Journal of Dental Sciences (2016) 11, 8–16

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

ORIGINAL ARTICLE

Increase in receptor activator of nuclear factor κB ligand/osteoprotegerin ratio in peri-implant gingiva exposed to *Porphyromonas gingivalis* lipopolysaccharide

Takahiro Shuto [a], Takanori Wachi [a], Yoshinori Shinohara [a], Hiroki Nikawa [b], Seicho Makihira [a]*

[a] *Section of Fixed Prosthodontics, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, Fukuoka, Japan*

[b] *Department of Oral Biology and Engineering, Integrated Health Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*

Received 12 June 2015; Final revision received 29 August 2015
Available online 6 January 2016

KEYWORDS
implant;
peri-implant mucositis;
peri-implantitis;
receptor activator of nuclear factor κB ligand/ osteoprotegerin

Abstract *Background/purpose:* The prevalence of peri-implant diseases, including peri-implant mucositis and peri-implantitis, is increasing. The aim of this study was to elucidate the pathological mechanisms of inflammation and alveolar bone resorption in peri-implant tissues. To do this, we fabricated inflamed gingiva around mini-implants in the palatine processes of rats using lipopolysaccharide derived from *Porphyromonas gingivalis* (*P.g*-LPS).
Materials and methods: Pure titanium mini-implants were implanted into the palatine processes of rats, and then intermittent injections of *P.g*-LPS were made into the gingival tissues surrounding the mini-implants. The expression patterns of tumor necrosis factor-α, interleukin-1β, chemokine (C–C motif) ligand 2, receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) in the tissues were examined using real-time reverse transcriptase polymerase chain reaction or enzyme-linked immunosorbent assays. Immunohistochemical analysis was also performed to compare the T and B cells expressing RANKL.
Results: P.g-LPS increased the expressions of tumor necrosis factor-α, interleukin-1β, chemokine (C–C motif) ligand 2, and RANKL in the gingival tissues surrounding the mini-implants. In contrast, the expression of OPG in the *P.g*-LPS samples was decreased. Consequently, the RANKL/OPG ratio was significantly increased. Moreover, cells stained positively for both anti-CD3 and anti-RANKL antibodies were only found in the samples treated with *P.g*-LPS.

* Corresponding author. Section of Fixed Prosthodontics, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan.
E-mail address: makihira@dent.kyushu-u.ac.jp (S. Makihira).

http://dx.doi.org/10.1016/j.jds.2015.10.005

Conclusion: These data revealed that *P.g*-LPS injections increased the RANKL/OPG ratio in the gingival tissues surrounding mini-implants in the rat model. In addition, the CD3-positive cells in the gingival tissues injected with *P.g*-LPS expressed RANKL. This suggests that the activated T cells capable of infiltrating gingival tissues affected by *P.g*-LPS may be one of the sources of RANKL and may also be involved in the disease progression from peri-implant mucositis to peri-implantitis.

Introduction

Dental implant therapy is currently viewed as a treatment modality with a high success rate. However, peri-implant mucositis and peri-implantitis caused by bacterial infections and excessive implant loading remain serious issues.[1–6] The emergence of peri-implantitis resulting in bone resorption has been observed and found problematic since the oral implant system, initially proposed by a Swedish group, was established. The number of reports on peri-implantitis has gradually increased. Although the general prevalence of peri-implant diseases is difficult to assess, it has been reported that 20% of patients undergoing implant treatment develop peri-implantitis,[7] and close to 80% of those patients present with symptoms of peri-implant mucositis.[8]

Peri-implant mucositis is an inflammation that is limited to the mucosa, while peri-implantitis affects the implant-supporting alveolar bone.[9] Peri-implant mucositis is thought to be a reversible condition, unlike peri-implantitis.[9] Because oral bacteria can colonize almost instantly after implantation, we focused on understanding peri-implant mucositis caused by bacterial infection for the purpose of finding preventive therapies. Although bacterial infections around implants are well known as the main causes of peri-implant mucositis and peri-implantitis,[3] the mechanisms of these diseases remain unclear.

Many microbiological studies of dental implants with healthy and diseased marginal peri-implant tissues in humans[10–12] and animals[13,14] have been published. These reports show that Gram-negative bacilli, such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, similar to those found in periodontal disease, can be detected in peri-implant pockets.[12–17] These microorganisms are strongly suspected of contributing to peri-implant diseases accompanied with inflammation and/or bone destruction.

Osteoclasts play an important role in the bone resorption associated with periodontitis, which is mediated by receptor activator of nuclear factor κB ligand (RANKL).[18] A decoy receptor of RANKL, osteoprotegerin (OPG) produced by osteoblasts, inhibits the RANKL/RANK signals and osteoclast formation.[19] Therefore, in this study, we assessed the RANKL/OPG expression ratio in the peri-implant tissues because it is crucial for the regulation of bone resorption.

Recently, interest in osteoimmunology, the study of interactions between immune cells and the skeletal system, has increased.[20] In fact, RANKL was already detected in the synovial fibroblasts and T cells in the inflamed joints of rheumatoid arthritis patients.[21] T cells expressing CD3 may also infiltrate the affected gingival tissue in peri-implantitis, as well as in periodontitis.[22] However, whether these activated T cells involved in peri-implantitis express RANKL or not remains unclear. CD20 antibody therapy for auto immune and inflammatory diseases has been tried over the past few years because of an increasing number of reports indicating that B cells are involved in those diseases.[23] Therefore, to determine whether lymphocytes expressing CD3 or CD20 in the tissues of peri-implant mucositis and peri-implantitis also express RANKL, we developed a rat model of peri-implant mucositis induced with lipopolysaccharide (LPS) derived from *P. gingivalis*, which has been previously reported to exist in the peri-implant pocket. The most often used method to induce alveolar bone resorption when creating a periodontitis model is ligature. However, an infection model using periodontal pathogens better mimics the clinical condition, allowing for more accurate evaluations of the disease. Thus, an LPS-induced model was used in the present study.

