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# Local osteoprotegerin gene transfer to periodontal tissue inhibits lipopolysaccharide-induced alveolar bone resorption

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*Background and Objective:* Osteoclastogenesis is primarily activated by receptor activator of nuclear factor κB ligand (RANKL) and is inhibited by osteoprotegerin (OPG). A previous study demonstrated that local OPG gene transfer to periodontal tissue inhibited RANKL-mediated osteoclastogenesis and experimental tooth movement. In the present study, we tested the hypothesis that local OPG gene transfer to the periodontium can neutralize RANKL activity induced by lipopolysaccharide injection, thereby inhibiting osteoclastogenesis and diminishing alveolar bone resorption in experimental periodontal disease.

*Material and methods:* Seven-week-old male Wistar rats received an injection of lipopolysaccharide or phosphate-buffered saline in the palatal gingiva of the upper first molars on both the right and left sides. An inactivated haemagglutinating virus of Japan (HVJ) envelope vector containing a mouse OPG expression plasmid [pcDNA3.1(+)-mOPG] or mock vector was injected periodically into the palatal periodontal tissue of the upper first molars.

*Results:* Lipopolysaccharide injection induced severe periodontal bone resorption. Local OPG gene transfer induced OPG production, and osteoclastogenesis was inhibited. Local OPG gene transfer significantly decreased alveolar bone resorption.

*Conclusion:* Osteoprotegerin gene transfer to periodontal tissue inhibited osteoclastogenesis and alveolar bone resorption in lipopolysaccharide-induced experimental periodontal disease.

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Recently, the number of adult orthodontic patients has been increasing. In these patients, the morbidity rate of periodontal disease is high, and alveolar bone resorption can often be seen. Orthodontic tooth movement during progressive periodontitis causes severe bone destruction, and thus control of the alveolar bone resorption induced by periodontal disease becomes very important for orthodontic treatment (1,2).

Alveolar bone resorption in periodontitis is the result of excessive osteoclastic activity, leading to an imbalance in bone remodelling that favours resorption (3–5). Osteoclastogenesis is primarily activated by receptor activator of nuclear factor κB ligand (RANKL) and is inhibited by osteoprotegerin (OPG; 6–8). Perio-

dontal ligament (PDL) cells, which exist between teeth and alveolar bone, regulate osteoclastogenesis through RANKL stimulation and OPG inhibition, and play important roles in processes such as periodontitis and orthodontic tooth movement (9). Furthermore, we have reported that OPG gene transfer to periodontal tissue inhibits RANKL-mediated osteoclastogenesis and inhibits experimental tooth movement (10). Combining this information, we hypothesized that local OPG induction in the periodontal tissue can neutralize RANKL induced by periodontitis and thereby inhibit osteoclastogenesis and alveolar bone resorption.

To test this hypothesis, we used an experimental model of periodontal disease induced by recurrent lipopolysaccharide (LPS) injection in rats, with or without local OPG gene transfer by means of a haemagglutinating virus of Japan (HVJ; Sendai virus) envelope vector gene delivery system, a method combining viral and non-viral features.

## Material and methods

### Animals

Sixteen 7-week-old male Wistar rats with an average weight of 167 g were used in this study. The animals were divided into four experimental groups, as shown in Table 1. In group 1, mock vector transfer and phosphate-buffered saline (PBS) injection were performed on the left side, and mock vector transfer and LPS injection were performed on the right side. The independent effect of LPS on alveolar bone resorption was investigated with this group. In group 2, LPS injection and mock vector transfer were performed on the left side, and LPS injection and OPG gene transfer were performed on the right side. This group was designed to investigate the effect of OPG gene transfer on alveolar bone resorption induced by LPS. In group 3, OPG gene transfer and PBS injection were performed on the left side, and OPG gene transfer and LPS injection were performed on the right side. In this group, the effect of LPS on alveolar bone resorption under OPG gene transfer was examined. In group 4, PBS injection and mock vector transfer were performed on the left side, and PBS injection and OPG gene transfer were performed on the right side. This group was designed to investigate the independent effect of OPG gene transfer on alveolar bone resorption.

Lipopolysaccharide injection and OPG gene transfer were performed on days 1, 3, 5, 7, 9 and 11. On day 14, the rats were killed using pentobarbital. The solutions were injected into the subperiosteal area, adjacent to the first molar. Osteoprotegerin gene transfer and LPS injection were performed on the animals under intraperitoneal anaesthesia of pentobarbital sodium 4 mg/100 g body weight (WAKO Jun-yaku, Tokyo, Japan) anaesthesia.

All animals were treated ethically, in compliance with the regulations of Tohoku University.

### Development of experimental periodontal disease

Purified lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma Chemical Co., St Louis, MO, USA) was injected directly into the palatal gingiva, adjacent to each first molar (10 μg per site) every 2 days (11,12).

*Table 1.* Experimental groups

| Group | Left side (control) | Right side (experimental) |
|---|---|---|
| 1 | Mock vector + PBS | Mock vector + LPS |
| 2 | LPS + mock vector | LPS + OPG vector |
| 3 | OPG vector + PBS | OPG vector + LPS |
| 4 | PBS + mock vector | PBS + OPG vector |

Animals were divided into four groups based on treatments. There were four animals in each group. OPG vector, OPG gene transfer; mock vector, (the original expression plasmid pc DNA3.1), the control for OPG gene transfer; LPS, LPS injection; and PBS, the control for LPS.

