary for osteoclast formation, RgpB, an arginine-specific gingipain produced by *P. gingivalis*, was added to co-cultures supplemented with a low concentration of 1α,25$(OH)_2D_3$ (0.1 nM) or LPS from *E. coli* (1 ng/ml). In contrast to Kgp, RgpB (100 nM) did not induce osteoclast formation in the co-cultures of osteoblasts and bone marrow cells, even in the presence of 1α,25$(OH)_2D_3$ and LPS from *E. coli* (Fig. 3A). To elucidate the involvement of the proteolytic activity of Kgp in the augmented osteoclastogenesis, the effect of Z-FK-ck, a catalytic-center-titrating inhibitor for Kgp, was examined in the co-culture system. Z-FK-ck at 2.5-times higher concentration used in the co-culture system did not affect either the viability of mouse osteoblasts (Fig. 3B) or the osteoclast formation induced by 1α,25$(OH)_2D_3$ in the co-culture of mouse osteoblasts and bone marrow cells (Fig. 3C). The same concentration of Z-FK-ck had no effect on the differentiation of mouse osteoclasts from bone marrow macrophages induced by RANKL (data not shown). Therefore, Kgp with the catalytic cysteine covalently modified by Z-FK-ck was used in an osteoclast formation assay. Osteoclasts did not appear in the co-cultures to which pre-inactivated Kgp was added along with *E. coli* LPS or 1α, 25$(OH)_2D_3$ (Fig. 3D). These results suggest that the proteolytic activity of Kgp is crucial for the induction of osteoclast formation by Kgp.

Kgp degraded OPG

To reveal the mechanism by which Kgp augments osteoclastogenesis, we examined the proteolysis of recombinant human OPG by Kgp. Kgp at 5 nM degraded 25 μg/ml OPG in Hanks' balanced salt solution in a time-dependent manner (Fig. 4A). Kgp at 5 nM partially and that at 100 nM completely cleaved 25 μg/ml OPG at least into five fragments in 15 h in αMEM containing 10% FBS, whereas the same concentration of RgpB did not (Fig. 4B). These results suggested that the enhancement of osteoclastogenesis by Kgp was at least in part via the proteolytic degradation of OPG by Kgp.

Then we quantified OPG in the co-culture media in the presence or absence of Kgp and LPS from *E. coli*. *E. coli* LPS at 1 ng/ml suppressed the secretion of OPG by osteoblasts in the co-culture system (Fig. 4C). Regardless of whether LPS exists or not, 100 nM Kgp significantly reduced the concentration of OPG in the culture supernatants (Fig. 4C), which is consistent with the degradation of recombinant OPG by Kgp in a cell-free reaction environments (Fig. 4A, B).

Kgp did not enhance LPS-induced osteoclast formation in co-culture of OPG-deficient cells

To ascertain whether the degradation of OPG by Kgp causes enhancement of osteoclast formation, we examined the effect of Kgp on osteoclast formation in a co-culture of OPG-deficient osteoblasts and bone marrow cells. A small number of multinucleated osteoclasts (12.2 ± 4.4 cells/well) were formed even in the absence of Kgp or LPS from either *E. coli* or *P. gingivalis* in the co-culture of OPG-deficient osteoblasts and bone marrow cells (Fig. 5A, B). While a lot of mononuclear, TRAP-positive cells were formed without any stimulation in OPG-deficient co-cultures, they were not counted as osteoclasts. LPS from *E. coli* at 1 ng/ml significantly increased the number of multinuclear osteoclasts. Kgp at 100 nM,

however, did not affect osteoclast formation either in the absence or presence of *E. coli* LPS (Fig. 5A). LPS from *P. gingivalis* was less potent than that from *E. coli* to induce osteoclast differentiation in the OPG-deficient co-culture. Kgp again did not promote osteoclast differentiation in the OPG-deficient co-culture either in the presence or absence of *P. gingivalis* LPS (Fig. 5B). In addition, osteoclast differentiation induced by 1 ng/ml LSP from *E. coli* and 100 nM Kgp in the co-culture of wild-type osteoblasts and bone marrow cells was inhibited to the level obtained in the absence of Kgp by human recombinant OPG in a concentration-dependent manner (Fig. 5C). Therefore, it is plausible that one of the major targets of Kgp is OPG and that the degradation of OPG by Kgp promotes osteoclastogenesis even if other mechanisms still exist unexplained.

