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Expression of receptor activator of nuclear factor kappa B ligand relates to inflammatory bone resorption, with or without occlusal trauma, in rats

Y. Yoshinaga, T. Ukai, Y. Abe, Y. Hara

Department of Periodontology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

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Background and Objective: Receptor activator of nuclear factor kappa B ligand (RANKL) is an important factor in osteoclast differentiation, activation and survival; however, its involvement in inflammatory bone resorption, with or without occlusal trauma, is unclear. The purpose of the present study was to investigate the distribution of RANKL-expressing cells in rat periodontium during lipopolysaccharide-induced inflammation with or without occlusal trauma.

Material and Methods: Lipopolysaccharide was injected into rat gingiva of the lower left first molar to induce inflammation. In addition, the occlusal surface of the upper left first molar of rat was raised by placing a gold inlay to induce occlusal trauma in the lower left first molars. The distribution of RANKL-expressing cells was immunohistochemically observed.

Results: In the inflammatory model, many osteoclasts were observed at the apical inter-radicular septum on day 5 and they were reduced by day 10. On the other hand, in the inflammatory model with occlusal trauma, many osteoclasts were still observed on day 10. RANKL expression was similar to the changes in osteoclast number. The expression of RANKL increased in endothelial cells, inflammatory cells and periodontal ligament cells.

Conclusion: These findings clearly demonstrated that RANKL expression on endothelial cells, inflammatory cells and periodontal ligament cells is involved in inflammatory bone resorption and the expression is enhanced by traumatic occlusion. These results suggest that RANKL expression on these cells is closely involved in the increase of osteoclasts induced by occlusal trauma.

Yoshitaka Hara, Department of Periodontology, Unit of Translational Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan
Tel: +81 95 8497683
Fax: +81 95 8497684
e-mail: harasen@net.nagasaki-u.ac.jp

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Osteoclastogenesis is mainly controlled by two cytokines, receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony-stimulating factor (1). RANKL is a member of the tumor necrosis factor superfamily that activates osteoclast differentiation, stimulates osteoclast activation and increases osteoclast survival (2–5). RANK, the receptor of RANKL, is present on osteoclast precursors and mature osteoclasts. In

normal bone homeostasis, RANK on the surface of osteoclast precursors is activated by cell-to-cell contact with RANKL on the surface of osteoblasts (6). Previous studies have shown that RANKL is produced by osteoblasts, periodontal ligament cells, T cells, B cells and endothelial cells, and that it supports osteoclastogenesis *in vitro* (1,7–10). In inflammatory bone resorption, bacteria or lipopolysaccharide (a major constituent of gram-negative bacteria) were shown to induce RANKL expression on T cells and the formation of osteoclast-like cells via an osteoblast-independent pathway (11). Lipopolysaccharide is implicated as a potent stimulator of inflammatory bone loss (12,13).

Periodontal tissue destruction, including bone resorption, is caused by dental plaque in periodontal disease. Generally, the bone resorption is thought to progress in the presence of occlusal trauma. Occlusal trauma alone, however, induces reversible bone resorption but does not cause periodontitis (14–20) Some studies have reported that RANKL mRNA expression is higher in the gingiva in advanced periodontitis than in gingivitis or healthy gingiva (21,22), and they suggested that up-regulation of RANKL expression might be associated with activation of bone destruction in periodontitis. However, little is known about the involvement of RANKL in bone resorption when inflammation co-exists with or without occlusal trauma. Furthermore, the cell types producing RANKL have not been clearly associated with inflammatory bone resorption, with or without occlusal trauma. The purpose of the present study was to investigate the distribution of RANKL-expressing cells in rat periodontium during lipopolysaccharide-induced inflammation, with or without occlusal trauma.

Material and methods

Experimental protocol

Thirty-five male 8-wk-old Lewis rats weighing 250–270 g were used in the present study. The rats were purchased from Charles River Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions in the Biomedical Research Center, Center for Frontier Life Sciences (Nagasaki University, Nagasaki, Japan). Animal care and experimental procedures were carried out in accordance with the Guidelines for Animal Experimentation of Nagasaki University and with approval from the Institutional Animal Care and Use Committee.

