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NF-κB-dependent induction of osteoprotegerin by *Porphyromonas gingivalis* in endothelial cells

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Abstract

Porphyromonas gingivalis is a major etiological pathogen of adult periodontitis characterized by alveolar bone resorption. Vascular endothelial cells supply many inflammatory cytokines into periodontal tissue. However, whether the cells contribute to bone metabolism in periodontitis remains unknown. In this study, we investigated the effect of *P. gingivalis* on osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) production, both of which are key regulators of bone metabolism, in human microvascular endothelial cells (HMVECs). We showed that *P. gingivalis* upregulated expression of OPG but not RANKL mRNA in HMVEC. *P. gingivalis* induced NF-κB activation, and the induction of OPG in HMVEC by the pathogen was blocked by the inhibitors of NF-κB. In addition, incubation of OPG with *P. gingivalis* supernatant resulted in loss of the protein. These results indicate that *P. gingivalis*-stimulated HMVEC secrete OPG via a NF-κB-dependent pathway, while the OPG is partly degraded by the bacteria. Thus, microvascular endothelial cells can act as a source of OPG and thereby may play an important role in regulating bone metabolism in periodontitis.

Keywords: *Porphyromonas gingivalis*; Periodontitis; Bone resorption; Osteoprotegerin; Receptor activator of NF-κB ligand; Endothelial cells; Protease; NF-κB; Lipopolysaccharide; Inflammatory cytokines

Porphyromonas gingivalis is a gram-negative, black-pigmented anaerobe that has been implicated in the etiology of periodontal disease [1,2]. This pathogen is speculated to strongly trigger the transmigration of neutrophils and monocytes from blood vessels into periodontal tissue [3,4]. We recently demonstrated that *P. gingivalis* induces the synthesis of monocyte chemotactic protein 1 (MCP-1) and interleukin 8 (IL-8) mRNA. These protein products cause leukocytes to adhere to endothelial cells, and the *P. gingivalis* fimbriae induce adhesion of a monocytic cell line to endothelial cells [5,6]. The dense infiltration of inflammatory cells in periodontal tissue induces an intense inflammatory reaction resulting in the destruction of tissue and alveolar bone surrounding the tooth root [2,7]. Thus, endothelial cells have been thought to play an essential role in periodontitis.

Osteoprotegerin (OPG) is a member of the tumor necrosis factor (TNF) receptor superfamily. OPG binds to the receptor activator of the nuclear factor-κB (NF-κB) ligand (RANKL), thereby neutralizing its functions and negatively regulating osteoclast differentiation, activity, and survival [8,9]. The fundamental role of RANKL and OPG in regulating bone metabolism has been demonstrated in transgenic and knockout mice [10,11]. Regarding periodontitis, it is generally accepted that periodontal pathogens that stimulate inflammation-induced osteoclast formation act through RANKL/OPG systems [12]. However, the influence of the periodontal pathogen on the expression of OPG and RANKL in the vascular system has not been previously examined.

Because microvascular endothelial cells may provide various cytokines and cells for bone resorption and formation across the endothelial cell layer from the circulating blood under pathological conditions [13–15], we investigated the effect of *P. gingivalis* on microvascular endothelial cells in terms of OPG and RANKL expression. Our observations indicate that endothelial cells secrete

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OPG in response to *P. gingivalis* stimulation and that they therefore may play an important role in the RANKL/OPG system in periodontal tissue during periodontitis.

Materials and methods

Bacterial culture and preparation of bacterial components. *Porphyromonas gingivalis* strain 381 was grown anaerobically (70% N_2, 15% H_2, and 15% CO_2) at 37 °C for 24 h in brain–heart infusion (BHI) broth (Merck, Darmstadt, Germany) containing hemin (5 μg/ml) and menadione (1 μg/ml). The bacteria were pelleted, washed twice with phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Tokyo, Japan), and resuspended in PBS or endothelial cell medium (EGM-2MV; Clonetics, San Diego, CA, USA) to appropriate concentrations (10^6–10^8 CFU/ml). Inactivation of *P. gingivalis* was accomplished by heat treatment (Heat-killed cells; 100 °C for 10 min). *P. gingivalis* lipopolysaccharide (LPS) was extracted by the hot-phenol method and purified as previously described [16,17]. *P. gingivalis* fimbriae were prepared and purified from cell washings as described previously [6]. LPS (Difco Laboratories, Detroit, MI, USA) derived from *Escherichia coli* O111:B4 was used as an additional control.