Materials and methods

Materials

Pure titanium mini-implants (diameter: 2 mm, length: 4 mm; Kondo Technology, Tokyo, Japan) were purchased and used in this study.[24] The mini-implants were washed with acetone and ethanol in an ultrasonic bath for 30 minutes and autoclaved before use. LPS derived from *P. gingivalis* (*P.g*-LPS) was purchased from InvivoGen (San Diego, CA, USA). The LPS was diluted with sterile endotoxin-free water and stored at −20°C until use.

Implantation of the mini-implant into a rat palatine process

Eight-week-old Wistar rats (Kyudo. Co., LTD, Fukuoka, Japan) were used for this animal study. The animal selection, management, anesthesia, and surgical and analysis procedures were approved by the Animal Care and Use Committee of Kyushu University (No. A25-138; Fukuoka, Japan). The *in vivo* experiments using the rat model were performed in accordance with the procedures allowed by the committee. In brief, the rats were carefully anesthetized with sevoflurane and pentobarbital. Prior to

beginning surgery, the implantation site was disinfected with 10% iodine. A mini-implant was then placed into the palatine process of the maxilla using micro drivers, and the recipient gingival site was tightly sutured. After the gingival tissues had healed, tridaily intermittent injections of *P.g*-LPS (100 μL of 1 mg/mL per injection) were made into the gingival tissues surrounding the mini-implant ($n = 6$ rats for each experimental endpoint). The same volume of phosphate-buffered saline (PBS) was injected instead of *P.g*-LPS into the control samples ($n = 6$ rats for each experimental endpoint). Each animal received only one implant. For real-time reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) analysis, the gingival tissues and alveolar bone from the samples were collected and divided into halves after 6 hours (6 h), 4 days (4 d), and 14 days (14 d). Each of the following analysis experiments were independently repeated using the two sample halves to ensure accuracy (i.e., $n = 6$ biological replicates and $n = 2$ technical replicates for each assay per group per endpoint).

Real-time quantitative RT-PCR analysis

Total ribonucleic acid (RNA) was extracted from the gingival tissues and alveolar bone from the *P.g*-LPS samples collected after 6 h, 4 d, and 14 d using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand complementary deoxyribonucleic acid (cDNA) was synthesized from the total RNA (100 ng) using ReverTra Ace (Toyobo, Osaka, Japan). The cDNA was then amplified with BIOTAQ DNA polymerase (Bioline, Randolph, MA, USA).[25] Real-time quantitative RT-PCR analyses for tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), chemokine (C—C motif) ligand 2 (CCL2), RANKL, OPG, and β-actin were performed using a Rotor-Gene 6000 (Qiagen, Tokyo, Japan). Beta-actin was chosen as an internal control to normalize the variability in amplification caused by slight differences in the total starting RNA concentrations. The sequences of primers and probes for TNF-α, IL-1β, CCL2, RANKL, and OPG used in the present study are listed in Table 1. The sequences of the primers and probe used for β-actin have been described previously.[26]

Table 1 Primers and probes used for real-time RT-PCR.

Target name	Primer and probe	Sequence(5'-3')
TNF-α	Primer F	CAGACCCTCACACTCAGATCATC
	Primer R	CGCTTGGTGGTTTGCTACGA
	Probe	6FAM-AAACTCGAGTGACAAGCCCGTAG CCCA-TAMRA
IL-1β	Primer F	GGCTTCGAGATGAACAACAAAAATG
	Primer R	GCTCATGGAGAATACCACTTGTTG
	Probe	6FAM-CTCGTGCTGTCTGACCCATGTG AGCTGA-TAMRA
CCL2	Primer F	TGTCCCAAAGAAGCTGTAGTATTTG
	Primer R	GACTGTAGTTTCTGATCTCACTTGG
	Probe	6FAM-CTCAAGAGAGAGATCTGTGCTGA CCCCA-TAMRA
RANKL	Primer F	TGCTCACCTCACCATCAATGC
	Primer R	GTTGCTTAACGTCATGTTAGAGATC
	Probe	6FAM-CCGACATCCCATCGGGTTCCCAT AAAGT-TAMRA
OPG	Primer F	CGGAAACAGAGAAGCAACTCAAA
	Primer R	CTCAGCCAATTCGGTATAATCTTGG
	Probe	6FAM-AATGCCTCTTCGCACAGGGT GACATCTA-TAMRA

Note. Forward primers (Primer F). reverse primers (primer R) and probes are listed.
CCL2 = chemokine (C—C motif) ligand 2; IL-1β = interleukin-1β; OPG = osteoprotegerin; RANKL = receptor activator of nuclear factor κB ligand; RT-PCR = reverse transcriptase polymerase chain reaction; TNF-α = tumor necrosis factor-α.

ELISA

The samples collected from the gingival tissues and alveolar bone surrounding the mini-implants were placed in RIPA buffer (Thermo Fisher Scientific, Yokohama, Japan). Tissue debris was removed by centrifugation at 12,000*g* for 5 minutes at 4°C. Then, the supernatants were transferred to new microtubes. Next, the contents of CCL2, RANKL, and OPG in the samples were assessed using commercially available ELISA kits (DuoSet ELISA Development System, R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. The protein content was estimated using a Coomassie Plus Assay Kit (Thermo Fisher Scientific), and the optical density was measured at 595 nm in a microplate reader (Infinite M200, Tecan, Kanagawa, Japan) according to the manufacturer's instructions. The concentrations of CCL2, RANKL, and OPG in the supernatant of the tissue homogenates were normalized to the total protein level.

