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An acute injection of *Porphyromonas gingivalis* lipopolysaccharide modulates the OPG/RANKL system and interleukin-6 in an ovariectomized mouse model

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Background/aims: In the present study, we attempted to develop a simulated model to explore the causal effects of periodontal pathogens on skeletal homeostasis in postmenopausal osteoporosis.
Methods: Fifty-three female adult ICR mice were randomly assigned to an experimental group (ovariectomized) or a control group. A single injection of *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis*-LPS, ATCC 33277) or *Escherichia coli* lipopolysaccharide (*E. coli*-LPS) was administered intraperitoneally 4 weeks after an ovariectomy. Concentrations of interleukin-6 (IL-6), osteoprotegerin (OPG), and the receptor activator of nuclear factor-κB ligand (RANKL) in serum were subsequently analyzed using an enzyme-linked immunosorbent assay (ELISA).
Results: Under stimulation with *P. gingivalis*-LPS or *E. coli*-LPS, the concentration of OPG rose in both groups. The serum level of RANKL showed a decreasing trend 24 h after the injection in both groups. After injection of *P. gingivalis*-LPS in both the experimental and control animals, the OPG : RANKL ratio increased 24 h after the booster (22.26–620.99, $P < 0.05$). The serum level of IL-6 in the experimental group significantly increased 1–6 h after administration of *E. coli*-LPS and 1–3 h after administration of *P. gingivalis*-LPS ($P < 0.05$).
Conclusions: A single booster injection of *P. gingivalis*-LPS induced short-term changes in OPG, RANKL, and IL-6 serum levels in this ovariectomized mouse model.

Key words: interleukin-6; osteoporosis; osteoprotegerin; *Porphyromonas gingivalis* lipopolysaccharide; receptor activator of nuclear factor-κB ligand

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A number of studies have generally suggested that osteopenia plays a role in the progression of periodontal diseases (28, 29, 32, 33). However, several cross-sectional studies showed no significant correlation between the clinical parameters of periodontitis and the systemic bone mass; nor was a significant relation observed between the bone mass measurements and alveolar bone height (7, 39). Further study of interactions between osteoporosis and periodontal disease is needed. Periodontitis and osteopenia may share common risk factors, including smoking, nutritional deficiencies, age,

and corticosteroid use leading to immune dysfunction, which may either directly influence or modulate both disease processes (34, 35).

Recent findings have suggested that osteoclastogenesis is directly regulated by the receptor activator of the nuclear factor-κB ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG) (1, 15, 19). In one study, we attempted to determine the profiles of OPG, RANKL, interleukin-6 (IL-6), and oncostatin M in human gingival crevicular fluid, and tried to establish an initial model of regulation of the OPG and RANKL system by IL-6 family cytokines during the progression of periodontal inflammation. Our data suggested that from evidence of the positive correlations between gingival crevicular fluid RANKL and IL-6/oncostatin M, crosstalk between members of the tumor necrosis factor (TNF) family and glycoprotein 130 (gp130) family (IL-6 and OSM) might play a certain role in the progression of periodontal disease (24). Several lines of inquiry have indicated that altered homeostasis in the OPG/RANKL/RANK signaling pathway may contribute to osteoporosis resulting from a menopause-induced estrogen deficiency (12, 26, 41). Not only RANKL, but also TNF-α in the TNF-ligand family was found to play a crucial role in regulating osteoclast differentiation and activity (40).

The incidences of bacteremia following dental procedures such as tooth extraction, endodontic treatment, periodontal surgery, and ultrasonic scaling have been well documented (2, 5, 6, 23), including in 100% of patients after dental extractions, in 70% after dental scaling, in 55% after third-molar surgery, and in 20% after endodontic treatment (10). The incidences of bacteremia in individuals with periodontitis, gingivitis, and clinically healthy periodontium after chewing, tooth brushing, and scaling were investigated, and *Porphyromonas gingivalis* was isolated from the blood of more than one-third of the patients with periodontitis, suggesting that those patients might suffer from bacteremia (8). In addition, there is evidence that dental procedures may cause bacteremia in adults and that components of the causative bacteria of oral infections, particularly lipopolysaccharide (LPS), may promote atherosclerosis, and affect blood coagulation and the function of platelets (30). *P. gingivalis*-LPS enhances the production of inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α in gingival fibroblasts and induces periodontal bone resorption (31). It is conceivable that *P. gingivalis*-LPS may also enter the blood circulation during ultrasonic scaling and play a certain role in causing transient bacteremia.