Endothelial cell culture. Human microvascular endothelial cells (HMVECs) were purchased from Clonetics and cultured in EGM-2MV supplemented with 1% gentamicin–amphotericin mix (GA-1000, Clonetics) according to the manufacturer's instructions. The cells were grown in 25-cm^2 flasks (Corning Costar, Cambridge, MA, USA), passaged every 3 or 4 days, and generally used after 4–7 passages. Fresh medium without antibiotics was added to the HMVEC before treatment with bacteria.

Treatment of HMVEC with P. gingivalis and its components. HMVEC monolayers grown in 96-well tissue culture plates (Corning) were cocultured with *P. gingivalis* (10^6–10^7 CFU/ml) suspended in EGM-2MV without antibiotics for 3, 6, 18, and 24 h. The multiplicity of infection (MOI) was calculated based on the number of HMVEC per well at confluence (1.28×10^4 cell/well). The amount of bacteria was quantified with a spectrophotometer (600 nm) based on a standard curve established by colony formation on bacterial plates. The cell-free supernatant was obtained following centrifugation. The supernatants were stored at −20 °C until use. To examine the ability of various *P. gingivalis* membrane components to stimulate HMVEC, we incubated HMVEC with heat-killed *P. gingivalis* (10^8 CFU/ml), *P. gingivalis* fimbria (20.0 μg/ml), *P. gingivalis* LPS (10.0 μg/ml), or *E. coli* LPS (1.0 μg/ml) for 24 h. In the NF-κB activation inhibition assays, endothelial cells were preincubated with 10 or 50 μM pyrrolidinedithiocarbamate (PDTC; Sigma Chemical, St. Louis, MO) or 10 mM *N*-acetyl-L-cystine (NAC; Sigma Chemical) [18] for 1 h before *P. gingivalis* was added (MOI 1:20). Inhibitors were maintained at their initial concentrations during the course of the inhibition assays. Supernatant samples were removed at 24 h post-addition and stored at −20 °C until use.

Analysis of OPG degradation. Bacterial supernatants were collected from cultures grown to an optical density of 1.0 at 600 nm and passed through 0.45-μm filters (Millipore, Bedford, MA, USA). *N*α-Tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor of the active sites of serine and cysteine proteases [19], was purchased from Sigma (St. Louis, MO, USA). Equal volumes (150 μl) of EGM-2MV containing recombinant OPG (5.0×10^2 pg/ml; PeproTech House, London, UK) and *P. gingivalis* culture supernatants (50 μl) with or without TLCK (0, 0.2 or 2 mM) were combined in each well of a 96-well plate and incubated for 24 h. The remaining OPG was analyzed by enzyme-linked immunosorbent assay (ELISA).

ELISA. OPG levels in cell-free supernatants were assayed with ELISA kits (Immundiagnostik AG, Bensheim, Germany), and the resulting data are presented as means ± SD of three experiments.

