Long-term sequential receptor activator of NF-κB ligand (RANKL) and osteoprotegrin (OPG) expression in lipopolysaccharide-induced rat periapical lesions

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BACKGROUND: Long-term sequential expression of receptor activator of NF-κB ligand (RANKL) and osteoprotegrin (OPG) in lipopolysaccharide (LPS)-induced rat periapical lesions has not been studied.
MATERIALS: Seventy-two 4-week-old Wistar rats were divided into eight experimental groups and one control group (eight animals in each).
METHODS: Lipopolysaccharide-induced periapical lesions were produced in rats by occlusal exposure of the pulp of their lower first molars in all experimental groups but not the control group. The extent of periapical destruction was measured by radiographic imaging. RANKL and OPG mRNA were measured in all tissue sections containing the periapical lesions as well as the control group every week from week 1 to week 8 by real-time quantitative reverse transcription polymerase chain reaction. RANKL and OPG protein were determined by immunohistochemistry. Osteoclasts were identified by enzyme histochemistry.
RESULTS: The sequential changes in the mRNA and protein expression of RANKL and OPG were largely compatible with the occurrence of osteoclasts histologically and enzymes histochemically, as well as the mean areas of the periapical lesions radiographically during long-term observation of the LPS-induced rat periapical lesions.
CONCLUSION: This study may be the first to demonstrate the long-term RANKL and OPG expression every week from week 1 to week 8 using LPS to produce periapical infection in a Wistar rat model. The long-term findings of high expressions of RANKL and OPG further extend the potential application of the Wistar rat model for future experimental trials using RANKL inhibitor to evaluate the treatment outcome for LPS-induced rat periapical lesions.

J Oral Pathol Med (2012) **41**: 186–193

Introduction

Periapical lesions, which are formed as a result of root canal infection, are accompanied by an immune response to the invading microbes and periapical bone destruction (1–3). Bony destruction is the distinctive function of osteoclasts. Members of the tumor necrosis factor superfamily including receptor activator of NF-κB ligand (RANKL) and its receptors, receptor activator of NF-κB (RANK) and osteoprotegrin (OPG) are involved in the formation of osteoclasts (i.e. osteoclastogenesis) (4, 5). Osteoclast activation and hence bony destruction occurs as a result of RANKL binding to the RANK receptor on the cell surface of preosteoclasts and mature osteoclasts (6). OPG is a decoy receptor for RANKL (7) and hence competes with RANK, which can attenuate bony destruction (8, 9). Osteoclastogenesis is thus mediated via a balance between RANKL and OPG (10).

Short-term studies of RANKL and OPG expression have examined periapical lesions induced in a Wistar rat model (11–13), but only short-term observation of experimentally induced rat periapical lesions limits the usage and further application of this rat model. To our

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Accepted for publication June 22, 2011

knowledge, long-term observation of the expression of these two cytokines has not been completed in a Wistar rat model.

Lipopolysaccharide (LPS) is an endotoxin and is a major component of the outer membrane of gram-negative bacteria (14). Reviewing the English-language literature, experimental application of LPS to the dental pulp has been found to be able to initiate and sustain apical periodontitis in animal models (15–17), but not in a Wistar rat model. Previous reports have documented that LPS is capable of directly stimulating osteoblasts to express RANKL, resulting in osteoclast production (18) without the involvement of RANK. Recent data have also suggested that LPS might be directly implicated in osteoclast differentiation via a pathway partly independent of the aforementioned RANKL/RANK interaction (19, 20). Additionally, it has been reported that LPS-induced osteoclast formation occurs directly if dental pulp cells are previously treated with RANKL (21); RANKL treatment was mandatory for at least the first 24-h, as LPS alone did not stimulate osteoclast formation (20).

Hence, the aim of this study was to analyze the long-term sequential expression of RANKL and OPG in experimentally induced rat periapical lesions administered with LPS, not only to enhance our understanding but also to extend the potential clinical application of this animal model. In addition, the enzyme histochemistry of osteoclasts and imaging analysis of periapical bone destruction were also studied for periapical lesions induced by LPS in a Wistar rat model in the present study.

Materials and methods
Induction of periapical lesions
Seventy-two out-bred, 4-week-old male Wistar rats purchased from the National Science Council Animal Breeding Center, Taipei, Taiwan, weighing about 100 g each at the commencement of the experiment, were randomly divided into eight experimental groups (groups A–H) and one control group (group I) (eight rats in each group). The animals were housed under constant conditions (22°C; 12-h light/dark cycle) and fed tap water and standard Purina laboratory chow *ad libitum*. The animal-handling protocol ensured that humane practices were adhered to throughout the experimental process.

Subsequent to 4 weeks of acclimatization to their new surroundings, periapical lesions were induced in all animals of the experimental groups A–H using the procedure documented by Stashenko et al.(22) with the modification of using LPS to augment periapical infection. Under ketamine-HCl (5 mg/kg, intramuscular) anesthesia, an occlusal class I cavity of the right mandibular first molars of rats of the experimental groups A–H was generated with magnification using a #1/4 round high-speed bur until the head of the bur penetrated into the pulp chamber. With magnification, a #8 endodontic file was subsequently introduced into the distal root canals downwards to the root apices to remove the pulp tissues. A paper point soaked thoroughly with LPS (*E. Coli* LPS, serotype 026: B6, Sigma, St. Louis, MO, USA, 25 μg/0.1 ml) was then inserted into the distal root canal up to the root apices. Finally, the cavity was sealed with reinforced zinc oxide eugenol cement (Dentsply Caulk Co., Milford, DE, USA) (23, 24).