DISCUSSION

It has been reported that LPS derived from bacteria colonized in periodontal tissue is critically involved in bone loss associated with periodontitis [9, 26], while it was also shown that osteoblasts play a crucial role in LPS-induced bone loss [9]. LPS stimulates the expression of RANKL and downregulates that of OPG in osteoblasts [8], which causes the promotion of osteoclast differentiation. On the other hand, it has been reported that microbial components including LPS inhibit osteoclast differentiation via stimulation of TLRs distributed on osteoclast precursor cells [27]. Therefore, it remains controversial whether LPS is the sole factor crucial for osteoclast-dependent bone resorption in periodontitis.

In the present study, we found that Kgp facilitated osteoclast differentiation induced by 1α,25$(OH)_2D_3$ and LPS in a co-culture system. More specifically, in the presence of 100 nM Kgp, a physiological concentration (0.1 nM) of 1α,25$(OH)_2D_3$ induced the differentiation and activation of osteoclasts (Fig. 1). Osteoclast differentiation and pit formation were also observed in co-cultures in medium containing 1 ng/ml of LPS from *E. coli* and 100 nM Kgp (Figs. 2A–D). The concentrations of 1α,25$(OH)_2D_3$ and *E. coli* LPS required for osteoclastogenesis in the presence of Kgp were 100-fold lower than those required in the absence of this gingipain. Enhanced formation of osteoclasts was also observed in the co-culture with LPS from *P. gingivalis* at 1 ng/ml and 100 nM Kgp, even though the effect of *P. gingivalis* LPS was less potent than that of *E. coli* LPS at the same concentration (Fig. 2E). Furthermore, Kgp in the present co-culture systems also enhanced osteoclast differentiation induced by other microbial components, such as unmethylated CpG DNA, PGN, and poly(I:C) RNA (Fig. 2F). It is known that 1α,25$(OH)_2D_3$, *E. coli* LPS, unmethylated CpG DNA, and PGN induce the expression of RANKL in osteoblasts via activation of the vitamin D receptor, TLR-4, TLR-9, and nucleotide-binding oligomerization domain 2 pathways, respectively [2, 8, 28, 29]. However, while it is known that poly(I:C) RNA is a ligand for TLR-3, to the best of our knowledge, induction of RANKL expression or osteoclast formation by poly(I:C) RNA has not been reported. Our findings that Kgp enhanced the osteoclast-inducing activity of various compounds with different signaling pathways suggest that it has an effect on some process other than the induction of RANKL expression in osteoblasts by these compounds.

The stimulatory effects of Kgp on osteoclastogenesis in the presence of 1α,25$(OH)_2D_3$ and *E. coli* LPS in the co-culture system were dependent on the protease activity of Kgp. A small number of osteoclasts were formed after incubation with active Kgp even in the absence of 1α,25$(OH)_2D_3$ or *E. coli* LPS, and that effect was completely inhibited by Z-FK-ck (Fig. 3D). The results shown in Figure 4 strongly indicate that the proteolytic elimination of OPG from the culture medium by Kgp is one of the major mechanisms of enhanced osteoclast formation in the presence of Kgp. This hypothesis is supported by the results obtained in the co-culture of cells from OPG deficient mice, where Kgp did not promote osteoclast formation (Fig. 5A, B). On the other hand, addition of excess amount of recombinant OPG suppressed osteoclast differentiation induced by *E. coli* LPS and Kgp (Fig. 5C), which again reinforced this hypothesis. These results, however, do not exclude other possible mechanisms responsible for the Kgp-dependent osteoclast differentiation. Kgp also induced osteoclastogenesis in the absence of bone resorbing factors such as 1α, 25$(OH)_2D_3$ and microbial components, even though its efficacy was much lower than that used in the combination with these factors (Figs. 1 and 2). It was previously reported that the expression of RANKL was induced in the osteoblasts by wild-type *P. gingivalis* via protease-activated receptors but not by the gingipains-deficient mutants [21]. It remains to be explored the role of gingipains in osteoclastogenesis other than degradation of OPG.

Various bacteria constitute the microflora in the human gingival crevice. In the animal models of periodontitis, the mixed infection of several bacteria including *P. gingivalis* induced the severer alveolar bone loss compared to the single infection of *P. gingivalis* [30, 31]. Therefore, it is plausible that the potentiation by Kgp of osteoclastogenesis in the presence of various microbial components (Fig. 2) underlies the alveolar bone loss associated with periodontitis.