For inducing inflammation in the furcation area, 125 μg of *Escherichia coli* lipopolysaccharide (from *E. coli* O111:B4; Sigma, St Louis, MO, USA) in 5 μL of phosphate-buffered saline was injected every 24 h into the buccal and lingual gingiva of the lower left first molar under ether anesthesia. The rats were killed 24 h after the 5th or 10th injection (lipopolysaccharide group). For excessive occlusal loading to the first molar, the occlusal surfaces of the upper left first molar were raised 1.0 mm by placing a gold inlay bonded with resin cements (Super-Bond C & B; Sun Medical, Shiga, Japan) under general anesthesia with sodium pentobarbital at 0.5 mL/kg of body weight. After the resin stiffened, a lipopolysaccharide injection was administered in the same way as described for the inflammatory model. The rats were killed 24 h after the 5th or 10th injection (lipopolysaccharide + trauma group). Another animal model with only excessive occlusal loading was made and the rats were killed after 5 and 10 d (trauma group). The remaining five rats were used as untreated controls (Fig. 1A).

Preparation of tissues

The left mandible of each rat was removed immediately after death and was fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C for 10 h, decalcified with 10% EDTA for 2 wk and then embedded in paraffin using the AMeX method (acetone, methyl benzoate and xylene) (23). Buccolingually oriented serial sections (4 μm thickness) at the level of the central roots of the lower left first molar were obtained.

Histochemical and immunohistological staining

Five groups of serial sections, each containing 10 subsections, were obtained from each specimen. The first subsections from each group of serial sections were stained with hematoxylin and eosin for histopathological observation. In order to identify the osteoclasts, the second subsections from each group were stained with tartrate-resistant acid phosphatase (24). Sections were counterstained with hematoxylin. Osteoclasts, identified as tartrate-resistant acid phosphatase-positive multinucleated cells, at the apical inter-radicular septum of the 250-μm square region were counted (Fig. 1B).

To detect the production of RANKL, the third subsections were used for the immunohistological staining of RANKL-expressing cells. Sections were deparaffinized and treated with 0.1% trypsin for 30 min at 37°C. Endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol for 30 min, followed by incubation in normal rabbit serum for 30 min at room temperature. These sections were then immersed in goat polyclonal anti-RANKL (N-19) (sc-7628; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. Sections were then incubated with biotinylated rabbit antigoat polyclonal immunoglobulin (Dako, Glostrup, Denmark) for 30 min. These sections were finally incubated with peroxidase-conjugated streptavidin (Dako) for 30 min, then incubated with diaminobenzidine tetraoxide solution and counterstained with hematoxylin. The RANKL-positive cells in a 250-μm square region in the soft tissue of the apical inter-radicular septum were counted (Fig. 1B) and classified into four types: endothelial cells, which constitute a vascular lumen; periodontal ligament cells, which have a spindle cytoplasm and an oval nucleus; osteoblastic cells, which have contact with a bone surface; and inflammatory cells including polymorphonuclear cells, lymphocytes and macrophages.