### *In vivo* gene transfer

The OPG expression plasmid was prepared according to a previously described method (10). For *in vivo* transfection, an HVJ envelope-vector kit (GenomONE®, Ishihara-sangyo Kaisha Ltd, Osaka, Japan) was used, according to the manufacturer's instructions (13). Administration of the HVJ envelope-vector containing the mouse OPG expression plasmid constructed using pc DNA 3.1 (pcDNA-mOPG; Invitrogen, Carlsbad, CA, USA) by an overdose of intraperitoneal pentobarbital anaesthesia was performed at the same time and sites as LPS or PBS injection. Osteoprotegerin vector solution (containing pcDNA-MORG enveloped by HVJ) (5 μl) was injected under anaesthesia, into the subperiosteal area adjacent to the first molar on days 1, 3, 5, 7, 9 and 11. Mock vector (the original expression plasmid pc DNA3.1) (5 μl) was injected into the corresponding area as a control in the same way.

### Measurement of bone mineral density (BMD) of the tibia

The rats were killed by an overdose of intraperitoneal pentobarbital anaesthesia on day 14, and the tissues were fixed by perfusion with 4% paraformaldehyde in PBS. The bilateral tibiae were excised, and the soft tissues were removed. Lateral radiographs of the tibiae were taken along with hydroxyapatite standards using a soft X-ray apparatus (Sohuron Inc., Tokyo, Japan) set at 4.5 mA, 40 cm, 60 s and 25 kV. The densitometric patterns of the proximal tibiae, especially the cancellous bone area, were measured by ScionImage® (Fig. 1). In brief, the standardized area (1.5 mm × 2.5 mm) in the tibiae was determined as 1.0 mm away from the growth plate and not containing cortical bone at the distal area, in a slight modification of our previous method (10).

### Tissue preparation

After fixation, the upper jaws, including the molars, were dissected out, further fixed overnight, decalcified with

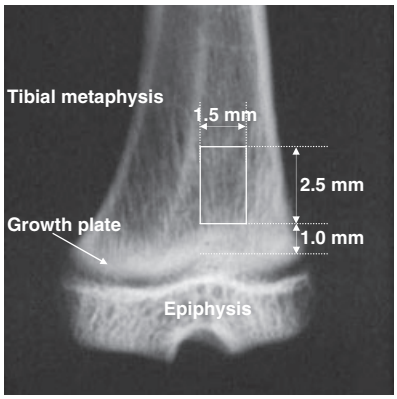


*Fig. 1.* X-ray photograph of proximal rat tibia. The densitometric patterns were measured inside the standardized area (1.5 mm × 2.5 mm), which was 1.0 mm away from the growth plate.

10% ethylenediaminetetraacetic acid in 0.01 M PBS (pH 7.4) for 9 weeks at 4°C, dehydrated, and embedded in paraffin. Eight micrometre serial frontal sections were prepared. To ensure the precision of the measurement values, all samples were set as the occlusal plane perpendicular to the horizontal plane during section cutting.

### Measurement of alveolar bone resorption

Periodontal bone resorption on the palatal surface of the maxillary molars was observed with haematoxylin and eosin staining. The alveolar bone loss was measured as the distance between the cemento-enamel junction (CEJ) and the alveolar ridge (12,14,15). The distance from the CEJ to the alveolar ridge of the palatal side was measured in the upper right and left molars at about 100 μm intervals, at ×10 magnification (Adobe Photoshop 6.0, San Jose, CA, USA). Each sample was measured three times, and the measurement error was calculated as 6.4 μm by the same investigator.

### Cytochemical examinations

For osteoclast detection, tartrate-resistant acid phosphate (TRAP) staining of the sections was performed with a leukocyte acid phosphatase kit (Sigma-Diagnosis®, St Louis, MO, USA). Osteoclasts were identified as multinucleated (> 3 nuclei) dark red cells. The TRAP-positive cells that formed resorption lacunae on the alveolar bone surface adjacent to the mesiopalatal roots of the upper first molars were counted.

### Quantitative immunohistochemical analysis

In brief, sections for immunohistochemical staining were deparaffinized and unmasked by incubation with liberate antibody binding solution (Polysciences Inc., Warrington, England) for 1 min. Then, the sections were preincubated in 5% bovine serum albumin (Dako, Glostrup, Denmark) in PBS for 30 min and subsequently incubated with anti-OPG antibody (1:250 dilution; sc-8468; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with anti-RANKL antibody (1:100 dilution; sc-7627; Santa Cruz Biotechnology) overnight at 4°C. After being thoroughly rinsed, the sections were incubated with fluorescein isothiocyanate-conjugated antigoat IgG (1:1000 dilution; sc-2024; Santa Cruz Biotechnology), washed, mounted in PBS–glycerol, and observed under a fluorescence microscope (Leica, Heidelberg, Germany). The same areas around the alveolar ridge of the palatal side of the upper right and left molars were photographed at ×10 magnification with the same exposure time and latitude.

To compare the expression of RANKL and OPG in periodontal tissues, the immunofluorescence intensity for RANKL and OPG was measured by ScionImage®.