Immunofluorescent analysis

Gingival tissue samples isolated from the areas surrounding the mini-implants were fixed with 4% paraformaldehyde and then rinsed three times with PBS. After dehydration in graded alcohol concentrations, the samples were embedded in paraffin, serial sectioned (10-μm thick), and mounted on glass slides. The deparaffinized sections were incubated in a heated antigen retrieval solution (HistoVT One, Nacalai Tesque, Kyoto, Japan) for 20 minutes at 90°C and then blocked with EzBlock BSA (ATTO, Tokyo, Japan) for 30 minutes. Next, the sections were incubated with primary antibodies against CD3 and CD20, which are surface molecules specifically expressed on T and B cells, soluble RANKL (sRANKL), and toll-like receptor 4 (TLR-4) overnight at 4°C and then with fluorescent-conjugated secondary anti-mouse immunoglobulin (Ig)M (*Alexa Fluor555, Invitrogen*, Carlsbad, CA, USA) to CD3, anti-mouse IgG (DyLight549, KPL, Baltimore, MD, USA) to CD20 and to TLR-4, and anti-rabbit IgG (CF488A, Biotium, Hayward, CA, USA) to sRANKL for 30 minutes at room temperature. After rinsing three times with PBS, the tissue sections were counterstained with 4′,6-diamidino-2-phenylindole (Dojindo, Kumamoto, Japan) for 10 minutes. Control IgG (Mouse IgG, whole molecule; Rockland, Philadelphia, PA,

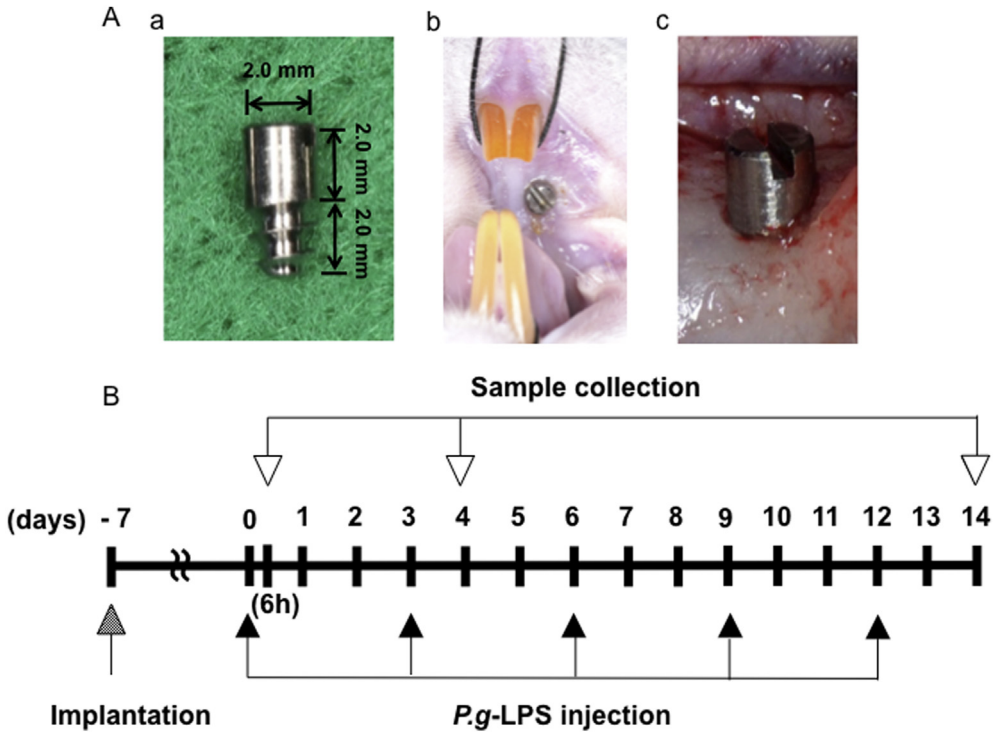

Figure 1 (A, a) Photograph of the mini-implant used in the present study. (b, c) One mini-implant was placed into the palatine process of the maxilla of each rat. (B) Summary of the timeline for implantation, injection of lipopolysaccharide derived from *Porphyromonas gingivalis* (*P.g*-LPS), and collection of samples is shown. Implantation was performed 7 days before the injection of *P.g*-LPS (−7 days and 0 days, respectively). Samples were collected at 6 hours (6h), 4 days (4d), and 14 days (14d) after the first injection of *P.g*-LPS.

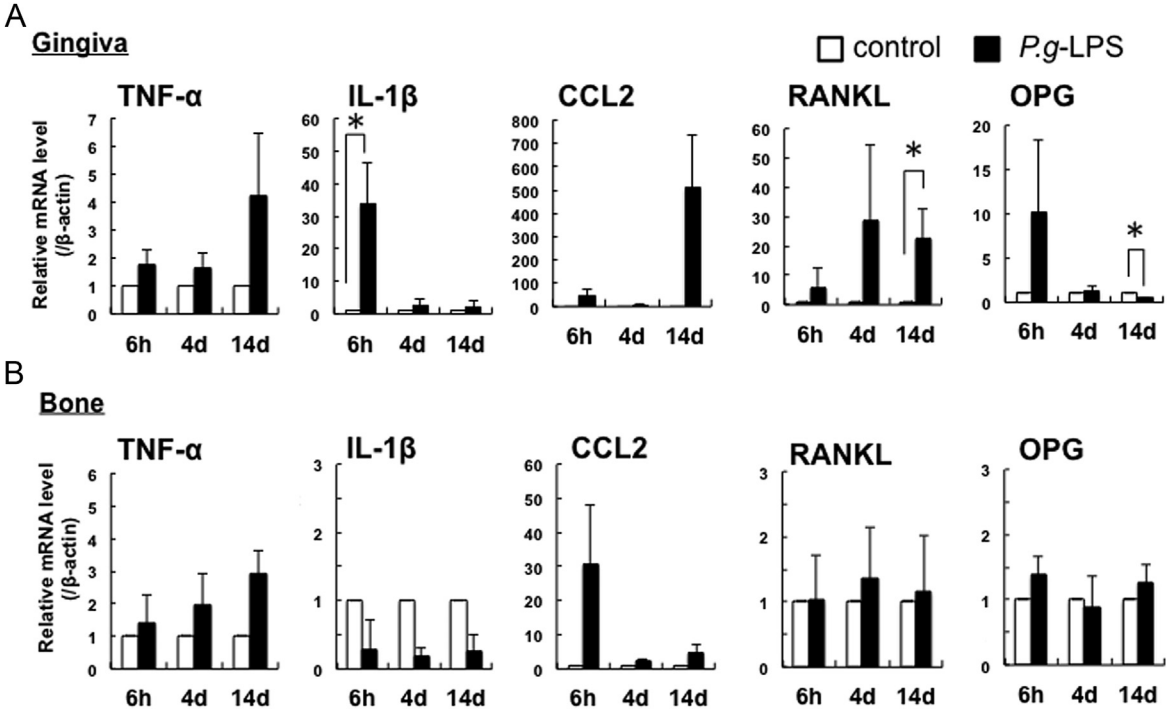

Figure 2 Effects of lipopolysaccharide derived from *Porphyromonas gingivalis* (*P.g*-LPS) on the mRNA expression levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), chemokine (C−C motif) ligand 2 (CCL2), receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) in the (A) gingival tissue and (B) bone surrounding the implants were examined by real-time quantitative reverse transcriptase polymerase chain reaction. The mRNA levels were normalized to the expression levels of β-actin mRNA. One site per animal was prepared, and six animals were assessed per group per endpoint. Two independent experiments, each using the samples in triplicate, were performed. Data are presented as mean $\pm$ standard deviation. *P $<$ 0.05.