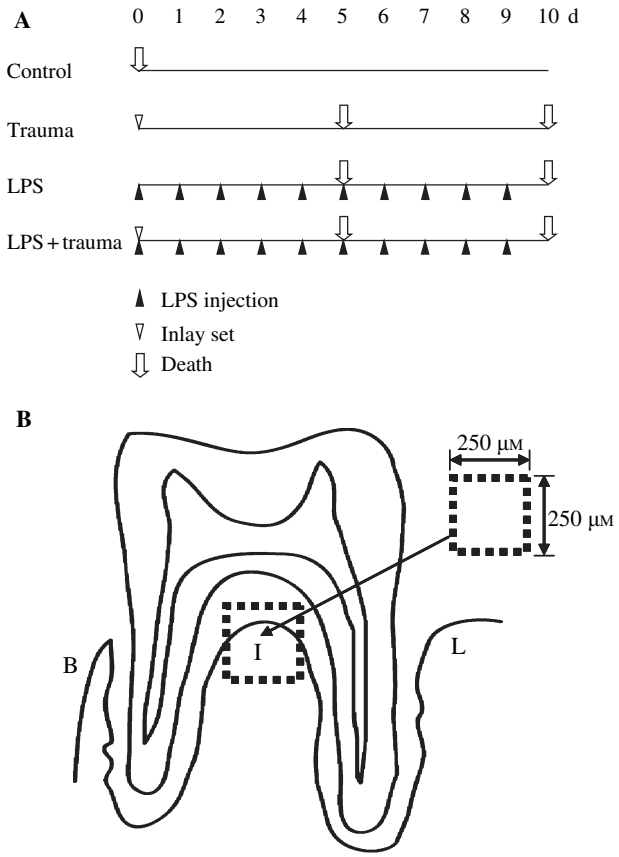

Fig. 1. (A) Experimental schedule. Black arrowhead: 125 μg of lipopolysaccharide in 5 μL of phosphate-buffered saline was injected into the buccal and lingual gingiva of the lower left first molar. White arrowhead: the occlusal surfaces of the upper left first molar were raised by 1.0 mm by setting a gold inlay bonded with resin cements. White arrow: the rats were killed 24 h after the last injection in the lipopolysaccharide and lipopolysaccharide + trauma groups. In the trauma group, the rats were killed 5 or 10 d after setting a gold inlay. (B) Schema of counting area of tartrate-resistant acid phosphatase-positive or receptor activator of nuclear factor kappa B ligand (RANKL)-positive cells. The observed area is indicated by a square in the furcation area. B, buccal side; I, inter-radicular septum; L, lingual side; LPS, lipopolysaccharide.

Statistics

Data were analyzed for statistical differences using one-factor analysis of variance with Fisher's protected least significant difference *post hoc* test. A probability level of < 0.05 was considered to be significant. The values are expressed as mean ± standard deviation.

Results

Histopathological and histometrical findings

In the control group, the periodontal ligament space showed a constant width and few osteoclasts existed at the apical inter-radicular septum (Fig. 2A).

In the trauma group, there were many osteoclasts with bone loss in the furcation area with few inflammatory cells on day 5. Many dilated blood vessels were observed around the bone crest (Fig. 2B). On day 10, osteoclasts disappeared and there were few dilated blood vessels (Fig. 2C).

In the lipopolysaccharide group, large numbers of infiltrated inflammatory cells with bone loss in the furcation area were observed on day 5. Many osteoclasts appeared at the inner side of the alveolar bone (Fig. 3A). On day 10, however, few osteoclasts existed on the bone surface, although bone loss and many inflammatory cells were observed (Fig. 3C).

In the lipopolysaccharide + trauma group, many inflammatory cells had infiltrated with bone loss in the furcation area on day 5. Many osteoclasts were observed on the bone surface, similar to the results obtained for the lipopolysaccharide group on day 5. Many dilated blood vessels were observed around the alveolar bone (Fig. 3B). On day 10, many osteoclasts were widely observed with bone resorption in the alveolar bone at the apical inter-radicular septum, in contrast to the lipopolysaccharide group of day 10. Many dilated blood vessels and inflammatory cells were observed in the furcation area (Fig. 3D).

The number of tartrate-resistant acid phosphatase-positive cells, and their corresponding standard deviation values, are presented in Table 1. Osteoclasts at the apical inter-radicular septum were present at higher numbers in the trauma, lipopolysaccharide and lipopolysaccharide + trauma groups on day 5 as compared with the control group. By day 10, the number of osteoclasts in the trauma and lipopolysaccharide groups had decreased to a level similar to that observed in the control group. There were significantly more osteoclasts in the lipopolysaccharide + trauma group on day 10 than in the other groups.

Immunohistological findings of RANKL

There were significantly more RANKL-positive cells in the lipopolysaccharide and lipopolysaccharide + trauma groups on day 5 than in the control and trauma groups. There were significantly more RANKL-positive cells in the lipopolysaccharide + trauma group than in the trauma and lipopolysaccharide groups on day 10 (Table 2).