### Statistics

Student's paired *t* test was used to compare bone resorption, osteoclast induction, and RANKL and OPG expression between the left and right sides within each group. The other data were analysed for statistical differences by Kruskal–Wallis analysis, followed by a Bonferroni-type multiple comparison (Tukey type). Values are expressed as means ± SD. A value of $P < 0.05$ was considered statistically significant.

## Results

### Animal status

The LPS injection and local administration of HVJ envelope-vector containing pcDNA-mOPG did not affect the growth of the animals. Initially, the average weights of the rats in the four groups were 167.5 ± 6.8, 167.0 ± 4.4, 161.3 ± 2.2 and 172.5 ± 11.5 g, respectively. Although the rats were randomly assigned into the groups, the average weight was higher in group 4 than in the other groups. On day 11, the average weights in the four groups were 191.5 ± 3.5, 197.2 ± 5.8, 200.5 ± 23.3 and 206.5 g ± 15.1 g, respectively. The body weights of the rats decreased just after the first LPS injection and OPG gene transfer, and then increased throughout the remainder of the experimental period. The local administration of 5 μl of either vector solution or PBS caused no appreciable macroscopic changes at the injection site.

Local gene transfer did not affect the BMD of the tibiae. The average BMD values in the four groups were 1201.0 ± 69.2, 1237.2 ± 103.6, 1251.1 ± 83.0 and 1396.5 ± 91.0 μg/mm$^2$, respectively. There were no significant pairwise differences among groups 1, 2 and 3. The significant differences between group 4 and the other three groups were thought to be related to the higher body weight in group 4.

### Lipopolysaccharide injection induced RANKL expression in periodontal tissue

Lipopolysaccharide injection significantly induced RANKL expression in periodontal tissue, regardless of OPG gene transfer ($P < 0.05$) (Fig. 2A–C, G–I). Local OPG gene transfer did not affect RANKL expression on the sides, regardless of the injection of LPS ($P > 0.05$; Fig. 2D–F,J–L).

### Local OPG expression in periodontal tissue was facilitated by OPG gene transfer but was not affected by LPS injection