Unlike LPS from *E. coli*, that from *P. gingivalis* is a ligand for TLR-2 [32]. The contribution of TLR-2 was observed in the tumor necrosis factor-α-dependent/RANKL-independent osteclastogenesis in response to *P. gingivalis* infection [33]. It was also reported that LPS from *P. gingivalis* induced the expression of RANKL in cementoblasts via TLR-2 [34]. On the other hand, contribution of TLR-2 was denied in the osteoclast differentiation induced by muramyl dipeptide, the minimal essential unit of PGN, in the mouse co-culture system [29]. The less potent activity of LPS from *P. gingivalis* to promote osteoclast differentiation in the presence of Kgp in comparison to those of the other ligands of TLR families such as *E. coli* LPS (TLR-4), unmethylated CpG DNA (TLR-9), and poly(I:C) RNA (TLR-3) suggests the possibility that TLR-2 has a minor contribution to the induction of osteoclast differentiation in the co-culture system we employed in this study (Fig. 2). Further investigation is required to elucidate the role of *P. gingivalis* LPS and/or TLR-2 in the osteoclastogenesis in the co-culture of osteoblasts and osteoclast precursor cells.

One of the important findings of this study is that Kgp enhances the decalcifying activity of osteoclasts as well as their differentiation induced by the combined use of a low concentration of either 1α,25$(OH)_2D_3$ or LPS from *E. coli*. Even in the absence of 1α, 25$(OH)_2D_3$ or LPS, a small number of osteoclasts formed by incubation with 100 nM Kgp yielded resorption pits on dentin slices (Figs. 1C and 2C). Physiological concentration of 1α, 25$(OH)_2D_3$ (0.1 nM) or *E. coli* LPS at 1 ng/ml or lower dramatically elevated the number of

resorption pits formed in the presence of 100 nM Kgp (Figs. 1C, 1D, 2C, and 2D), indicating that Kgp is able to support osteoclastic bone resorption *in vivo* in *P. gingivalis*-infected periodontitis sites. It has been reported that Kgp activates pro-MMP-1 and pro-MMP-9 by limited proteolysis [35]. Along with elimination of the inhibitory effects of OPG due to its degradation by Kgp, it is possible that Kgp is involved in the formation of resorption pits on dentin slices by direct hydrolysis of dentin-matrix components or by activating pro-MMPs secreted by osteoclasts.

In contrast to Kgp, RgpB did not have an effect on osteoclast differentiation in the same experimental settings (Fig. 3A), which could be explained by poor degradation of OPG in the presence of 10% FBS (Fig. 4B). Since OPG was fragmented after incubation with RgpB in the buffer without serum (data not shown), protease inhibitors in the serum likely suppressed the protease activity of RgpB. It has been reported that Rgps but not Kgp were inhibited by α2-macroglobulin in human plasma [12, 36], and it is possible that inhibitors in bovine serum such as α2-macroglobulin effectively inhibit RgpB, whereas they do not suppress the proteolytic activity of Kgp. Therefore, the contrasting activities of RgpB and Kgp to enhance osteoclastogenesis in the presence of $1\alpha,25(OH)_2D_3$ or LPS in the present co-culture systems might have been the result of different levels of inhibition of these proteases by serum protease inhibitors, though other mechanisms cannot be excluded. A recent study by Pathirana *et al.* that used gingipain-deficient mutants of *P. gingivalis* indicated that gingipains were involved in the induction of alveolar bone loss in a murine periodontitis model. Interestingly, the activity of Kgp was found to contribute more significantly to bone loss than other gingipains [22]. Their findings are consistent with ours and can be explained, at least in part, by enhanced osteoclastogenesis by Kgp in combination with other bacterial components.

In this study, we found that Kgp and bone-resorbing factors such as $1\alpha,25(OH)_2D_3$, LPS, and other bacterial components synergistically induce osteoclast differentiation, and function in a setting where both osteoblasts and osteoclast precursor cells co-exist. While LPS released from periodontal bacteria has been regarded as the major factor for osteoclastogenesis and alveolar bone loss in periodontitis, these findings reveal that Kgp is another factor involved those associated with *P. gingivalis* infection. Our observations also suggest targeted nullification of Kgp activity as a possible strategy for treatment and/or prevention of bone loss in patients affected by periodontitis.