Accordingly, we assumed that *P. gingivalis*-LPS might penetrate the periodontium after scaling and affect the bone homeostasis of osteoporotic individuals. The purpose of the present study was to set up a reliable *in vivo* model for investigating a transient attack of *P. gingivalis*-LPS on modulation of the serum OPG/RANKL system and IL-6 in an ovariectomized mouse model.

Materials and methods

Animal design for the ovariectomy

Fifty-three female adult ICR mice (Animal Center, National Taiwan University Hospital, Taipei, Taiwan) were maintained for 10 weeks at the Animal Center of Taipei Medical University. Mice were randomly assigned to a treatment group (E group, $n = 27$) for an ovariectomy or to a control group (C group, $n = 26$) for a sham operation. The study protocol was approved by the Institutional Animal Care and Use Committee of Taipei Medical University. On day 0 of the study, a serum sample was taken from the intraorbital cavity, consisting of 0.4 ml whole blood, which was injected into 2-ml Eppendorf tubes. The sample was allowed to remain at room temperature for 2 h and then centrifuged at 15,000 g for 10 min to collect the serum. The concentrations of IL-6, OPG, and RANKL in the serum were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D System, Minneapolis, MN, and Biomedica Medizinprodukte, Wien, Austria, respectively) to quantify the protein level and to establish pre-surgical data. The ICR mice were then anesthetized with ether to allow exposure of the ovaries by a dorsal approach; the gonads were removed in the experimental group but only manipulated without removing the organs in the sham-operated cohort.

Efficacy of the ELISA bioassay

A bilateral ovariectomy in mice was conducted 4 weeks before the second round of serum samples was taken. For ELISA, 50 μl assay diluent was added to each well of a 96-well plate before the standard or sample serum was pipetted into the wells. After the solution was washed out, the cytokine polyclonal anti-rat IL-6 OPG/RANKL conjugate (100 μl) was coated on each well and shaken for 2 h. Then, 100 μl substrate solution (including 50 μl hydrogen peroxidase and 50 μl chromogen) was added, and the mixture was protected from light for 30 min. Stop solution (100 μl) was added to stop the reaction. A spectrophotometer was used to determine the optical density with the wavelength set at 450 nm. The detection limit for ELISA was 0.2 ng/ml (the mean minimum detectable dose of IL-6 was 1.6 pg/ml, that of OPG was 4.5 pg/ml, and that of RANKL was <5 pg/ml).

LPS preparation

LPS derived from a *P. gingivalis* strain (ATCC33277, *P. gingivalis*-LPS) was extracted following the method of Westphal et al. (38). Before use, the LPS was diluted in pyrogen-free distilled water (1 mg/ml). The LPS endotoxin level was examined using *Limulus* amebocyte lysate (Biowhitakker; BMA, Vallensbaek Strand, Denmark). LPS from *Escherichia coli* 0127:B8 (*E. coli*-LPS; Sigma, St Louis, MO) was used as the control.

Acute injection of *P. gingivalis*-LPS

Four weeks after the ovariectomy operation, 100 μg *P. gingivalis*-LPS and 100 μg *E. coli*-LPS (Sigma) were independently injected into the peritoneum of assigned animals in both the E and C groups. Serum was collected 1, 3, 6, 24, and 48 h after the injection. Serum concentrations of IL-6, OPG, and RANKL were quantified using a sandwich ELISA.

Biostatistics

SPSS version 10.0 software (SPSS, Inc., Chicago, IL) was used to carry out statistical analyses of the variances of IL-6, OPG, and RANKL concentrations in the serum, and the OPG : RANKL ratio. Since the initial data above were not drawn from a normally distributed population, as estimated by the Shapiro–Wilk test, it was decided to analyze them using the non-parametric Mann–Whitney *U*-test, and the *P*-value was set at 0.05. The Kruskal–Wallis test was used to verify differences between baseline data and those data derived after an acute injection of either *P. gingivalis*-LPS or *E. coli*-LPS. Spearman rank correlation coefficients were used to examine changes in IL-6 and correlations with OPG, RANKL, and the OPG : RANKL ratio. A statistical correlation was considered significant when the *P*-value was < 0.05.