Reverse transcription-polymerase chain reaction analysis. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on HMVEC treated or untreated with *P. gingivalis* (MOI 2:1, 4:1 or 20:1) for 18 h. Total RNA was extracted from these cells with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of RNA extracted from each sample was determined spectrophotometrically. The RNA was reverse-transcribed (SuperScript reverse transcriptase, Gibco-BRL, Grand Island, NY, USA) according to the manufacturer's instructions by using oligo$(dT)_{12-18}$ primers (Gibco-BRL). PCR amplification was performed with Ready-to-Go PCR beads (Amersham–Pharmacia Biotech, Piscataway, NJ, USA) and a thermocycler (model MIR-D40, Sanyo, Tokyo, Japan). The PCR conditions were set at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min for 24 cycles for OPG and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or for 32 cycles for RANKL. The primers used to amplify OPG were 5′-GGGGACCACAATGAACAAGTTG-3′ and 5′-AGCTTGCACCACTCCAAATCC-3′ [15]. The primers used for RANKL were 5′-TGCCAACATTTGCTTTCGAC-3′ and 5′-TTGGGATTTTGATGCTGGTTT-3′ [20]. The primers used for GAPDH were 5′-AGTCAACGGATTTGGTCGTAT-3′ and 5′-CCAGTGGACTCCACGACGTAC-3′ [5].

Analysis of NF-κB subunit composition. The activation of the p50 and p65 subunits of NF-κB in HMVEC treated with *P. gingivalis* (MOI 1:20) for 24 h was determined with an NF-κB family transcription factor assay kit (Active Motif, Carlsbad, CA, USA). This kit uses ELISA to measure transcription factor activities. The detecting antibodies recognize epitopes on either p50 or p65 that are accessible only when NF-κB is activated and bound to its target DNA [21] (containing the NF-κB consensus binding site 5′-GGGACTTTCC-3′) attached to 96-well plates. Nuclear protein extracts were obtained using the Nuclear/Cytosol Fractionation Kit (BioVision Research Products, Mountain View, CA, USA) and NF-κB ELISA was conducted according to protocols supplied by the manufacturers.

Data analysis. All experiments were carried out in duplicate or triplicate and the results are expressed as means ± SD. Student's *t* test was used and the significance level was set at $P < 0.05$.

Results

Porphyromonas gingivalis induces OPG mRNA expression in a dose-dependent manner in HMVEC

To assess the effects of *P. gingivalis* treatment on the OPG/RANKL system in endothelial cells, we first measured the levels of OPG and RANKL mRNA in HMVEC by RT-PCR analysis. The PCR products from treated and untreated HMVEC yielded bands of 408 bp (OPG), 101 bp (RANKL), and 278 bp (GAPDH). Up-regulation of OPG mRNA occurred at MOIs of 4:1 and 20:1 but not at a MOI of 2:1 (Fig. 1). In contrast, for RANKL mRNA, no significant difference was observed in response to bacterial treatment compared with the control. Treatment with these pathogens did not result in endothelial cell death, as measured by trypan blue uptake (data not shown).

Porphyromonas gingivalis induces OPG production in a time-dependent manner in HMVEC

We cocultured HMVEC with the bacteria for 3, 6, 18, or 24 h and used ELISA to analyze the amounts of OPG produced (Fig. 2). Addition of the bacteria at a MOI of 20:1 increased OPG production at 18 and 24 h compared

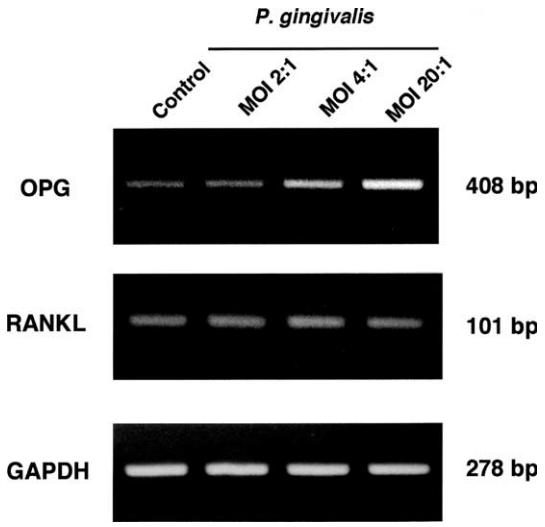

Fig. 1. Dose-dependent effects of *P. gingivalis* on OPG and RANKL mRNA expression in HMVEC. HMVEC were incubated for 18 h with medium alone (control) or with *P. gingivalis* at MOIs from 2:1 to 20:1. Total RNA was isolated from HMVEC and subjected to RT-PCR analysis. Oligonucleotides specific for GAPDH were used as controls for mRNA and cDNA synthesis. PCR-amplified products were analyzed on 2% agarose gels containing ethidium bromide.