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Abbreviations used are

RANKL	receptor activator of NF-κB ligand
OPG	osteoprotegerin

RANK	receptor activator of NF-κB
1α, 25(OH)$_2$D$_3$	1α,25-dihydroxyvitamin D$_3$
PGN	peptidoglycan
Rgp	arginine-specific gingipain
Kgp	lysine-specific gingipain
MMP	matrix metalloprotease
Z-FK-ck	benzyloxycarbonyl-L-phenylalanyl-L-lysyl-acyloxyketone
TRAP	tartarate-resistant acid phosphatase

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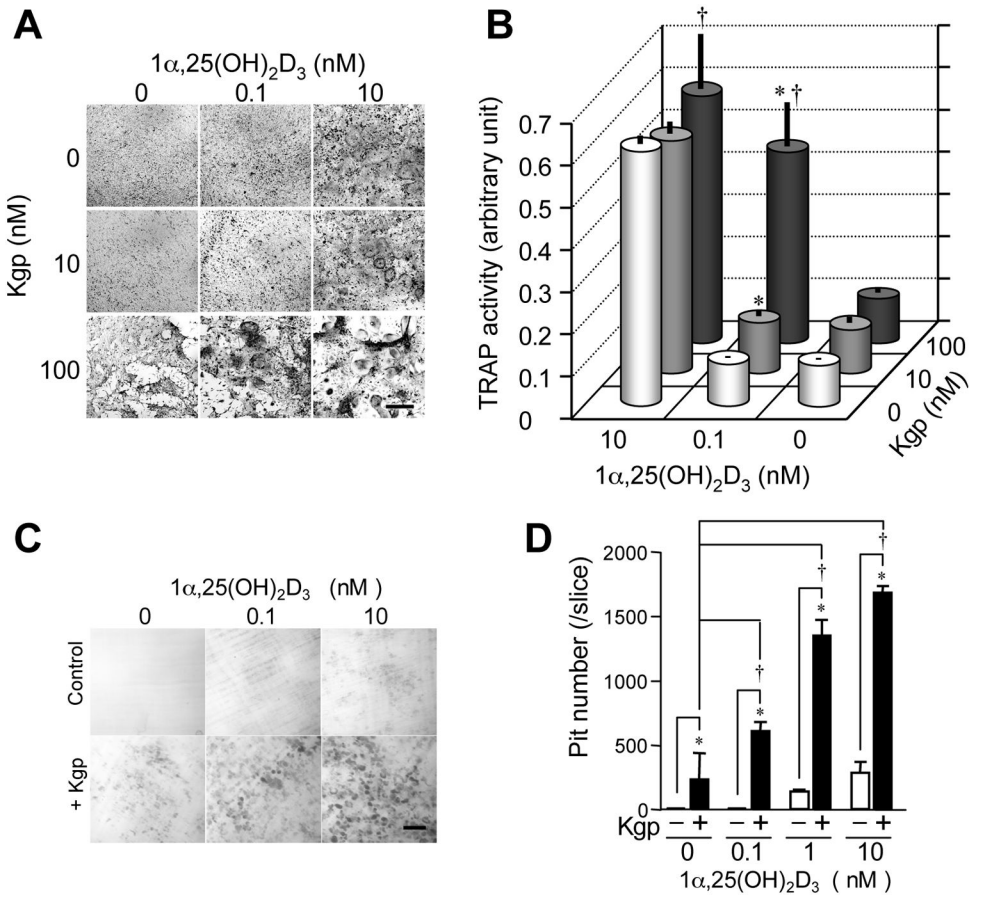

Figure 1. Kgp enhanced 1α,25(OH)$_2$D$_3$-induced osteoclast formation and activation