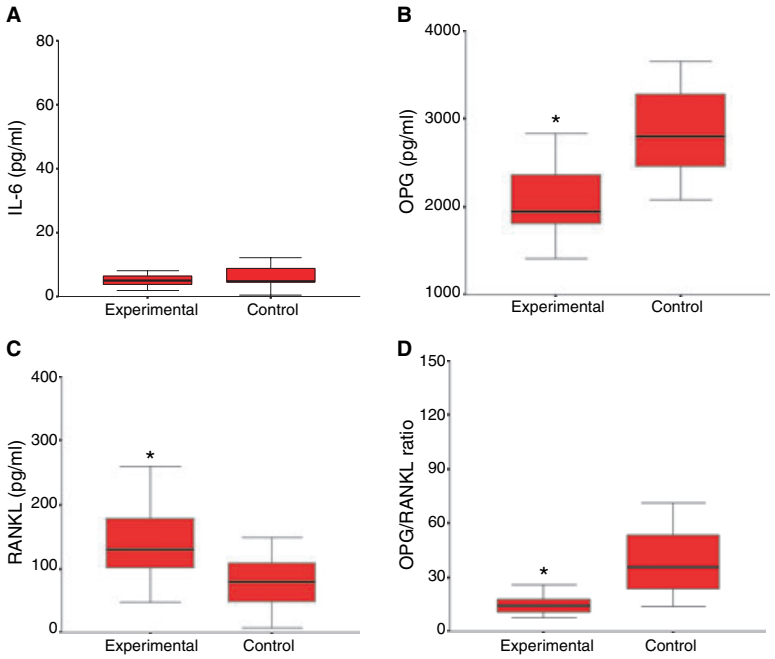

Fig. 1. (A) There was no difference in serum IL-6 concentrations between the experimental and sham-operated groups. (B) Effect of an ovariectomy on OPG production in the serum of the simulation model. (C) Effects of ovariectomy on the expression of RANKL in serum. (D) Ratio of OPG : RANKL showing a significant difference between the experimental and control groups 4 weeks after ovariectomy.

Results

Effects of an ovariectomy on IL-6, OPG, RANKL, and the OPG : RANKL ratio

After ovariectomy, no difference was found between the changes in IL-6 concentrations in the sera of ovariectomized mice and in sham-operated mice 4 weeks after gonad dissection (Fig. 1A, $P = 0.739$). As shown in Fig. 1B, the expression of OPG in the serum of the experimental group ranged from 1410.19 to 2830.96 pg/ml. However, the mean data were significantly lower than those of the control group (2080.14–3648.62 pg/ml) ($P < 0.001$). On the other hand, the concentration of RANKL in the experimental group ranged from 47.01 to 258.53 pg/ml (Fig. 1C), which was higher than that in the control group (6.69–149.79 pg/ml) ($P < 0.001$). The OPG : RANKL ratio of the control group (13.90–493.97) was larger than that of the experimental group (7.77–50.32) at 4 weeks after the ovariectomy (Fig. 1D, $P < 0.001$).

Effects of bacterial LPSs on IL-6, OPG, RANKL, and the OPG : RANKL ratio

Under stimulation with *P. gingivalis*-LPS or *E. coli*-LPS, the concentrations of OPG rose in both groups with a higher amplitude of increase in the *E. coli*-LPS-stimulated group than in the *P. gingivalis*-LPS-stimulated group (Fig. 2A). Compared to the baseline data, the level of RANKL in the serum showed a decreasing trend 24 h after the injection of *P. gingivalis*-LPS in the ovariectomized group (from 144.16 to 36.43 pg/ml, $P < 0.05$) and the control group (from 79.12 to 33.06 pg/ml, $P < 0.05$) (Fig. 2B). Twenty-four hours after injection of *P. gingivalis*-LPS in the experimental group, the OPG : RANKL ratio increased (from 22.26 to 620.99, $P < 0.05$) (Fig. 3A). The expression of IL-6 sharply increased at 1–6 h in the *E. coli*-LPS group and at 1–3 h in the *P. gingivalis*-LPS group compared with baseline data ($P < 0.05$) (Fig. 3B). In addition, changes in OPG, RANKL, the OPG : RANKL ratio, and IL-6 in the experimental group did not significantly differ from those in the control group with injection of either *E. coli*-LPS or *P. gingivalis*-LPS.

Correlations of IL-6 with OPG, RANKL, and the OPG : RANKL ratio

The data analyzed with Spearman rank correlation coefficients showed that regardless of which bacterial LPS was