USA), rabbit IgG, or IgM (Purified Mouse IgM, κIsotype Ctrl; BioLegend, San Diego, CA, USA) was used as a control immunoglobulin for each antibody. Fluorescence images were acquired with a BZ-9000 (Keyence, Osaka, Japan).

Data analysis

The significance of differences between the mean group values was assessed using Student *t* tests. SPSS software (version 20.0; IBM, Tokyo, Japan) was used for this statistical analysis.

Results

Effect of P.g-LPS on the expression of cytokines related to inflammation and bone resorption in the gingival tissue and bone surrounding the mini-implants

To examine the effect of *P.g*-LPS on the cytokine expression associated with inflammation and bone resorption, we developed a model in which mini-implants were placed into a rat maxilla (Figure 1A). The timeline of *P.g*-LPS injection and sample collection are shown in Figure 1B. The messenger RNA (mRNA) expression profiles of TNF-α, IL-1β, CCL2, RANKL, and OPG in the tissues surrounding the mini-implants were investigated after the gingival tissues were injected with *P.g*-LPS. At 6 h after *P.g*-LPS injection, the mRNA expression of the inflammation-inducing factors TNF-α and IL-1β and the osteoclast-inducing factors CCL2 and RANKL in the gingival tissues injected with *P.g*-LPS was increased compared with those in the gingival tissues without *P.g*-LPS, but only the increase in IL-1β was significant (*t* test, $P < 0.05$; Figure 2A). After 14 d, the mRNA expression of RANKL was significantly increased with *P.g*-LPS (*t* test, $P < 0.05$; Figure 2A). By contrast, OPG was significantly decreased at 14 d after the *P.g*-LPS-injection (*t* test, $P < 0.05$). In bone, although all of the cytokines examined, except IL-1β, were increased, and CCL2 was highly increased, by *P.g*-LPS stimulation at 6 h, these increases were not significant (*t* test, $P > 0.05$; Figure 2B).

The ELISA data show that CCL2 protein levels in the gingival tissues exposed to *P.g*-LPS were significantly higher than those in the control samples not injected with *P.g*-LPS 4 d after *P.g*-LPS injection (*t* test, $P < 0.05$; Figure 3A). By contrast, the concentration of OPG in the gingival tissue was significantly decreased in the *P.g*-LPS-injected tissues compared with the control samples at 14 d after *P.g*-LPS