A and **B**, Osteoblasts and bone marrow cells were co-cultured for 5 days with the indicated concentrations of 1α,25(OH)$_2$D$_3$ and Kgp. **A**, Representative photographs of cells after TRAP-activity staining. Bar=200 μm. **B**, TRAP activity in the cell lysates was determined spectrophotometrically. Values in each column indicate the mean ± SD of 4 experiments. The symbols * and † indicate that the values are significantly higher than that obtained at 0.1 nM 1α,25(OH)$_2$D$_3$ without Kgp and that at 100 nM Kgp without 1α,25(OH)$_2$D$_3$, respectively. **C** and **D**, Osteoblasts and bone marrow cells were co-cultured for 6 days on dentin slices with the indicated concentrations of 1α,25(OH)$_2$D$_3$ with or without 100 nM Kgp. Resorption pits formed on the dentin slices were stained with toluidine blue. **C**, Resorption pits appear dark in the photographs. Bar=200 μm. **D**, The numbers of pits were counted under a microscope. Solid and open columns show results obtained in cultures with and without Kgp, respectively. Data are expressed as the mean ± SD of 4 cultures. *The value is significantly higher than the control at each concentration of 1α,25(OH)$_2$D$_3$. †The value is significantly higher than that obtained at 100 nM Kgp without 1α,25(OH)$_2$D$_3$.

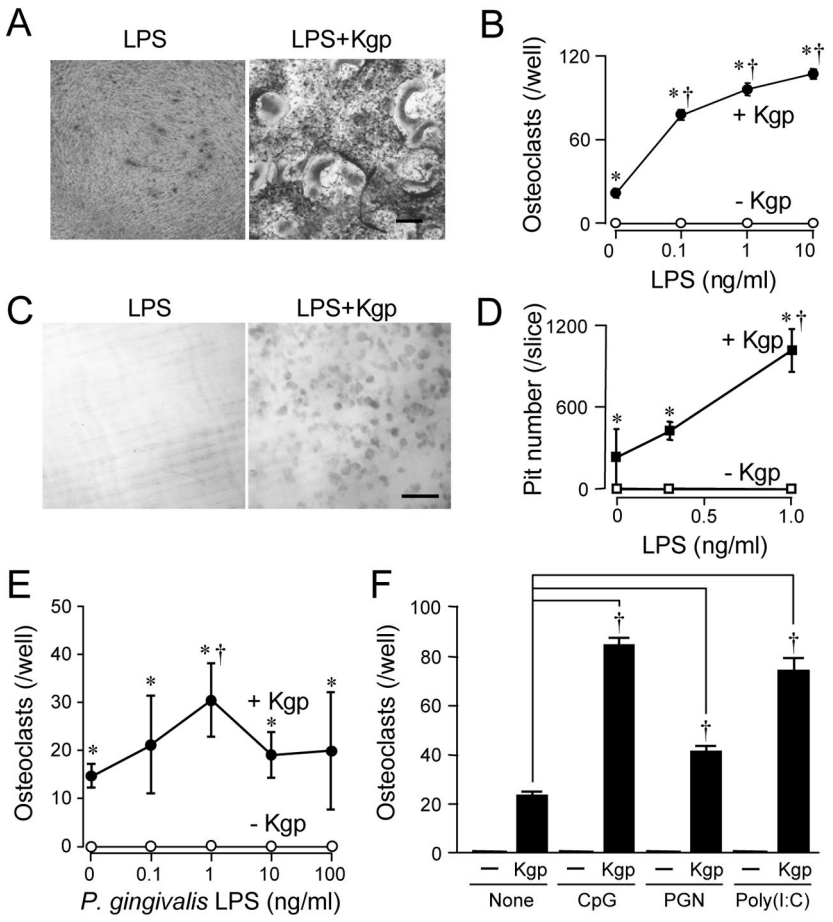

Figure 2. Synergistic effects of Kgp and microbial conponents on osteoclast and pit formation in co-cultures of osteoblasts and bone marrow cells