On day 5, the number of RANKL-positive cells in endothelial cells and periodontal ligament cells was higher in the trauma, lipopolysaccharide, and lipopolysaccharide + trauma groups than in the control group. Further-

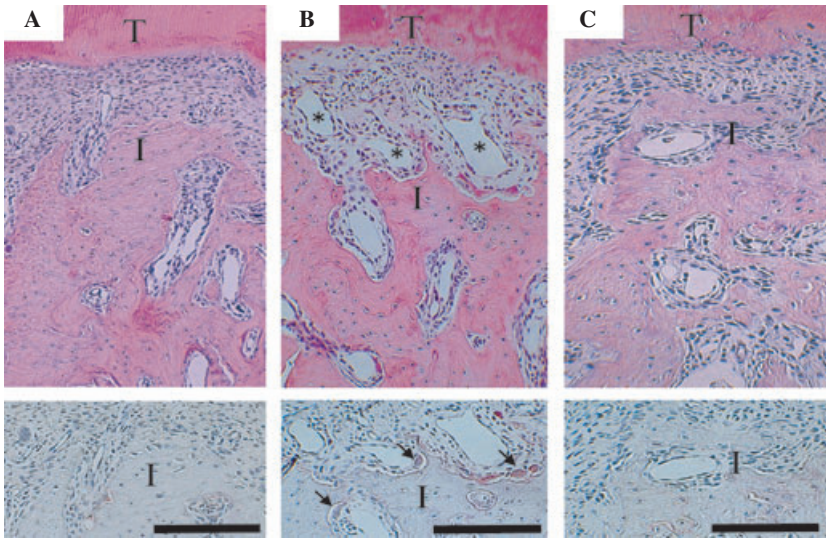

Fig. 2. Bucco-lingual sections of the inter-radicular zone, stained with hematoxylin and eosin or tartrate-resistant acid phosphatase, in the control and trauma groups. (A) Control group. The periodontal ligament space showed a constant width and few osteoclasts existed at the apical inter-radicular septum. (B) Day 5 in the trauma group. There were few inflammatory cells, but many osteoclasts were observed with bone loss in the furcation area. Many dilated blood vessels (*) were observed around the crest. (C) Day 10 in the trauma group. Osteoclasts and bone loss disappeared and there were few dilated blood vessels. The bottom panel shows tartrate-resistant acid phosphatase staining appearance. I, inter-radicular septum; T, teeth. Scale bar, 200 μm.

more, the number of RANKL-positive cells in inflammatory cells was also higher in the lipopolysaccharide and lipopolysaccharide + trauma groups than in the control group. On the other hand, the number of RANKL-positive osteoblastic cells in the trauma, lipopolysaccharide and lipopolysaccharide + trauma groups decreased compared with those in the control group (Table 3). On day 10, the number of RANKL-positive cells in endothelial cells, inflammatory cells and periodontal ligament cells was higher in the lipopolysaccharide + trauma group than in the other groups. In contrast, the number of RANKL-positive osteoblastic cells in the trauma, lipopolysaccharide and lipopolysaccharide + trauma groups had decreased compared with that of the control group (Table 4).

Discussion

Some studies have reported that traumatic occlusion is a codestructive factor in inflammatory bone resorption in the periodontium (15,18–20). In the present study, we established an inflammatory model by repeated injections of lipopolysaccharide, and excessive occlusal force was applied using an inlay. These *in vivo* models were useful for investigating the relationship between inflammation and occlusal trauma in bone resorption. On day 5, many osteoclasts existed at the apical inter-radicular septum in the experimental groups. On day 10, few osteoclasts were observed in the trauma and lipopolysaccharide groups; on the other hand, many osteoclasts were observed in the lipopolysaccharide + trauma group. This result demonstrates that inflammatory bone resorption with traumatic occlusion is more destructive than inflammation alone. This is in agreement with previous reports (15,20). Glickman *et al.* reported that occlusal trauma affected the pathway of gingival inflammation (14). In the present study it was found that occlusal trauma may influence the spread of lipopolysaccharide in the furcation area. As a result, the numbers of osteoclasts were significantly different between the lipopolysaccharide group and the lipopolysaccharide + trauma group on day 10, although there was no difference in the number of inflammatory cells (data not shown).