When the OPG expression plasmid was administered by the HVJ envelope-

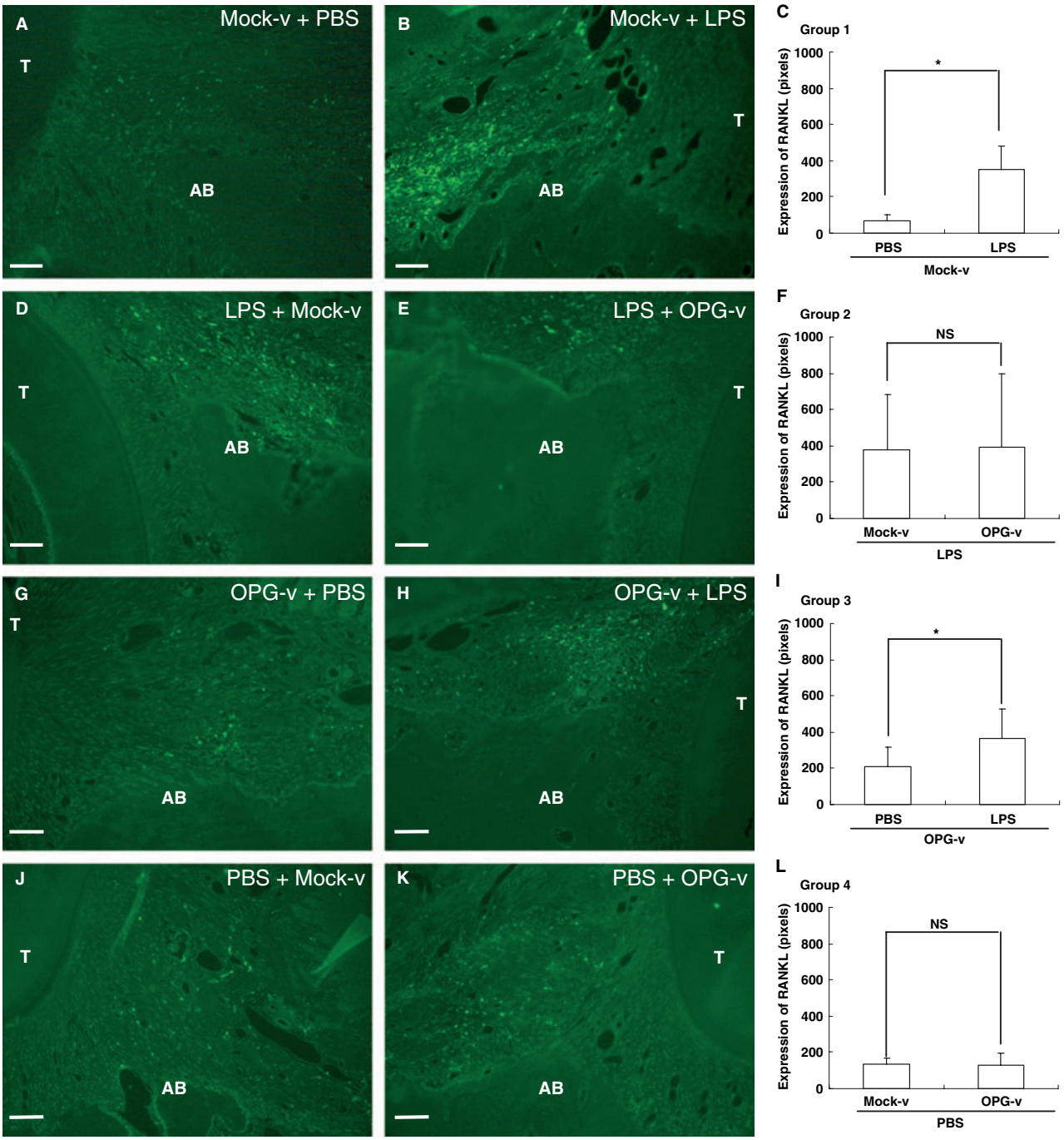


*Fig. 2.* Quantitative immunohistochemical analysis of RANKL. Immunofluorescence analysis of serial sections was performed with anti-RANKL antibodies. Representative photographs of the palatal sides of first molars on both sides are shown. AB, alveolar bone; T, tooth. Scale bar represents 100 μm. (A–C) Group 1: (A) left side (mock vector + PBS); (B) right side (mock vector + LPS). (D–F) Group 2: (D) left side (LPS + mock vector); (E) right side (LPS + OPG vector). (G–I) Group 3: (G) left side (OPG vector + PBS); (H) right side (OPG vector + LPS). (J–L) Group 4: (J) left side (PBS + mock vector); (K) right side (PBS + OPG vector). The immunofluorescence intensity was measured in samples from six photographs taken under the same exposure conditions for each animal. Within each group, the right-side averages of each animal were compared with the left-side averages using Student's paired *t* test. $^*P < 0.05$; NS, not significant; $n = 4$. (C,F,I,L) The expression of RANKL in the periodontium.

vector system, OPG expression was induced locally in periodontal tissue, especially in fibroblastic cells of the periodontium ($P < 0.01$; Fig. 3J–L). Mock vector did not induce OPG expression in periodontal tissue (Fig. 3A–C). There was no significant difference in OPG expression in periodontal tissue between the side with LPS