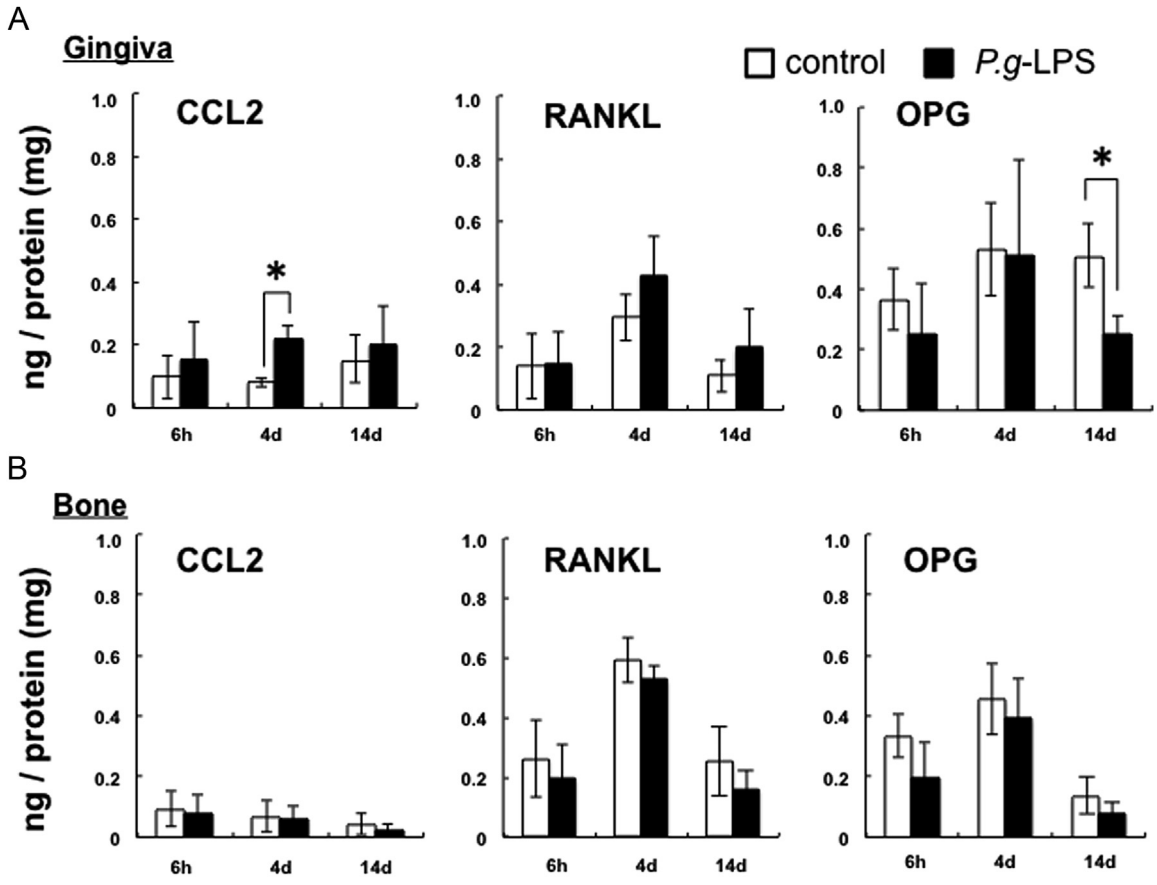

Figure 3 Effects of lipopolysaccharide derived from *Porphyromonas gingivalis* (*P.g*-LPS) on the protein levels of chemokine (C—C motif) ligand 2 (CCL2), receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) in the (A) gingival tissue and (B) bone surrounding the implants were measured with enzyme-linked immunosorbent assay and normalized to the total protein content. One site per animal was prepared, and six animals were assessed per group per endpoint. Two independent experiments, each using the samples in triplicate, were performed. Data are presented as mean ± standard deviation. *$P < 0.05$.

injection (*t* test, $P < 0.05$; Figure 3A). In the bone, no significant effect of *P.g*-LPS injection was found on the CCL2, RANKL, or OPG content (Figure 3B). Consequently, the RANKL/OPG ratio in the gingival tissues injected with *P.g*-LPS was greater than that in the control tissues. Especially at 14 d after stimulation, the increase in the RANKL/OPG ratio was significant (*t* test, $P < 0.05$; Figure 4A). By contrast, no significant increase in the RANKL/OPG ratio was found in the bone samples (*t* test, $P > 0.05$; Figure 4B).

Expression patterns of RANKL in the gingival tissues injected with P.g-LPS

To determine the expression patterns of RANKL in the gingival tissue inflamed by *P.g*-LPS injection, dual-color fluorescence microscopy was performed after staining for RANKL and lymphocyte-specific CD markers. Images of the RANKL- and CD marker-positive cells were digitally merged, and the double-positive cells are displayed as yellow staining (Figure 5). At 14 d, CD3-positive cells were observed in the subepithelial connective tissue lesions of tissue samples both in the presence and absence of *P.g*-LPS, but the number of these cells in the *P.g*-LPS samples was higher than that in the control samples. In addition, RANKL-expressing cells were observed in both samples. In the gingival tissues injected with *P.g*-LPS, yellow-stained RANKL and CD3 double-positive cells were observed, but they were not found in the *P.g*-LPS-free sections (Figure 5A). Many CD20- or RANKL-positive cells were observed in the *P.g*-LPS samples, but only a small number of CD20- and RANKL-double-positive cells were found (Figure 5B). When the control mouse, rabbit IgG, or mouse IgM was used, no specific staining was seen (data not shown).

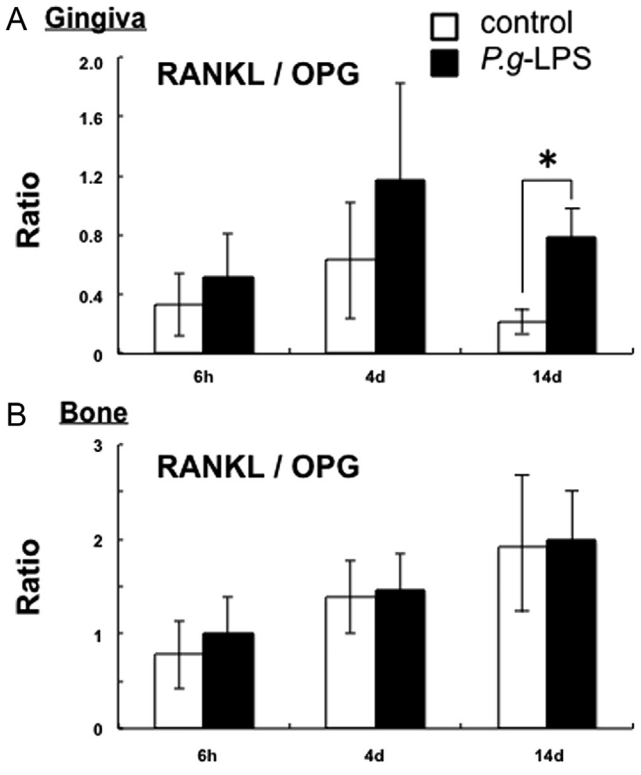

Figure 4 Receptor activator of nuclear factor κB ligand/osteoprotegerin (RANKL/OPG) ratio in the (A) gingival tissue and (B) bone surrounding the implants was calculated. Two independent experiments, each using the samples in triplicate, were performed. Data are presented as mean ± standard deviation. *$P < 0.05$.

Discussion

We used LPS derived from *P. gingivalis* in this study to induce inflammation around a mini-implant placed in the palatine process of rats. Several previous reports induced experimental periodontitis using LPS at concentrations of <10 mg/mL.[27–29] Based on a preliminary experiment, we decided to use 1 mg/mL *P.g*-LPS for the present study. *P. gingivalis* is one of the bacteria most strongly involved in periodontitis,[30] and *P.g*-LPS is an agonist for TLR-2 and TLR-4.[31] The presence of TLR-2 and TLR-4, which is an LPS receptor, was found in the mucosa, particularly in the area near the epithelium. Further, the expression of TLR-4 was increased by *P.g*-LPS.[31,32] Similarly, we found that TLR-4 was expressed by the cells in the gingival tissues surrounding the mini-implants injected with *P.g*-LPS (Figure S1). Therefore, it is possible that the rat model in the present study was responding to the *P.g*-LPS. However, the infection mechanisms in peri-implant diseases may be different from those found in periodontitis because the peri-implant and periodontal tissue structures, including the organization of collagen fibers and blood vessels, are different.

Peri-implant mucositis is a disease often managed initially with dental treatment and does not always advance to peri-implantitis.[9] However, to inhibit the cellular reactions of peri-implant tissues to endotoxin containing LPS, preventive treatment beginning as early as possible is necessary because the endotoxin can induce bone resorption-related molecules, as well as periodontal disease.