A, Representative photographs showing TRAP activity staining of cells cultured for 5 days with 1 ng/ml of LPS from *E. coli* in the presence or absence of 100 nM Kgp. **B**, Osteoblasts and bone marrow cells were co-cultured for 5 days in media containing various concentrations of *E. coli* LPS in the presence (●) or absence (○) of 100 nM Kgp. **C**, Representative photographs showing dentin slices on which osteoblasts and bone marrow cells were co-cultured for 6 days with 1 ng/ml of *E. coli* LPS in the presence or absence of 100 nM Kgp. The dark spots indicate resorption pits stained with toluidine blue. **D**, Osteoblasts and bone marrow cells were co-cultured for 6 days with the indicated concentrations of *E. coli* LPS in the presence (■) or absence (□) of 100 nM Kgp. Resorption pits formed on dentin slices were counted. **E**, Osteoblasts and bone marrow cells were co-cultured for 5 days in media containing various concentrations of LPS from *P. gingivalis* in the presence (●) or absence (○) of 100 nM Kgp. **F**, Osteoblasts and bone marrow cells were co-cultured for 5 days in media containing 1 nM unmethylated CpG DNA (CpG), 1 ng/ml of proteoglycan (PGN) from *S. aureus*, or 1 ng/ml of Poly(I:C) RNA [Poly(I:C)] in the presence or absence of 100 nM Kgp. **A** and **C**, Bars=200 μm. **B**, **E**, and **F**, TRAP-positive multinuclear cells with 3 or more nuclei were counted as osteoclasts. **B**, **D**, **E**, and **F**, Data are expressed as the mean ± SD of 4 cultures. The value with * is significantly higher than that obtained without Kgp (**B**, **D**, **E**), and that with † is significantly

higher than that obtained with 100 nM Kgp in the absence of microbial components (**B**, **D**–**F**).

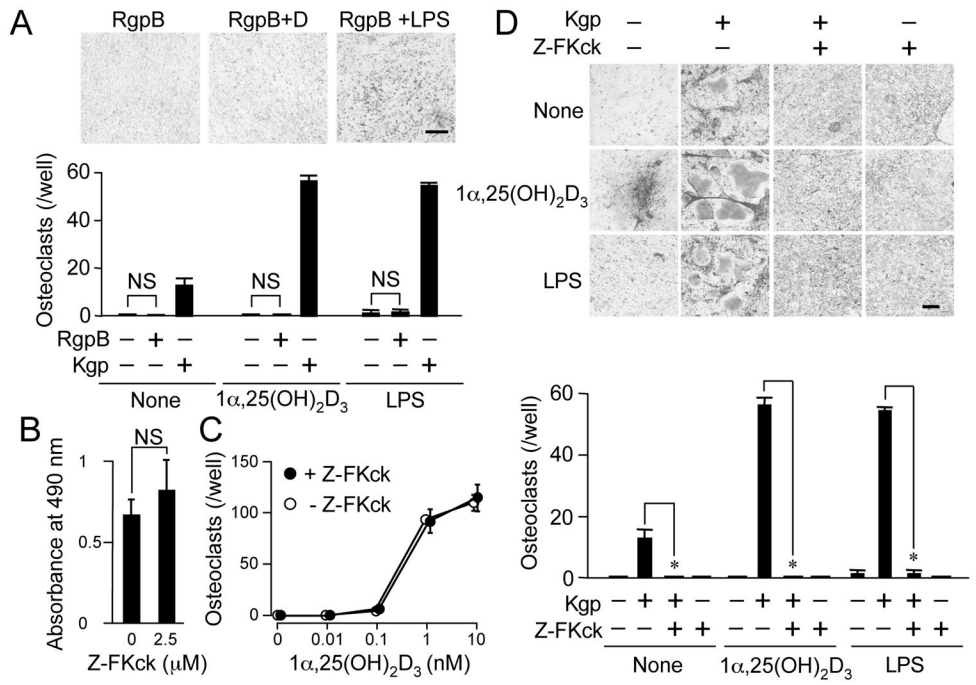

Figure 3. Kgp enhanced osteoclastogenesis through its proteolytic activity

A (upper panel), TRAP-activity staining was performed after 5-day cultures of osteoblasts and bone marrow cells with 100 nM RgpB in the absence (RgpB) or presence of 0.1 nM 1α, 25(OH)$_2$D$_3$ (RgpB+D), or 1 ng/ml of LPS from *E. coli* (RgpB+LPS). Photographs show TRAP-activity staining. Bar=200 μm. **A** (lower panel), Osteoblasts and bone marrow cells were co-cultured for 5 days in the absence (None) or presence of 0.1 nM 1α,25(OH)$_2$D$_3$, or 1 ng/ml of LPS from *E. coli* with or without 100 nM RgpB or Kgp. TRAP-positive cells with 3 or more nuclei were counted as osteoclasts. **B**, Mouse osteoblasts were cultured for 3 days in the presence or absence of 2.5 μM Z-FK-ck, and their viability was assessed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay. **C**, Osteoblasts and bone marrow cells were co-cultured for 5 days in the media containing 0.1 nM 1α,25(OH)$_2$D$_3$ in the presence (●) or absence (○) of 2.5 μM Z-FK-ck. **D**, Osteoblasts and bone marrow cells were incubated for 5 days with 0.1 nM 1α,25(OH)$_2$D$_3$ or 1 ng/ml of LPS from *E. coli* in the presence or absence of 100 nM Kgp, 100 nM Kgp pretreated with Z-FK-ck, or Z-FK-ck, with the final concentration of Z-FK-ck in the culture medium at 1 μM. Cells were stained for TRAP activity (upper panel). Bar=200 μm. TRAP-positive multinuclear cells with 3 or more nuclei were counted as osteoclasts (lower panel). *Significantly lower than the value obtained in the experiment with intact Kgp in each condition. **A–D**, Values indicate the mean ± SD of 4 experiments. NS, Difference was not significant.