with controls, but we did not observe the induction of OPG production in HMVEC at a MOI of 4:1. These results reveal that although *P. gingivalis* upregulated the mRNA levels of OPG at MOIs of 4:1 and 20:1, OPG production at a MOI of 4:1 did not differ significantly from that of the control.

Porphyromonas gingivalis protease degrades recombinant OPG

To determine whether soluble proteases released from *P. gingivalis* into the culture supernatant are capable of degrading OPG secreted from HMVEC, we incubated recombinant OPG with both *P. gingivalis* culture supernatants and EGM-2MV in the presence or absence of TLCK, an inhibitor of the active sites of serine and cysteine proteases. As a control, recombinant OPG was incubated with endothelial cell medium only. When recombinant OPG was incubated with bacterial supernatant and endothelial cell medium, the OPG level was reduced to 43.8% of that of the control (Table 1). In the presence of bacterial supernatant and 2 mM TLCK, the OPG level remained at 91.2% of the control level (Table 1). These results indicate that *P. gingivalis* serine and/or cysteine proteases degrade OPG.

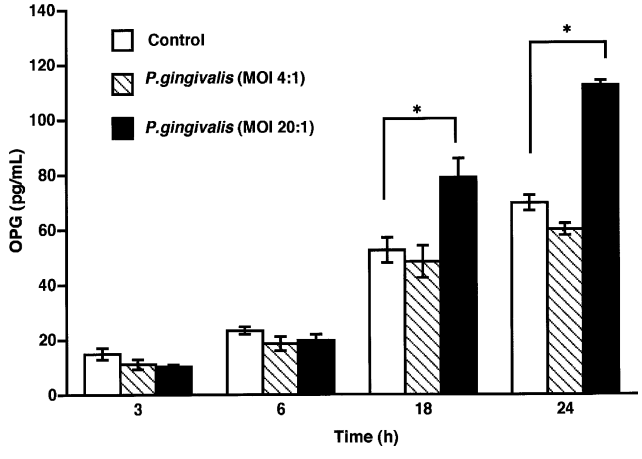

Fig. 2. Time-dependent effects of OPG production in HMVEC treated with *P. gingivalis*. HMVEC were incubated with medium alone (control) or with *P. gingivalis* at an MOI of 4:1 or 20:1. At the times indicated, sample supernatants were collected and OPG production was analyzed by ELISA. The data are shown as means ± SD ($n = 3$; $^*P < 0.05$ versus control value).

Table 1
Effects of protease inhibitors on osteoprotegerin (OPG) degradation in culture supernatant from *P. gingivalis*

TLCK (mM)	Bacterial culture supernatant	% of OPG remaining
0	–	100
0	+	43.8 ± 4.5
0.2	+	38.3 ± 1.5
2	+	91.2 ± 1.5

See Materials and methods for experimental details. OPG levels remaining after the 24-h incubation period were measured by ELISA. The data presented are means ± 1 SD of three experiments.

Porphyromonas gingivalis LPS and heat-killed whole cells stimulate OPG production in HMVEC

To examine the ability of different *P. gingivalis* membrane components to stimulate HMVEC, we added heat-killed *P. gingivalis* whole cells, LPS, and fimbriae to HMVEC and used ELISA to assay OPG production in the culture supernatants. The addition of heat-killed *P. gingivalis* whole cells or *P. gingivalis* LPS stimulated the production of OPG (Fig. 3), but we did not observe induction of OPG in HMVEC incubated with *P. gingivalis* fimbriae. LPS derived from *E. coli* was used as a positive control.