During inflammation, proinflammatory cytokines, such as TNF-α and IL-1β are secreted in abundance by immune cells including macrophages and result in the activation of T cells expressing RANKL and osteoclasts. TNF-α and IL-1β are involved in both peri-implant mucositis and peri-implantitis.[33] In the present study, the gingival tissues highly expressed those genes in response to *P.g*-LPS stimulation, but bone did not. This suggests that the rat model created here shows partial simulation of inflamed gingiva without inflammation of the bone, which may correspond to the early or acute stage of the disease. CCL2 and RANKL are also known as monocyte chemoattractant factor and osteoclastic differentiation-inducing factor, respectively.[34–36] *P.g*-LPS accelerated the expressions of CCL2 and RANKL in the gingival tissues. However, the RANKL expressions were not significantly changed during the experimental period. The expression of OPG, which is a decoy receptor for RANKL,[37] was significantly decreased by *P.g*-LPS at 14 d. Previous reports have suggested that the RANKL/OPG or OPG/RANKL ratio is associated with bone resorption in rheumatoid arthritis, osteoarthritis, hip-joint disease, and periodontitis.[38,39] In the present study, the

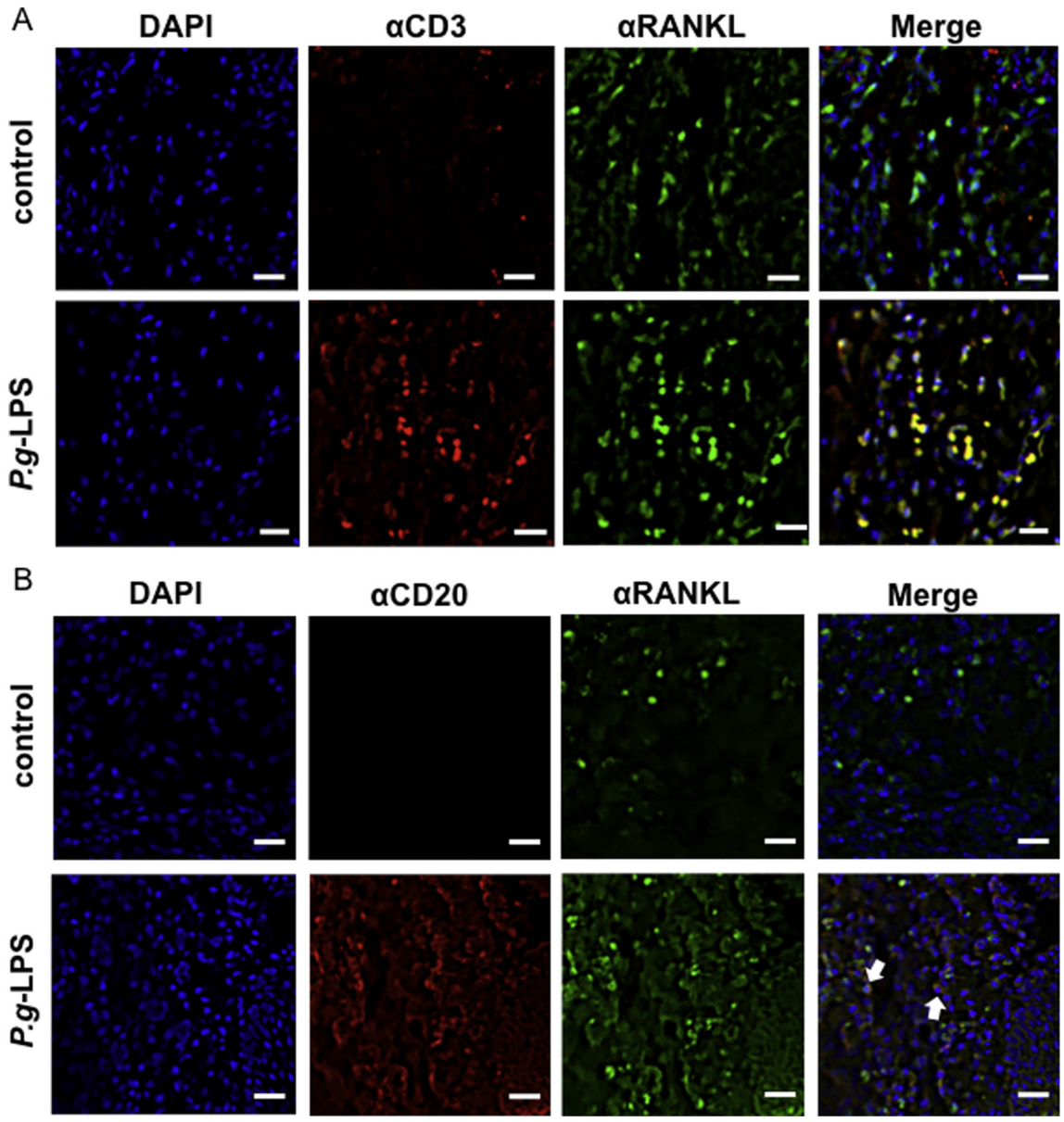

Figure 5 Fluorescence microscopic observation of the gingival tissue surrounding the implants. CD3, CD20, and receptor activator of nuclear factor κB ligand (RANKL) expression were visualized by staining with specific antibodies. RANKL- (green), CD3- (red), or CD20-positive (red) cells were identified in the gingival tissues of both control and lipopolysaccharide derived from *Porphyromonas gingivalis* (*P.g*-LPS) samples. Cells double-positive for (A) CD3 and RANKL and (B) for CD20 and RANKL are shown in yellow. 4′,6-diamidino-2-phenylindole (DAPI: blue) was used for staining nuclei. Scale bars: 20 μm.

RANKL/OPG ratio in the gingival tissues was significantly increased by *P.g*-LPS at 14 d (*t* test, $P < 0.05$; Figure 4A), but the ratio in bone did not increase (*t* test, $P > 0.05$; Figure 4B), which may lead to bone resorption. This also suggests that the rat model with *P.g*-LPS injections was only partly stimulated at 14 d with inflamed gingiva, which may correspond to the late or chronic stage of disease. A longer duration of *P.g*-LPS injections would be likely to stimulate a more severe inflammation accompanied by alveolar bone resorption. Moreover, while *P.g*-LPS was used to investigate the inflammation in the tissues surrounding the mini-implants in the present study, the cellular reactions in the tissues exposed to LPS from other bacteria may be different. Therefore, to determine the mechanisms of peri-implant inflammation and alveolar bone resorption, further research is needed.