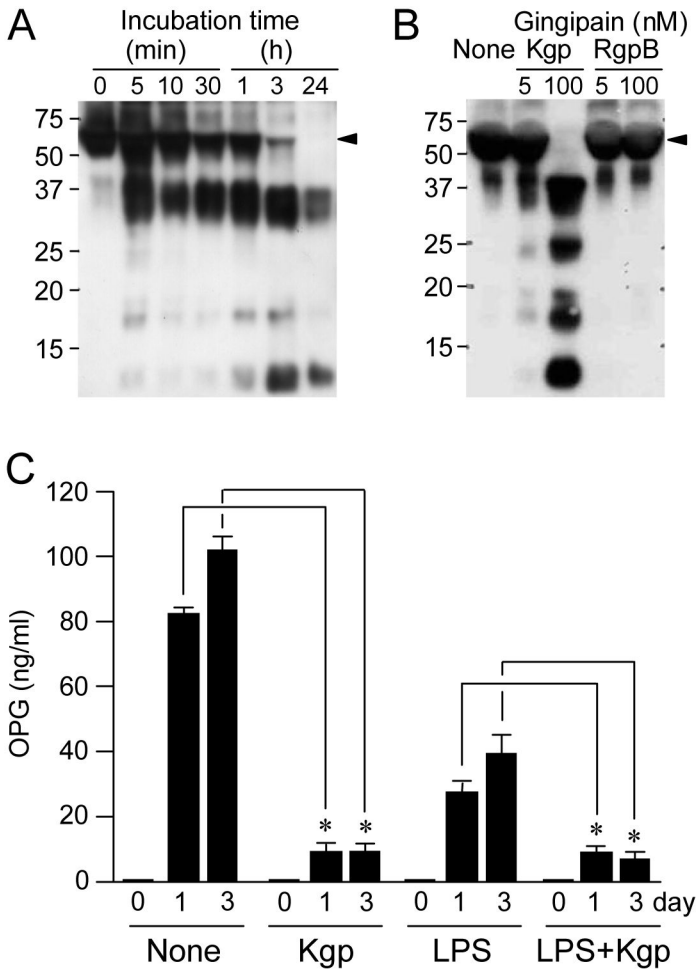

Figure 4. Degradation of OPG by Kgp

A, Recombinant human OPG (25 μg/ml) was treated with 5 nM Kgp for indicated periods at 37°C in Hanks' balanced salt solution containing 0.1 mM L-Cys. Reaction products were analyzed by Western blotting using the specific antibody for OPG after separation by 10% polyacrylamide gel electrophoresis. The arrowhead indicates immunoreactive bands for intact OPG. **B**, Recombinant human OPG (25 μg/ml) was treated with 0, 5, and 100 nM Kgp or RgpB for 15 h at 37°C in α-MEM plus 10% FBS. Reaction products were analyzed by western blotting using anti-OPG antibody as described above. Arrowhead, intact OPG. **C**, Osteoblasts and bone marrow cells were co-cultured for indicated periods in the absence (None) or presence of 100 nM Kgp (Kgp), 1 ng/ml of LPS from *E. coli* (LPS), and their combination (LPS+Kgp). OPG in culture supernatants was determined by ELISA. The results are shown as the means ± SD of 4 experiments. *The concentration of OPG was significantly lower than that in each corresponding culture without Kgp.