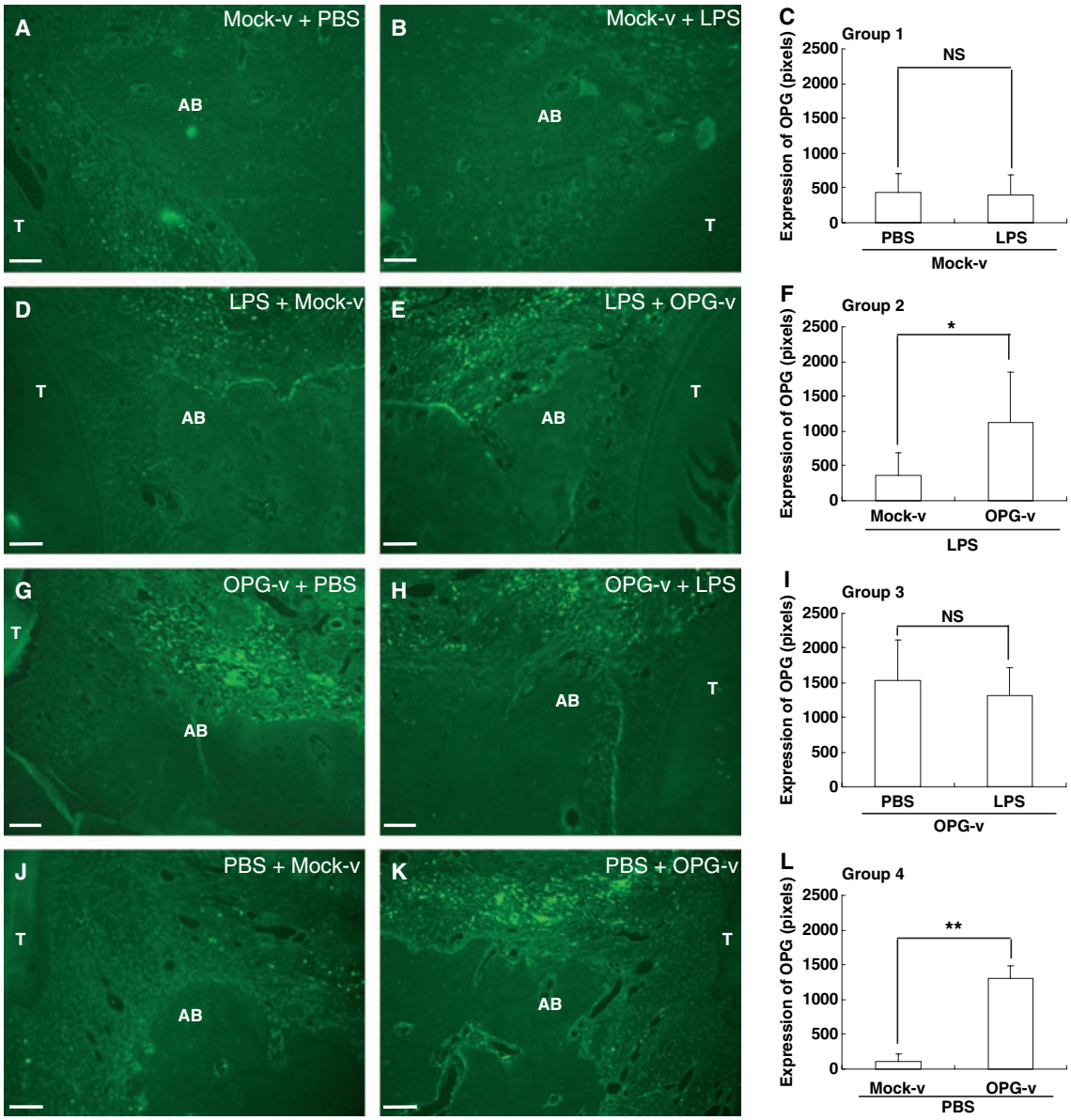


*Fig. 3.* Quantitative immunohistochemical analysis of OPG. Immunofluorescence analysis of serial sections was performed with anti-OPG antibodies. Representative photographs of the palatal sides of first molars on both sides are shown. AB, alveolar bone; T, tooth. Scale bar represents 100 μm. (A–C) Group 1: (A) left side (mock vector + PBS); (B) right side (mock vector + LPS). (D–F) Group 2: (D) left side (LPS + mock vector); (E) right side (LPS + OPG vector). (G–I) Group 3: (G) left side (OPG vector + PBS); (H) right side (OPG vector + LPS). (J–L) Group 4: (J) left side (PBS + mock vector); (K) right side (PBS + OPG vector). The immunofluorescence intensity was measured in samples from six photographs taken under the same exposure conditions for each animal. Within each group, the right-side averages of each animal were compared with the left-side averages using Student's paired *t* test. $*P < 0.05$; $**P < 0.01$; NS, not significant; $n = 4$. (C,F,I,L) The expression of OPG in the periodontium.

injection and the side without LPS injection (Fig. 3G–I).

### Local OPG gene transfer significantly decreased bone resorption

As shown in Fig. 4, repetitive LPS injection induced significant alveolar bone resorption compared with the control sides (473.3 ± 38.7 vs. 588.3 ± 27.9 μm, respectively; $P < 0.01$; Fig. 4A). Local OPG gene transfer significantly inhibited the bone resorption (522.9 ± 16.7 μm) induced by LPS injection (Fig. 4B).

### Local OPG gene transfer reduced osteoclastogenesis in periodontal tissue

The number of osteoclasts was increased by LPS injection, from 4 ± 2 to 15 ± 2 cells ($P < 0.01$; Fig. 5E), and local OPG gene transfer inhibited

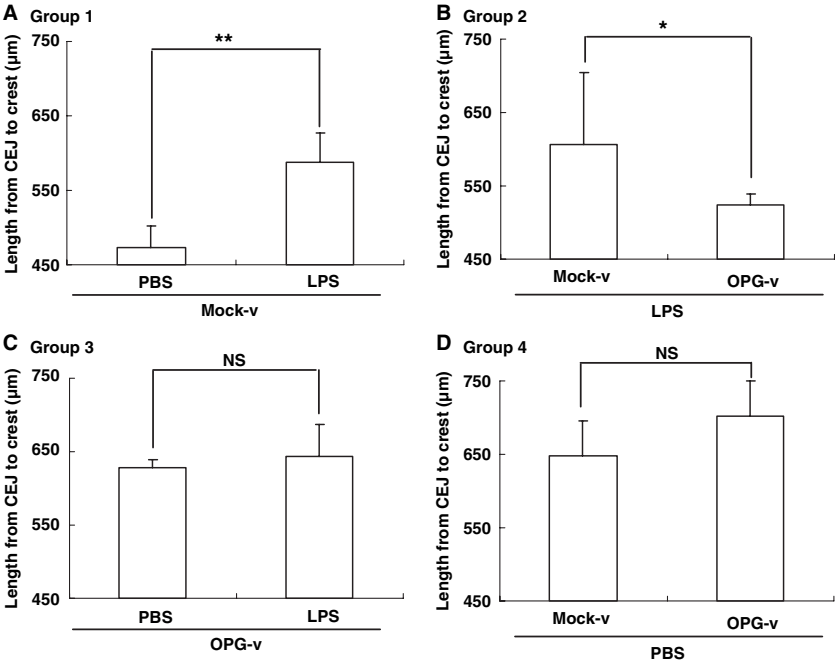


*Fig. 4.* Comparison of alveolar bone resorption. Alveolar bone resorption was evaluated by measuring the distance from the cemento-enamel junction (CEJ) to the alveolar ridge using haematoxylin and eosin staining. The values are expressed as the means ± SD. Student's paired *t* test was performed separately within each group for comparison of bone loss between the left and right sides. $*P < 0.05$; $**P < 0.01$; NS, not significant; $n = 4$. (A) Group 1: left (mock vector + PBS), right (mock vector + LPS). (B) Group 2: left (LPS + mock vector), right (LPS + OPG vector). (C) Group 3: left (OPG vector + PBS), right (OPG vector + LPS). (D) Group 4: left (PBS + mock vector), right (PBS + OPG vector).

the osteoclast induction stimulated by LPS injection (from 15 ± 2 to 7 ± 1 cells; $P < 0.05$; Fig. 5F). When OPG gene transfer was performed on both sides (group 3), there was no significant difference in the number of osteoclasts between the side with and the side without LPS injection (6 ± 2 vs. 3 ± 1 cells, respectively; $P > 0.05$; Fig. 5G).