NF-κB regulation of OPG production

To elucidate the intracellular signaling pathway that regulates *P. gingivalis* induction of OPG expression, we examined whether the transcription factor NF-κB was involved in this signaling process. Treatment of HMVEC with *P. gingivalis* resulted in a significant increase of both p65 and p50 compared with that of untreated cells (Fig. 4). These results indicate that *P. gingivalis* upregulates transcription of the NF-κB pathway. In addition, we examined the effect of two NF-κB activation inhibitors, PDTC and NAC, on the production of OPG in HMVEC. As

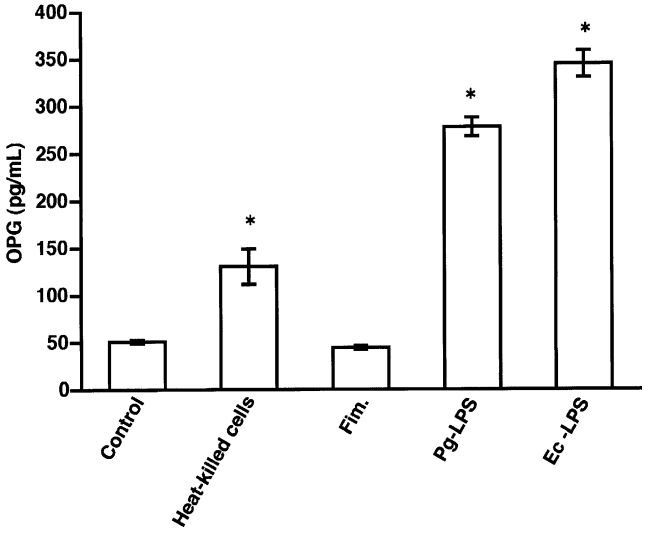

Fig. 3. Effects of heat-killed *P. gingivalis* whole cells, *P. gingivalis* fimbriae and LPS on OPG production in HMVEC. Cells were incubated with medium alone (control), heat-killed whole-cell preparations of *P. gingivalis* (Heat-killed cells; corresponding to 10^8 CFU/ml), *P. gingivalis* fimbriae (Fim; 20.0 μg/ml), *P. gingivalis* LPS (Pg-LPS; 10.0 μg/ml), or *E. coli* LPS (Ec-LPS; 1.0 μg/ml). Supernatant samples were collected at 24 h post-addition and analyzed by ELISA for OPG production. Data are shown as means ± SD ($n = 3$; $^*P < 0.05$ versus control value).

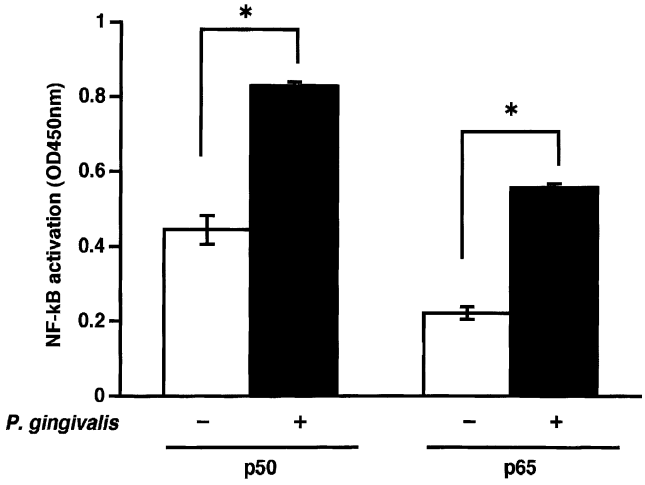

Fig. 4. NF-κB p50 and p65 activation in HMVEC treated with *P. gingivalis*. Treatment with *P. gingivalis* was performed at MOI of 1:20 for 24 h and then NF-κB activation in cellular extracts was analyzed with an NF-κB family ELISA-based assay kit. Data are shown as means ± SD ($n = 3$; $^*P < 0.05$ versus control value).

shown in Fig. 5, *P. gingivalis*-stimulated OPG production in HMVEC was significantly reduced in the presence of PDTC and NAC. Moreover, OPG production in un-stimulated HMVEC was suppressed by the inhibitors. These results indicate that NF-κB regulates not only *P. gingivalis*-stimulated OPG production in HMVEC, but also constitutive OPG expression levels.