RANKL is an important factor in osteoclast differentiation, activation and survival (2–5). It has been shown *in vivo* that the number of osteoclasts increases with an increase in RANKL expression in peri-apical lesions (25). The change in RANKL expression corresponded to the changes in osteoclast number in the present study. These findings clearly demonstrate that the expression of RANKL affects osteoclastogenesis in the inflammatory bone resorption, with or without traumatic occlusion. Furthermore, highly expressed RANKL seemed to result in an elevated number of osteoclasts in the lipopolysaccharide + trauma group, in contrast to the lipopolysaccharide group, on day 10, suggesting that occlusal trauma enhances bone resorption by up-regulating RANKL expression.

In this study, RANKL expression in endothelial cells, periodontal ligament cells and inflammatory cells was up-regulated on day 5 in all experimental groups, except for the trauma group. Furthermore, on day 10, when inflammation co-existed with traumatic occlusion, the increase in RANKL-expressing cells was maintained, although the expression was decreased in both the trauma and lipopolysaccharide groups. These findings suggested that traumatic occlusion influences RANKL expression and inflammatory bone resorption in the present model. Traumatic occlusion may cause mechanical stress in components of the periodontium. Mechanical stress, in turn, induces the production of inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β (26,27). *In vitro* studies have shown that RANKL is expressed in endothelial cells, T cells and periodontal ligament cells in response to these inflammatory cytokines (7,28–30). Moreover, mechanical stress directly induces RANKL expression in periodontal ligament cells *in vitro* (27,31). Therefore, mechanical stress might induce RANKL expression via direct and indirect mechanisms in the present model. It was difficult to distinguish