## Discussion

Human periodontitis is associated with irreversible loss of alveolar bone. There are two approaches to controlling the loss of alveolar bone. One is enhancement of alveolar bone formation and the other is inhibition of alveolar bone resorption. Numerous reports have described the pharmacological control of alveolar bone resorption induced in periodontitis; some have focused on the regulation of osteoclasts (3,16,17). However, because these drugs are rapidly flushed by circulating blood, daily systemic administration or daily injection is needed. Since the advent of gene therapy in dentistry, significant progress has been made in treating periodontal disease and reconstructing the dentoalveolar apparatus (18,19). However, gene therapy methods have not yet been developed for controlling periodontal disease because of the multifactorial origin and complex genetic predisposition of the disease and the potential risks associated with gene therapy (19). Here, we showed that local OPG gene transfer inhibited LPS-induced alveolar bone resorption.

Osteoprotegerin is a potent inhibitor of osteoclast differentiation and activation (6,20), and OPG gene transfer or OPG administration have been confirmed to be feasible and effective therapeutic candidates for treating or preventing bone loss in osteoclast-dependent skeletal disorders such as osteolysis and arthritis (20–22). However, the effects of OPG gene transfer on bone loss in periodontitis have rarely been reported. The inhibition of RANKL function with a decoy receptor OPG diminished alveolar bone destruction and reduced the number of periodontal osteoclasts after microbial challenge (15,23), but the injection of OPG protein did not completely block alveolar bone destruction. Given that the administered protein is easily flushed by circulating blood, protein administration to periodontal lesions has limitations, including the exceeding cost of obtaining the highly purified protein, the need for highly concentrated injections and difficulty in maintaining an effective concentration at the injection site over the long term (24). In the present study, we used local OPG gene transfer instead of OPG protein administration to show, for the first time, that local OPG gene transfer successfully inhibited bone resorption in this animal periodontitis model.

Local gene transfer has two advantages (25). First, it can maintain a locally effective concentration and prolonged protein expression, regardless of blood circulation. Second, protein expression occurs at the local site, so systemic effects can be avoided. Although numerous viral and non-viral methods of gene transfer have been developed and improved (26,27), viral gene transfer vectors such as adenoviral vector have been proven to have problems, especially regarding immunogenicity and cytotoxicity (28). Non-viral vectors are less toxic and less immunogenic but provide less efficient gene transfer. Therefore, a haemagglutinating virus of Japan (HVJ)–liposome method, combining viral and non-viral features, was chosen for OPG gene transfer in the present study (13). We were able to induce local OPG expression in the periodontal tissue without any systemic effects. One transfection can produce sustained protein expression for about 4 days (10). Furthermore, no local inflammation was observed at the sites of gene transfer with the HVJ–liposome method, which is consistent with other reports (10,13).

Lipopolysaccharide, which is exclusively produced by gram-negative bacteria, is an important factor in the pathogenesis of periodontal disease