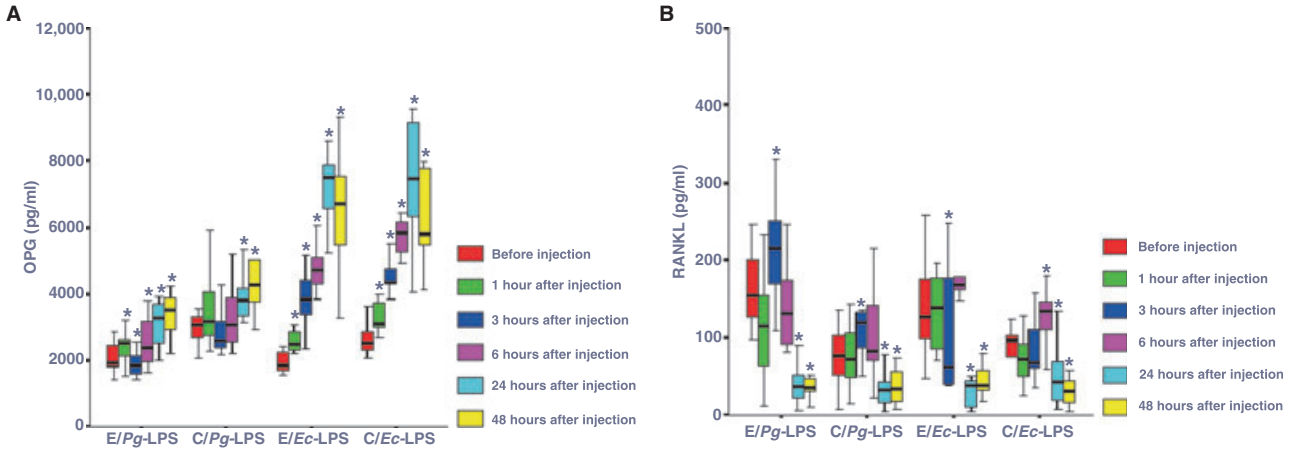

Fig. 2. (A) Changes in osteoprotegerin (OPG) of the experimental (E) ($n = 45$) and control (C) ($n = 45$) groups individually injected with *Porphyromonas gingivalis* LPS and *Escherichia coli*-LPS (ELISA, $*P < 0.05$). Each value represents the box extending from the 25th to the 75th percentiles. The line within the box is the median. Whiskers extend to the largest and smallest values within 1.5 box lengths plotted as pg OPG/ml. $*P < 0.05$, statistically significantly different compared to baseline data. (B) Changes in the RANKL of the experimental (E) and control (C) groups individually injected with *P. gingivalis*-LPS and *E. coli*-LPS (ELISA, $*P < 0.05$). Values are presented as for (A).

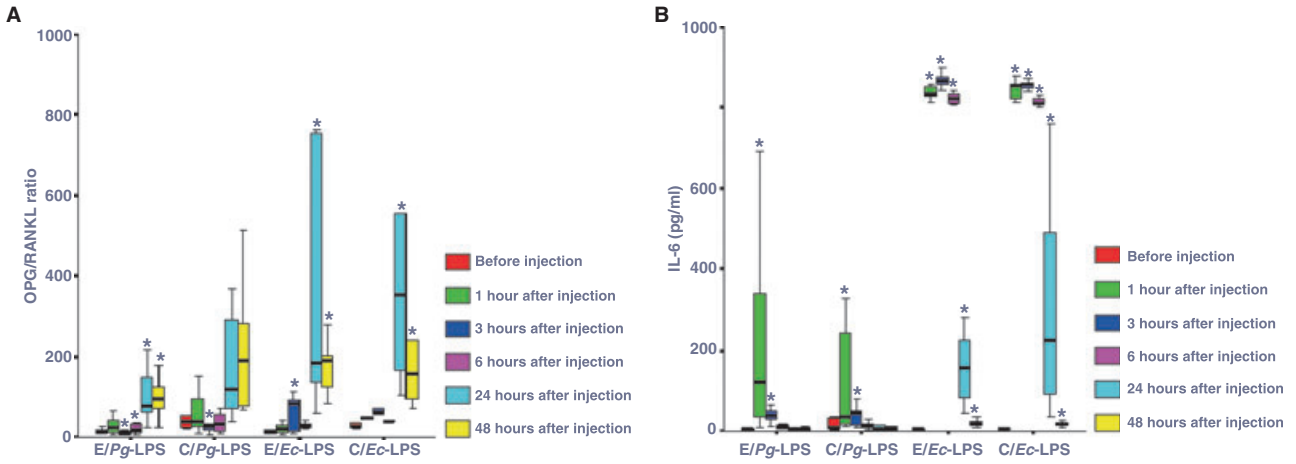

Fig. 3. (A) Changes in the OPG/RANKL ratio of the experimental (E) and control (C) groups individually injected with *Porphyromonas gingivalis*-LPS and *Escherichia coli*-LPS (ELISA, $*P < 0.05$). Values are presented as in Fig. 2A. (B) Changes in the IL-6 levels of the experimental (E) and control (C) groups individually injected with *P. gingivalis*-LPS and *E. coli*-LPS (ELISA, $*P < 0.05$). Values are presented as in Fig. 2A.

used, there were no significant correlations in changes of IL-6 with OPG, RANKL, or the OPG : RANKL ratio after the LPS injections ($P = 0.266$ to $P = 0.971$), except for OPG and IL-6 in the control group ($P < 0.01$).