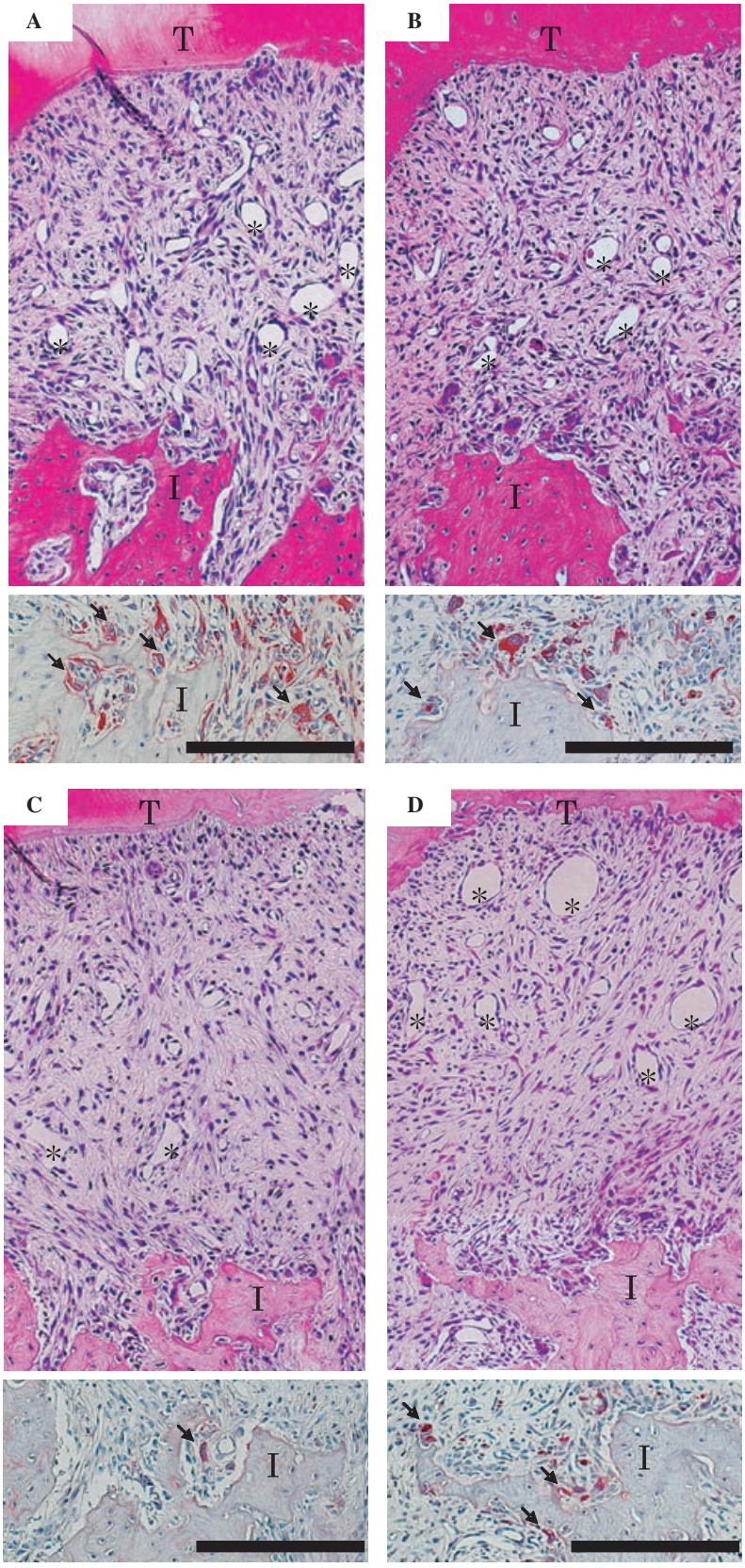

Fig. 3. Bucco-lingual sections of the inter-radicular zone stained with hematoxylin and eosin or tartrate-resistant acid phosphatase in the lipopolysaccharide and lipopolysaccharide + trauma groups. (A) Day 5 in the lipopolysaccharide group. Large numbers of infiltrated inflammatory cells with bone loss. Many osteoclasts (arrows) appeared on the inner side of the alveolar bone. (B) Day 5 in the lipopolysaccharide + trauma group. Many inflammatory cells had infiltrated in the furcation area with bone loss and many osteoclasts (arrows) were observed on the bone surface. Many dilated blood vessels (*) were observed. (C) Day 10 in the lipopolysaccharide group. Although bone loss was observed, few osteoclasts existed on the bone surface. (D) Day 10 in the lipopolysaccharide + trauma group. Bone resorption and many osteoclasts (arrows) were widely observed in the alveolar bone at the apical inter-radicular septum. Many dilated blood vessels (*) were observed in the furcation area. The bottom panel shows the tartrate-resistant acid phosphatase staining appearance. I, inter-radicular septum; T, teeth. Scale bar, 200 μm.

microscopically between macrophages and periodontal ligament cells in both the lipopolysaccharide and lipopolysaccharide + trauma groups. No report has clearly shown that macrophages produce RANKL *in vitro*. Although Crotti *et al.* reported, by immunohistochemistry, RANKL expression in macrophages, they could not be certain that these macrophages were the source of this RANKL because these macrophages may have had RANKL on their surface bound to RANK (21). But we assume that this would not affect the result, because no report has clearly shown that macrophages produce RANKL *in vitro*.

In this study, more dilated blood vessels were observed in both the trauma and lipopolysaccharide + trauma groups than in the lipopolysaccharide group, indicating that traumatic occlusion affected blood vessels. Vascular endothelial growth factor not only plays a crucial role in angiogenesis but also induces RANK expression in osteoclast precursors (32) and prolongs osteoclast survival (33). The production of vascular endothelial growth factor from endothelial cells is accelerated by shear stress (34). Traumatic occlusion has been reported to induce an increased blood flow in rats (35). These results suggest that endothelial cells are affected by shear stress under occlusal trauma and that vascular

Table 1. The number of tartrate-resistant acid phosphatase-positive multinucleated cells

	Control	Trauma	LPS	LPS + trauma
5 d	1.6 ± 1.3	4.1 ± 1.3	7.2 ± 0.3	6.5 ± 1.4
10 d	1.6 ± 1.3	1.5 ± 0.9	1.8 ± 0.8	3.4 ± 1.0

LPS, lipopolysaccharide.
Data represent mean ± SD.
**, $p < 0.01$, *, $p < 0.05$ represent the significant differences between each group. (Fisher's protected least significant difference *post hoc* test).