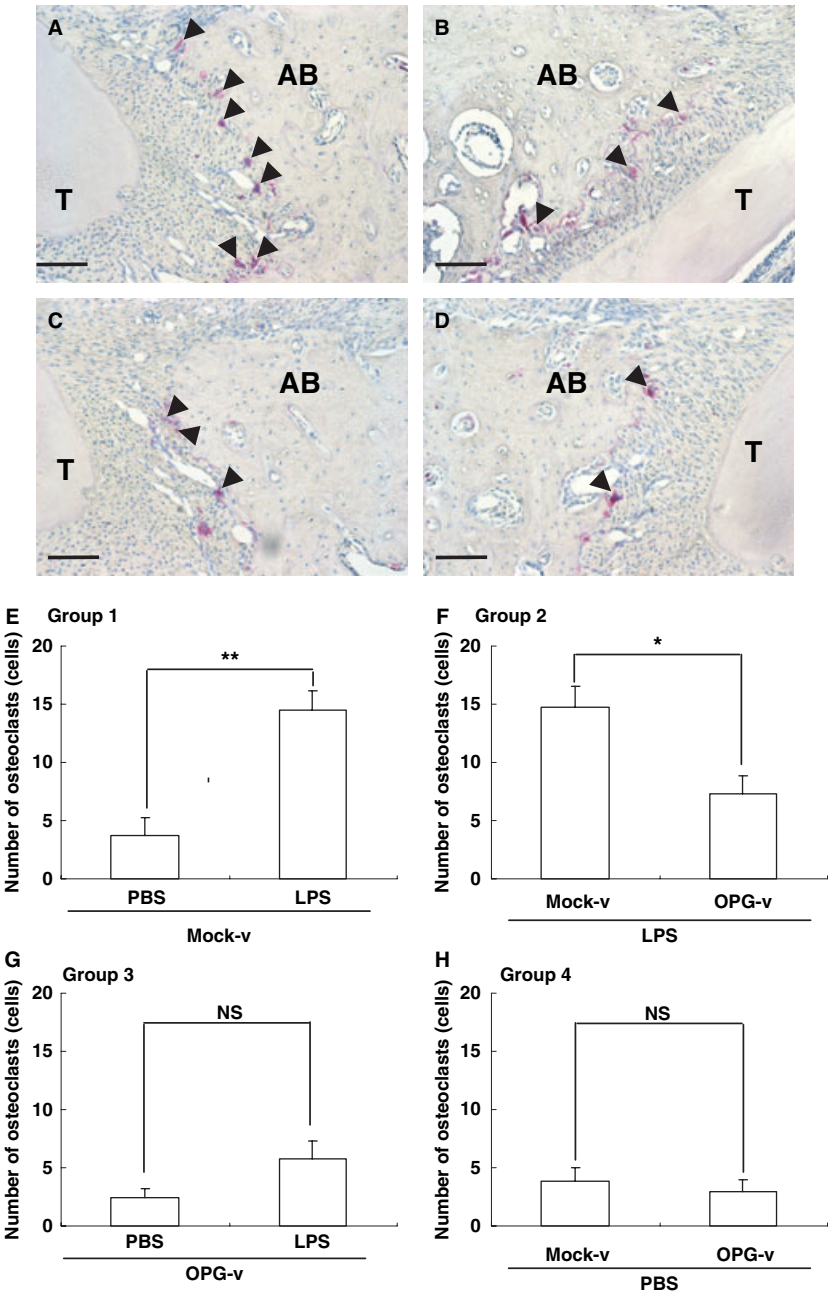


*Fig. 5.* Osteoclastogenesis in the periodontium. TRAP-positive multinucleated cells were observed around the teeth on the sides of: (A) LPS + mock vector; (B) LPS + OPG vector; (C) PBS + mock vector; and (D) PBS + OPG vector. The periodontal tissues of the mesiopalatal roots of the upper first molars were examined in serial cross-sections of the molars. The TRAP-positive multinucleated cells that formed resorption lacunae on the alveolar bone surface adjacent to the mesiopalatal roots of the upper first molars were counted. AB, alveolar bone; T, tooth. Scale bar represents 100 μm. (E–H) Comparison of the number of osteoclasts appearing in the periodontium. The numbers of osteoclasts were counted from six photographs of each animal. Student's paired *t* test was performed separately within each group for comparison of the average number of osteoclasts in each animal between the left and right side. $*P < 0.05$; $**P < 0.01$; NS, not significant; $n = 4$.

(29). Intragingival LPS injection induces a model of periodontal disease characterized by rapid bone loss with biochemical features similar to that of naturally occurring adult periodontitis (11,12,14). In the present study, repetitive local LPS injection induced bone resorption at the injection site through direct enhancement of RANKL expression in periodontal tissue. Local OPG gene transfer to the periodontal tissue was performed with an HVJ envelope-vector delivery system containing pcDNA-mOPG, resulting in OPG protein production in the periodontal tissue and significant inhibition of osteoclastogenesis. To diminish individual differences among the animals, different combinations of LPS injection or OPG gene transfer, as well as control injections, were simultaneously performed on both the right and left sides of the animal. Furthermore, Student's paired *t* test was used for statistical comparison between the right and left side within the same section so as to compensate the angulation differences of serial sections to the occlusal plane during section cutting. Lipopolysaccharide injection and OPG gene transfer did not affect body weight or bone mineral density.

It has been demonstrated that LPS stimulates the expression of RANKL; however, disparate findings have been reported for the effect of LPS on OPG expression. In one report, human periodontal cells stimulated with LPS inhibited osteoclastogenesis by producing more OPG than RANKL, via the induction of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α; 30). In another report, after treatment with LPS, increased RANKL expression was observed, whereas the level of OPG expression remained the same (31). In the present study, local OPG expression in periodontal tissue was facilitated by OPG gene transfer but was not affected by LPS injection, which was consistent with the latter report. Local OPG gene transfer significantly reduced the number of osteoclasts at the periodontium with LPS injection. Since RANKL expression in the periodontal tissue was not affected by OPG gene transfer, the effect of RANKL on osteoclastogenesis might be neutralized. Thus, OPG gene transfer almost completely inhibited the induction of osteoclastogenesis. These results proved the high efficiency of the transfection performed in this study. There was a slight increase in

the number of osteoclasts on the sides receiving LPS injections plus OPG gene transfer compared with the control sides, possibly owing to LPS induction of other cytokines such as IL-1β and TNF-α, which may directly stimulate osteoclastogenesis independent of the RANKL–RANK–OPG regulatory axis (29,31,32). Enforced OPG expression almost completely diminished the alveolar bone resorption induced by LPS injection. Our results are consistent with those of previous studies that used OPG gene transfer to control bone resorption in other skeletal disorders (20,22).

The induction of osteoclastogenesis in periodontal disease appears to be regulated primarily through RANKL signalling in periodontal tissue cells such as fibroblasts, osteoblasts and activated T cells (4,15). In this study, we demonstrated that OPG gene transfer to periodontal tissue inhibited RANKL-related osteoclastogenesis induced by LPS injection and inhibited experimental alveolar bone resorption without eliciting any systemic effects. Our results suggest that OPG gene transfer to periodontal tissue may be useful for the prevention of bone resorption in patients with periodontal diseases.