In addition to periodic radiographic examinations,[40] the early diagnosis by biochemical analysis may be required to reduce radiological exposure. RANKL and matrix metalloproteinase have been detected in peri-implant crevicular fluid.[41–44] Therefore, animal models, such as the one used in the present study, are very important and useful for developing biochemical tests related to the pathological conditions of peri-implant mucositis and peri-implantitis around short- and long-term implants of peri-implant crevicular fluid and/or tissues. Although CCL2, RANKL,

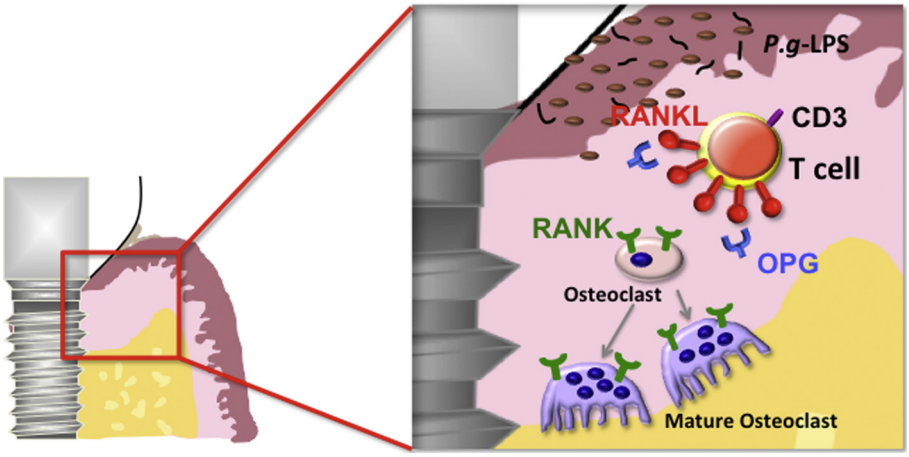

Figure 6 Schematic illustration of the hypothetical mechanisms of peri-implant disease caused by lipopolysaccharide derived from *Porphyromonas gingivalis* (*P.g*-LPS) infection. *P.g*-LPS binds to toll-like receptors expressed on the epithelium and fibroblast cells, causing these cells to secrete cytokines such as tumor necrosis factor-α and interleukin-1β. T cells infiltrate into the site of inflammation and are activated by these cytokines. The receptor activator of nuclear factor κB ligand produced by the activated T cells may promote osteoclastogenesis and bone resorption.

and OPG molecules were detected in the crevicular fluid in the present study, their contents varied widely (unpublished data). Further studies using larger animal models or a modified method of collection for crevicular fluid will help to clarify this.

The results of the present study revealed that *P.g*-LPS injection increased RANKL and decreased OPG, which led to an increased RANKL/OPG ratio in the gingival tissues surrounding mini-implants in the rat model. Additionally, CD3-positive cells expressing membrane-type RANKL molecules were found more frequently in the gingival tissues surrounding the mini-implants injected with *P.g*-LPS than in the control samples not injected with *P.g*-LPS. Therefore, the activated T cells in peri-implant mucositis may be one of the sources of membrane-type RANKL, and may be involved in the bone resorption associated with the progression from peri-implant mucositis to peri-implantitis (Figure 6).

Conflicts of interest

All of the authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgments

This study was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (grant No. 21390516, 24390437, and 15K11162).

We appreciate the technical support from the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jds.2015.10.005.

References

1. Bullon P, Fioroni M, Goteri G, Rubini C, Battino M. Immunohistochemical analysis of soft tissues in implants with healthy and peri-implantitis condition, and aggressive periodontitis. *Clin Oral Implants Res* 2004;15:553—9.
2. Kozlovsky A, Tal H, Laufer BZ, et al. Impact of implant overloading on the peri-implant bone in inflamed and non-inflamed peri-implant mucosa. *Clin Oral Implants Res* 2007;18:601—10.
3. Leonhardt A, Renvert S, Dahlen G. Microbial findings at failing implants. *Clin Oral Implants Res* 1999;10:339—45.
4. Miyata T, Kobayashi Y, Araki H, Ohto T, Shin K. The influence of controlled occlusal overload on peri-implant tissue. Part 3: a histologic study in monkeys. *Int J Oral Maxillofac Implants* 2000;15:425—31.
5. Rutar A, Lang NP, Buser D, Burgin W, Mombelli A. Retrospective assessment of clinical and microbiological factors affecting periimplant tissue conditions. *Clin Oral Implants Res* 2001;12:189—95.
6. Quirynen M, Peeters W, Naert I, Coucke W, van Steenberghe D. Peri-implant health around screw-shaped c.p. titanium machined implants in partially edentulous patients with or without ongoing periodontitis. *Clin Oral Implants Res* 2001;12:589—94.
7. Mombelli A, Müller N, Cionca N. The epidemiology of peri-implantitis. *Clin Oral Implants Res* 2012;23:67—76.
8. Roos-Jansåker AM, Lindahl C, Renvert H, Renvert S. Nine- to fourteen-year follow-up of implant treatment. Part II: presence of peri-implant lesions. *J Clin Periodontol* 2006;33:290—5.