Table 2. The number of receptor activator of nuclear factor kappa B ligand-positive cells

	Control	Trauma	LPS	LPS + trauma
5 d	49.8 ± 9.6	46.2 ± 4.9	73.1 ± 4.5	71.4 ± 6.0
10 d	49.8 ± 9.6	42.0 ± 8.4	36.2 ± 9.3	58.7 ± 9.2

LPS, lipopolysaccharide.
Data represent mean ± SD.
**, $p < 0.01$, *, $p < 0.05$ represent the significant differences between each group. (Fisher's protected least significant difference *post hoc* test).

Table 3. The number of receptor activator of nuclear factor kappa B ligand-positive cells on day 5

	Control	Trauma	LPS	LPS + trauma
Endothelial cells	0.5 ± 0.6	8.7 ± 2.5	8.6 ± 1.8	10.1 ± 3.4
Inflammatory cells	0.0 ± 0.0	0.0 ± 0.0	6.9 ± 3.2	6.7 ± 5.1
PDL cells	12.3 ± 2.7	21.6 ± 3.9	35.1 ± 11.3	28.5 ± 2.1
Osteoblastic cells	36.9 ± 8.9	15.9 ± 5.2	23.0 ± 9.1	26.1 ± 7.2

LPS, lipopolysaccharide.
Data represent mean ± SD.
**, $p < 0.01$, *, $p < 0.05$ represent the significant differences between each group. (Fisher's protected least significant difference *post hoc* test).

endothelial growth factor production increases in the furcation area in the trauma and lipopolysaccharide + trauma groups. Therefore, vascular endothelial growth factor may have induced an increase of osteoclasts in both the trauma and the lipopolysaccharide + trauma groups. Thereafter, there were no findings of dilated blood vessels in the trauma group on day 10. The decrease of dilated blood vessels may be related to the reduction of osteoclasts in the trauma group on day 10.

Several studies have reported that RANKL expression on osteoblasts decreases in response to mechanical stress, but increases in response to lipopolysaccharide and inflammatory cytokines *in vitro* (36–41). In the present study, RANKL expression on the osteoblastic cells decreased under inflammatory condition and/or mechanical stress. This indicates that RANKL expression on osteoblastic cells was not affected in the present model. Thammasitboon *et al.* reported that lipopolysaccharide indirectly induced the apoptosis of osteoblasts through the induction of tumor necrosis factor-α released from macrophages (42). The total number of osteoblastic cells was decreased in the experimental models in the present study (data not shown). Lipopolysaccharide might indirectly induce the apoptosis of osteoblasts in this model.

Osteoprotegerin, which is the soluble decoy receptor for RANKL, plays a central role in the regulation of bone resorption by inhibiting osteoclastogenesis. The balance between RANKL and osteoprotegerin is important for regulating osteoclastogenesis (3,43). In addition, Tsuji *et al.* reported that the combination of lipopolysaccharide and mechanical stress reduced the expression of osteoprotegerin in periodontal ligament cells *in vitro* (44). Further studies should be carried out to investigate changes in osteoprotegerin expression in inflammatory bone resorption, with and without excessive occlusal force, as the present study was unable to do this.

Collectively, in the present study we showed that endothelial cells, inflammatory cells and periodontal ligament cells expressed RANKL in inflammatory bone resorption *in vivo*. The expression was enhanced by traumatic occlusion. These results clearly indicate that RANKL expression on these cells relates to osteoclastogenesis in inflammatory alveolar bone resorption, with or without occlusal trauma *in vivo*. Furthermore, our results suggest that RANKL expression on these cells is involved in the increase of the osteoclast population induced by occlusal trauma.

Table 4. The number of receptor activator of nuclear factor kappa B ligand-positive cells on day 10

	Control	Trauma	LPS	LPS + trauma
Endothelial cells	0.5 ± 0.6	1.4 ± 0.7	0.7 ± 0.6	2.7 ± 0.6
Inflammatory cells	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 1.5	5.2 ± 1.5
PDL cells	12.3 ± 2.7	14.7 ± 4.0	15.9 ± 7.1	30.9 ± 5.5
Osteoblastic cells	36.9 ± 8.9	25.9 ± 7.2	16.4 ± 3.8	19.9 ± 7.0

LPS, lipopolysaccharide.
Data represent the mean ± SD.
**, $p < 0.01$, *, $p < 0.05$ represent the significant differences between each group. (Fisher's protected least significant difference *post hoc* test).

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