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## References

1. Cardaropoli D, Re S, Corrente G, Abundo R. Intrusion of migrated incisors with infrabony defects in adult periodontal patients. *Am J Orthod Dentofacial Orthop* 2001;**120:**671–675.
2. Frank CA, Long M. Periodontal concerns associated with the orthodontic treatment of impacted teeth. *Am J Orthod Dentofacial Orthop* 2002;**121:**639–649.
3. Bezerra MM, de Lima V, Alencar VB *et al.* Selective cyclooxygenase-2 inhibition prevents alveolar bone loss in experimental periodontitis in rats. *J Periodontol* 2000;**71:**1009–1014.
4. Crotti T, Smith MD, Hirsch R *et al.* Receptor activator NFκB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. *J Periodont Res* 2003;**38:**380–387.
5. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003;**423:**337–342.
6. Simonet WS, Lacey DL, Dunstan CR *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997;**89:**309–319.
7. Udagawa N, Takahashi N, Jimi E *et al.* Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor. *Bone* 1999;**25:**517–523.
8. Yasuda H, Shima N, Nakagawa N *et al.* A novel molecular mechanism modulating osteoclast differentiation and function. *Bone* 1999;**25:**109–113.
9. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. *J Dent Res* 2001;**80:**887–891.
10. Kanzaki H, Chiba M, Takahashi I, Haruyama N, Nishimura M, Mitani H. Local OPG gene transfer to periodontal tissue inhibits orthodontic tooth movement. *J Dent Res* 2004;**83:**920–925.
11. Miyauchi M, Sato S, Kitagawa S *et al.* Cytokine expression in rat molar gingival periodontal tissues after topical application of lipopolysaccharide. *Histochem Cell Biol* 2001;**116:**57–62.
12. Ramamurthy NS, Xu JW, Bird J *et al.* Inhibition of alveolar bone loss by matrix metalloproteinase inhibitors in experimental periodontal disease. *J Periodont Res* 2002;**37:**1–7.
13. Kaneda Y, Nakajima T, Nishikawa T *et al.* Hemagglutinating virus of Japan (HVJ) envelope vectors as a versatile gene delivery system. *Mol Ther* 2002;**6:**219–226.
14. Kawai T, Eisen-Iev R, Seki M, Eastcott JW, Wilson ME, Taubman MA. Requirement of B7 costimulation for Th1-mediated inflammatory bone resorption in experimental periodontal disease. *J Immunol* 2000;**164:**2102–2109.
15. Teng YT, Nguyen H, Gao X *et al.* Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J Clin Invest* 2000;**106:**R59–R67.
16. Di Paola R, Mazzon E, Rotondo F *et al.* Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. *Eur J Pharmacol* 2005;**516:**151–157.
17. Lohinai Z, Mabley JG, Feher E, Marton A, Komjati K, Szabo C. Role of the activation of the nuclear enzyme poly(ADP-ribose) polymerase in the pathogenesis of periodontitis. *J Dent Res* 2003;**82:**987–992.
18. Jin QM, Anusaksathien O, Webb SA, Printz MA, Giannobile WV. Engineering of tooth-supporting structures by delivery of PDGF gene therapy vectors. *Mol Ther* 2004;**9:**519–526.
19. Karthikeyan BV, Pradeep AR. Gene therapy in periodontics: a review and future implications. *J Contemp Dent Pract* 2006;**7:**83–91.
20. Doran PM, Turner RT, Chen D *et al.* Native osteoprotegerin gene transfer inhibits the development of murine osteolytic disease induced by tumor xenografts. *Exp Hematol* 2004;**32:**351–359.
21. Lubberts E, van den Bersselaar L, Oppers-Walgreen B *et al.* IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-κB ligand/osteoprotegerin balance. *J Immunol* 2003;**170:**2655–2662.
22. Yang SY, Mayton L, Wu B, Goater JJ, Schwarz EM, Wooley PH. Adeno-associated virus-mediated osteoprotegerin gene transfer protects against particulate polyethylene-induced osteolysis in a murine model. *Arthritis Rheum* 2002;**46:**2514–2523.
23. Valverde P, Kawai T, Taubman MA. Selective blockade of voltage-gated potassium channels reduces inflammatory bone resorption in experimental periodontal disease. *J Bone Miner Res* 2004;**19:**155–164.
24. Jin QM, Anusaksathien O, Webb SA, Rutherford RB, Giannobile WV. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *J Periodontol* 2003;**74:**202–213.
25. Blesing CH, Kerr DJ. Intra-hepatic arterial drug delivery. *J Drug Target* 1996;**3:**341–347.
26. Marshall E. Gene therapy's growing pains. *Science* 1995;**269:**1052–1055.

27. Ledley FD. Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum Gene Ther* 1995;**6:**1129–1144.
28. Raper SE, Chirmule N, Lee FS *et al*. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003;**80:**148–158.
29. Daly CG, Seymour GJ, Kieser JB. Bacterial endotoxin: a role in chronic inflammatory periodontal disease. *J Oral Pathol* 1980;**9:**1–15.
30. Wada N, Maeda H, Yoshimine Y, Akamine A. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-κB ligand in periodontal ligament fibroblasts through the induction of interleukin-1β and tumor necrosis factor-α. *Bone* 2004;**35:**629–635.
31. Tiranathanagul S, Yongchaitrakul T, Pattamapun K, Pavasant P. *Actinobacillus actinomycetemcomitans* lipopolysaccharide activates matrix metalloproteinase-2 and increases receptor activator of nuclear factor-κB ligand expression in human periodontal ligament cells. *J Periodontol* 2004;**75:**1647–1654.
32. Wang PL, Ohura K. *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Crit Rev Oral Biol Med* 2002;**13:**132–142.