Discussion

The present *in vivo* study indicated that the OPG level in serum significantly increased with the administration of *P. gingivalis*-LPS or *E. coli*-LPS. In an investigation of the effect of *P. gingivalis* on OPG and RANKL production in human microvascular endothelial cells, results showed that *P. gingivalis* upregulated the expression of OPG but not RANKL messenger RNA (20). It was also shown that cultured human periodontal ligament cells stimulated with LPS inhibited osteoclastogenesis by more effectively producing OPG than RANKL via the induction of IL-1β and TNF-α (36). The increase in OPG seems to represent a defensive mechanism for binding to LPS-induced RANKL and inhibiting subsequent events, like osteoclastogenesis, in bone homeostasis.

The present baseline data after the ovariectomy and injection with the two types of bacterial LPS showed that IL-6 was not correlated with OPG and RANKL in bone homeostasis. Another study found that proinflammatory cytokines, but not IL-6, may stimulate osteoclastogenesis by inducing the expression of RANKL in human osteoblast cells (13). In contrast, IL-6 is directly capable of inducing the formation of multinucleated cells, the vitronectin receptor, and calcitonin receptor by a RANKL-independent mechanism (21). It can be postulated that the gp130 family and the reciprocation of OPG and RANKL are two independent modulatory systems for regulating osteoclastogenesis.

Most studies that analyzed the relationship between oral infections and systemic diseases implicated periodontal disease as a risk factor for systemic diseases (22). Subgingival biofilms constitute an enormous and continually present bacterial load. They represent continually renewing reservoirs of gram-negative bacteria and LPS with ready access to periodontal tissues and the circulation. In a novel *in vivo* murine calvarial model to assess the effects of oral pathogens on the expression of bone resorptive cytokines in host tissues, it was suggested that oral microorganisms with access to host tissues elicited a battery of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) (18). The study of patients with chronic periodontitis undergoing an episode of subgingival scaling or brushing also showed a significant elevation in circulating proinflammatory cytokines and IL-6 and the occurrence of transient bacteremia (8, 16). Similarly, our model showed that short-term challenge with gram-negative bacterial LPS induced transient changes in IL-6, OPG, and RANKL in the serum. However, these biomarkers have pleiotropic functions and can be expressed in numerous tissues; it is difficult to forecast their long-term effects on bone homeostasis, using this rat model of postmenopausal osteoporosis.

In general, the endotoxic activity of *P. gingivalis*-LPS is very low compared with that of the LPS isolated from enterobacteria (27). The LPS of *P. gingivalis* differs from that of other gram-negative bacteria in that its protein structure lacks a 4-*O*-phosphoryl group in the lipid A backbone. This may be the cause of the low toxicity of *P. gingivalis*-LPS (14, 25, 37). It has been shown that the production of cytokines induced by *P. gingivalis*-LPS was negligible when compared with that induced by *E. coli*-LPS (3, 11). It is conceivable that in this study the potency of *E. coli*-LPS was stronger than that of *P. gingivalis*-LPS in elevating serum levels of the OPG : RANKL ratio and IL-6 and would be a reasonable choice for the positive control LPS in this study.

It is well known that bacteria from the oral cavity may give rise to septicemia, but whether the magnitudes of bacteremia detected in serum are sufficient to elicit septicemia remains unknown (16). The clinically relevant dose of *P. gingivalis*-LPS is also unknown and probably varies greatly (4, 9, 16). It was verified by Isogai et al. (17) that a 100-μg injection of *P. gingivalis*-LPS was detected in macrophages and induced apoptosis of cells of the spleen and lymph organs in 176 C3H/HeN mice. Apoptosis reached its maximum between 1 and 2 days after an acute injection. A similar peak was detected in mice injected with *E. coli*-LPS. This implies that the use of 100 μg of *P. gingivalis*-LPS (1 μg/1 μl) was reasonable in the present study for the short-term evaluation of bacteremia in bone homeostasis.

It can be concluded that a single booster of *P. gingivalis*-LPS may induce short-term changes in OPG, RANKL, and IL-6 in the serum in this mouse model. Transient bacteremia caused by treatment of periodontal disease or tooth brushing may possibly affect fluctuations of both the OPG and RANKL system and IL-6 in the serum of patients with osteoporosis on a short-term basis. Determining the long-term effects of *P. gingivalis*-LPS requires further investigation.

Acknowledgments

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