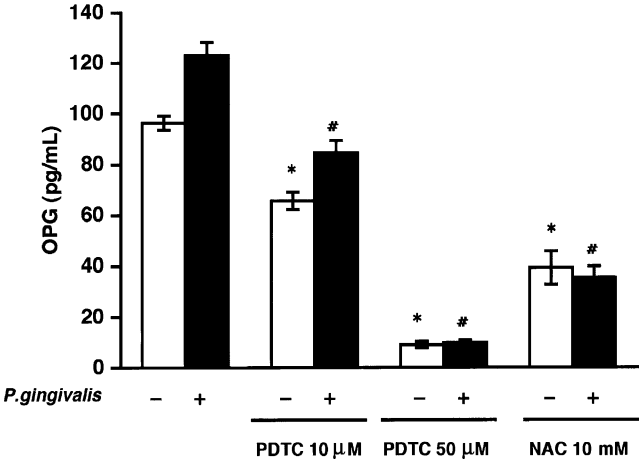

Fig. 5. Effects of PDTC and NAC on OPG production in HMVEC treated with *P. gingivalis*. After preincubation for 1 h with either PDTC (10 or 50 μM) or NAC (10 mM), HMVEC were treated with or without *P. gingivalis* for 24 h. Supernatant samples were collected and analyzed by ELISA for OPG. Data are shown as means ± SD ($n = 3$; $^*P < 0.05$ versus untreated HMVEC; $^{\#}P < 0.05$ versus HMVEC treated with *P. gingivalis* in the absence of inhibitors).

Discussion

Bacteria or their products cause inflammatory bone loss, and it has long been suggested that vascular endothelial cells contribute to bone metabolism by producing mediators such as IL-1, IL-6, fibroblast growth factors, and macrophage colony-stimulating factors against bacterial infections [7,22]. Observations presented here indicate that *P. gingivalis* induces OPG, but not RANKL, from HMVEC. This observation is the first to demonstrate that endothelial cells contribute to the OPG/RANKL system and may play an active role in bone metabolism under bacterial stimulation. Since osteoclast precursors reside in the peripheral circulation [23,24], these cells may be exposed to and potentially regulated by OPG and RANKL displayed on endothelial cells during and following their transmigration across the endothelial cell layer in response to microbial pathogens. Therefore, it is possible that endothelial cells might suppress or slow down bone resorption.

Human microvascular endothelial cells are one of the first lines of defense against invading microbial pathogens [25]. In this study, we documented that *P. gingivalis*-stimulated HMVEC secretes OPG protein. Interestingly, the inflammatory cytokines TNF-α and IL-1, which are essential for the initiation of inflammatory immune reactions and are produced by endothelial cells and leukocytes, upregulate OPG and RANKL mRNA levels in HMVEC [15]. It is likely that human microvascular endothelial cells act as inflammatory cells and may directly contribute to the progression of inflammatory bone loss in periodontitis. In addition, our results indicate that *P. gingivalis*-stimulated OPG production in HMVEC is NF-κB-dependent.

Because NF-κB activation by various stimuli leads to the upregulation of inflammatory mediators, this regulation by NF-κB suggests that OPG may be involved in the inflammatory functions of endothelial cells [26]. These possibilities are also supported by the fact that OPG-deficient mice show extensive inflammation of the aorta and renal arteries in addition to severe osteoporosis [10]. Taken together, these results suggest that OPG production from HMVEC might be one of the immune mechanisms against bacterial challenge.