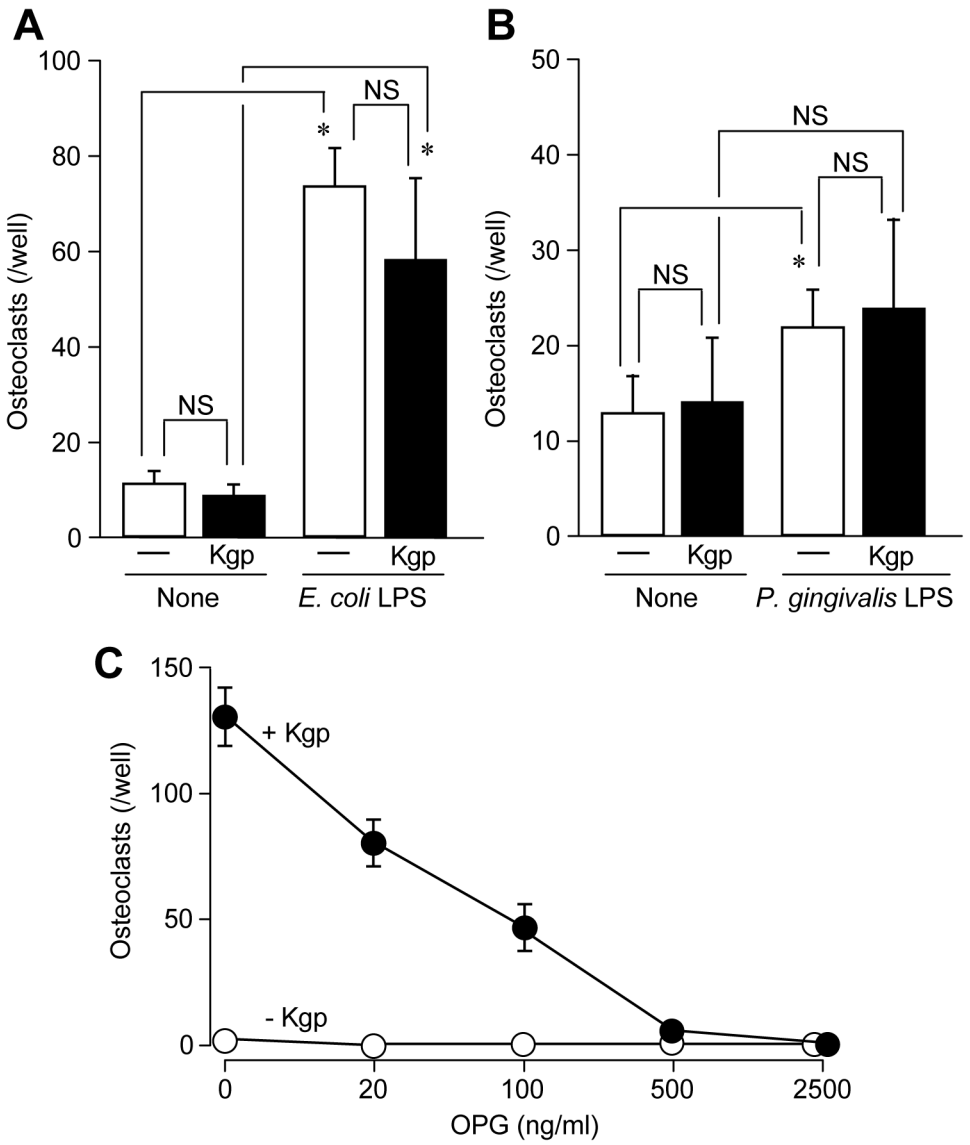

Figure 5. Role of OPG in the enhanced induction of osteoclast differentiation in the presence of Kgp

A, Osteoblasts and bone marrow cells were obtained from OPG-deficient mice, then co-cultured for 5 days in the presence or absence of 1 ng/ml of LPS from *E. coli* and 100 nM Kgp. **B,** Osteoblasts and bone marrow cells from OPG-deficient mice were co-cultured for 5 days in the presence or absence of 1 ng/ml of LPS from *P. gingivalis* and 100 nM Kgp. **C,** Osteoblasts and bone marrow cells from wild-type mice were co-cultured for 5 days in the presence (●) or absence (○) of 100 nM Kgp in the media containing 1 ng/ml of LPS from *E. coli*. **A–C,** TRAP-positive cells with 3 or more nuclei were counted as osteoclasts. Values in each column indicate the mean ± SD of at least 3 experiments. **A, B,** *Significantly different. NS, Difference is not significant.