9. Zitzmann NU, Berglundh T. Definition and prevalence of peri-implant diseases. *J Clin Periodontol* 2008;35:286—91.
10. Hultin M, Gustafsson A, Hallström H, Johansson LA, Ekfeldt A, Klinge B. Microbiological findings and host response in patients with peri-implantitis. *Clin Oral Implants Res* 2002;13: 349—58.
11. Lee KH, Tanner AC, Maiden MF, Weber HP. Pre- and post-implantation microbiota of the tongue, teeth, and newly placed implants. *J Clin Periodontol* 1999;26:822—32.
12. Renvert S, Roos-Jansaker AM, Lindahl C, Renvert H, Rutger Persson G. Infection at titanium implants with or without a clinical diagnosis of inflammation. *Clin Oral Implants Res* 2007; 18:509—16.
13. Shibli JA, Martins MC, Lotufo RF, Marcantonio Jr E. Microbiologic and radiographic analysis of ligature-induced peri-implantitis with different dental implant surfaces. *Int J Oral Maxillofac Implants* 2003;18:383—90.
14. Tillmanns HW, Hermann JS, Tiffee JC, Burgess AV, Meffert RM. Evaluation of three different dental implants in ligature-induced peri-implantitis in the beagle dog. Part II. Histology and microbiology. *Int J Oral Maxillofac Implants* 1998;13: 59—68.
15. Mombelli A, van Oosten MA, Schurch Jr E, Land NP. The microbiota associated with successful or failing osseointegrated titanium implants. *Oral Microbiol Immunol* 1987;2: 145—51.
16. Persson GR, Salvi GE, Heitz-Mayfield LJ, Lang NP. Antimicrobial therapy using a local drug delivery system (arestin) in the treatment of peri-implantitis. I: microbiological outcomes. *Clin Oral Implants Res* 2006;17:386—93.
17. Salcetti JM, Moriarty JD, Cooper LF, et al. The clinical, microbial, and host response characteristics of the failing implant. *Int J Oral Maxillofac Implants* 1997;12:32—42.
18. Crotti T, Smith MD, Hirsch R, et al. Receptor activator NF κB ligand (RANKL) and osteoprotegerin (OPG) protein expression in perioditis. *J Periodont Res* 2003;38:380—7.
19. Takayanagi H, Iizuka H, Juji T, et al. Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 2000;43:259—69.
20. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* 2007;7:292—304.
21. Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFκ ligand and tumor necrosis factor-α in bone destruction in rheumatoid arthritis. *Bone* 2002;30:340—6.
22. Gualini F, Berglundh T. Immunohistochemical characteristics of inflammatory lesions at implants. *J Clin Periodontol* 2003;30: 14—8.
23. Martin F, Chan AC. B cell immunobiology in disease:evolving concepts from the clinic. *Ann Rev Immunol* 2006;24:467—96.
24. Atsuta I, Yamaza T, Yoshinari M, et al. Changes in the distribution of laminin-5 during peri-implant epithelium formation after immediate titanium implantation in rats. *Biomaterials* 2005;26:1751—60.
25. Makihira S, Mine Y, Nikawa H, et al. Titanium ion induces necrosis and sensitivity to lipopolysaccharide in gingival epithelial-like cells. *Toxicol In Vitro* 2010;24:1905—10.
26. Mine Y, Makihira S, Nikawa H, et al. Impact of ions on osteoblast-, osteoclast- and gingival epithelial-like cells. *J Prosthodont Res* 2010;54:1—6.
27. Do MJ, Kim K, Lee H, et al. Development of animal experimental periodontitis models. *J Periodontal Implant Sci* 2013; 43:147—52.
28. Dumitrescu AL, Abd-El-Aleem S, Morales-Aza B, Donaldson LF. A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation. *J Clin Periodontol* 2004;31:596—603.
29. Rogers JE, Li F, Coatney DD, et al. *Actinobacillus actinetemcomitans* lipopolysaccharide-mediated experimental bone loss model for aggressive periodontitis. *J Periodontol* 2007;78: 550—8.
30. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol* 2000;2002(28):12—55.
31. Kinane DF, Shiba H, Stathopoulou PG, et al. Gingival epithelial cells heterozygous for toll-like receptor 4 polymorphisms asp299gly and thr399ile are hypo-responsive to porphyromonas gingivalis. *Genes Immun* 2006;7:190—200.
32. Mori Y, Yoshimura A, Ukai T, Lien E, Espevik T, Hara Y. Immunohistochemical localization of toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. *Oral Microbiol Immunol* 2003;18:54—8.
33. Petkovic AB, Matic SM, Stamatovic NV, et al. Proinflammatory cytokines (IL-1beta and TNF-alpha) and chemokines (IL-8 and MIP-1alpha) as markers of peri-implant tissue condition. *Int J Oral Maxillofac Surg* 2010;39:478—85.
34. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature* 2003;423:337—42.
35. Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor-B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. *J Biol Chem* 2005;280:16163—9.
36. Yasuda H, Shima N, Nakagawa N, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* 1998;95:3597—602.
37. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999;20:345—57.
38. Giner M, Rios MA, Montoya MA, Vazquez MA, Naji L, Perez-Cano R. RANKL/OPG in primary cultures of osteoblasts from post-menopausal women. Differences between osteoporotic hip fractures and osteoarthritis. *J Steroid Biochem Mol Biol* 2009;113:46—51.
39. Kajiya M, Giro G, Taubman MA, Han X, Mayer MP, Kawai T. Role of periodontal pathogenic bacteria in RANKL-mediated bone destruction in periodontal disease. *J Oral Microbiol* 2010;2: 5532.
40. De Bruyn H, Vandeweghe S, Ruyffelaert C, Cosyn J, Sennerby L. Radiographic evaluation of modern oral implants with emphasis on crestal bone level and relevance to peri-implant health. *Periodontol 2000* 2013;62:256—70.
41. Kivela-Rajamaki M, Maisi P, Srinivas R, et al. Levels and molecular forms of MMP-7 (matrilysin-1) and MMP-8 (collagenase-2) in diseased human peri-implant sulcular fluid. *J Periodontal Res* 2003;38:583—90.
42. Monov G, Strbac GD, Baron M, Kandler B, Watzek G, Gruber R. Soluble RANKL in crevicular fluid of dental implants: a pilot study. *Clin Implant Dent Relat Res* 2006;8:135—41.
43. Özçakır-Tomruk C, Chiquet M, Mericske-Stern R. Tenascin-C and matrix metalloproteinase-9 levels in crevicular fluid of teeth and implants. *Clin Implant Dent Relat Res* 2012;14: 672—81.
44. Severino VO, Napimoga MH. de Lima Pereira SA. Expression of IL-6, IL-10, IL-17 and IL-8 in the peri-implant crevicular fluid of patients with peri-implantitis. *Arch Oral Biol* 2011;56:823—8.