Porphyromonas gingivalis induces osteoclastic cell formation from murine leukocytes in the absence of osteoblasts [7]. This pathogen induces osteoclast formation directly and indirectly [7,27]. In contrast, *E. coli* LPS-stimulated human gingival fibroblasts produce OPG protein, and the culture supernatant of *E. coli* LPS-stimulated gingival fibroblasts inhibits the ability of soluble RANKL to stimulate monocyte differentiation into osteoclasts [28]. The structure of *P. gingivalis* LPS is different from *E. coli* LPS; it lacks ester-linked phosphate at the 4′ position of the glucosamine disaccharide and the presence of fatty acids with long acyl chains [29]. However, it is possible that *P. gingivalis* LPS also induces OPG production from gingival fibroblasts, because both LPS types are recognizable through Tall-like receptor 4 by gingival fibroblasts [30]. Periodontal ligament cells and dental pulp cells also express OPG mRNA [31,32]. These OPG/RANKL networks may prevent bone resorption induced by bacterial infection. Although the mechanism whereby bone resorption in periodontitis is not clear, we speculate that these bone protective mechanisms could function when periodontal tissue is exposed to a relatively low amount of LPS. Indeed, *P. gingivalis* LPS does not induce RANKL expression, but inflammatory cytokines, which are released by the endothelium and leukocytes, induce RANKL expression, and thus inflammatory cytokines can prompt osteoclast formation [15]. These OPG/RANKL networks might lead to bone resorption in the tissues with increasing inflammation.

Porphyromonas gingivalis possesses a number of virulent features, including fimbriae, vesicles, proteases, LPS, and peptidoglycans [33]. These factors have been demonstrated to facilitate the destruction of periodontal tissue. Our results showed that purified LPS derived from *P. gingivalis* induced OPG production from HMVEC. Furthermore, we observed a stimulatory effect of heat-killed *P. gingivalis* on OPG production in HMVEC, suggesting the participation of heat-resistant molecules. Taken together, these findings suggest that *P. gingivalis* LPS is a component responsible for OPG production in microvascular endothelial cells.

The present study showed that *P. gingivalis* reduced recombinant OPG, and that TLCK, an inhibitor of the active sites of serine and cysteine proteases, prevented the reduction of OPG. These results indicate that bacterial serine and/or cysteine proteases degrade OPG. Our previous study showed that *P. gingivalis* proteases degraded two inflammatory cytokines, MCP-1 and IL-8 [5]. Degradation of inflammatory cytokines decreases the host's ability to mount an effective response to the pathogen, because inactivation of cysteine proteases decreases *P. gingivalis* virulence [1]. *P. gingivalis* proteases may create an imbalance of cytokines including OPG and lead to bone resorption.

Our understanding of the OPG/RANKL system in vascular endothelial cells is still very limited. *E. coli* LPS does not change RANKL or OPG mRNA expression levels in umbilical vein endothelial cells [34]. However, our current results show that *E. coli* LPS induced OPG production in HMVEC. It is likely that the response of endothelial cells to LPS may be different depending on the sites at which they reside. In addition, OPG mRNA expression was strongly induced by osteopontin in rat aortic endothelial cells through the NF-κB-dependent pathway, and OPG is a potent survival factor for the endothelial cell lines [35]. To date, OPG not only functions as a decoy receptor for RANKL, but also for TNF-related apoptosis-inducing ligand (TRAIL) [36]. Moreover, it has been well documented that HMVEC express TRAIL receptors and TRAIL induces apoptosis and inflammatory gene expression in HMVEC [37]. Therefore, it would be interesting to conduct further studies to examine whether OPG functions as a survival factor of HMVEC by inhibiting the TRAIL-induced apoptosis in *P. gingivalis* infection.

In conclusion, we showed that HMVEC secrete OPG in response to *P. gingivalis* challenge through an NF-κB-dependent mechanism, and the bacterium degrades (at least in part) the protein produced. Our observations also suggest that *P. gingivalis* LPS is responsible for the stimulation of HMVEC. Induction of OPG in endothelial cells may play an important role in bone metabolism during periodontal inflammation.

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