One week after pulp exposure, all the animals of group A were humanely sacrificed, and the mandibles were dissected (Fig. 1A,B) and processed for image analysis using the procedures described in the next section.

After image analysis, the LPS-induced periapical lesions were carefully removed. A portion of each periapical specimen was immediately frozen in liquid nitrogen for real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis. Another portion was routinely processed for light microscopy by fixation in 10% neutral-buffered formalin solution, decalcification with 10% EDTA solution, dehydration in ascending concentrations of alcohol solution, cleaning in xylene, and finally embedding in paraffin. Serial sections of each specimen were prepared at a 5-μm thickness. One section was prepared for hematoxylin–eosin staining, while the other sections were used for enzyme histochemical staining for osteoclasts and immunohistochemical staining for RANKL and OPG. Then, all animals of the other experimental groups B–H were sacrificed, using a similar procedure, 2–8 weeks after pulp exposure, one group every week. Finally, all rats of the unexposed pulp group (group I) were also killed using a similar procedure.

Image analysis
Radiographs of the resected mandibles of the experimental and control groups were acquired using a
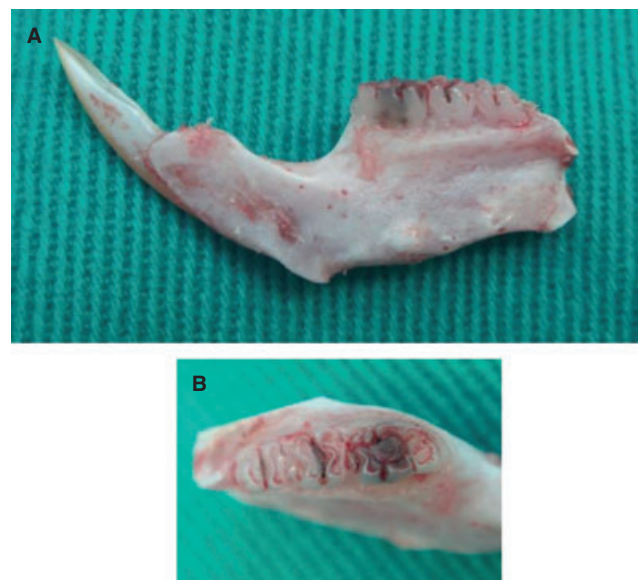
Figure 1 Lateral (A) and occlusal view (B) of a representative dissected rat mandibular specimen.

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microradiograph device and analyzed by a ScanX® image analysis system (Air Techniques, Melville, NY, USA). Areas of the periapical lesions at the distal root apices of the right mandibular first molars were enumerated in pixels, which were transformed to square millimeters using $1 \text{ mm}^2 = 1000$ pixels, as determined by measuring a standard known area.

Real-time QRT-PCR
Total RNA samples were prepared from all tissues samples of the experimental and control groups using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The quality and concentration RNA was determined from the optical density at a wavelength of 260 nm (using an OD_{260} unit equivalent to 40 μg/ml of RNA). The RNA was resuspended in 100 μl of diethlpyrocarbonate (DEPC)-treated water at a final concentration of 1 μg/ml. Reverse transcription was performed from 500 ng total RNA template using Taqman reverse transcription reagent. cDNA was amplified by PCR with oligoprimers for RANKL and OPG which were designed with reference to the published cDNA sequences in GenBank (Table 1). The reaction was performed on a thermal cycler (Mx3000P™; Stratagene, La Jolla, CA, USA) with Maxima™ SYBR Green qPCR Master Mix (2×) added to the PCR reaction mixture. After normalization to the expression level of β-actin mRNA, the relative expression levels of RANKL and OPG mRNA were presented as a percentage change compared with the control.

Immunohistochemistry
Staining was performed using the standard avidin–biotin peroxidase complex method (25). Rabbit polyclonal antibodies against mouse RANKL (Cat. no: ab9957; Abchem Corporation, Cambridge, UK) and OPG (Cat. no: BS1862; Bioworld Technology Inc., Minneapolis, MN, USA) were used. Tissue sections of the experimental and control groups containing the periapical areas were mounted on gelatin-chrome alum-coated slides. Following repeated deparaffinization in xylene and rehydration in a decreasing-concentration ethanol series (absolute, 95%, 70%, and 30% ethanol, and then water), tissue sections were microwave-treated thrice (5 min each) in a citrate buffer (10 mM; pH = 6.0) to retrieve antigenicity. Endogenous peroxidase activity was blocked by soaking the tissue sections in 3% hydrogen peroxide (H_2O_2) in methanol for 60 min. Before staining, a 10% solution of normal rabbit serum was applied for 60 min to tissue sections to inhibit non-specific staining. These sections were subse-

quently incubated with antibodies against RANKL and OPG (1:100, each) overnight at 4°C. Following subsequent rinsing with Tris-buffered saline (TBS, three times, 10 min each), tissue sections were then incubated for 60 min at room temperature with biotin-conjugated goat anti-rabbit IgG (1:100; Vector, Burlingame, CA, USA). Following this, all sections were washed with TBS again (three times, 10 min each) and then incubated with avidin–biotin complex conjugated with horseradish peroxidase (Dako, Santa Barbara, CA, USA) for a further 60 min. After washing with TBS (three times, 10 min each), peroxidase binding was visualized as brown reaction products via a benzidine reaction. Each set of experiments included a human squamous-cell carcinoma specimen known to express RANKL and OPG (26), which served as a positive control and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments. In each specimen, RANKL- and OPG-positive cells in the LPS-induced rat periapical tissue sections were observed microscopically.

Enzyme histochemistry
Tartrate-resistant acid phosphatase (TRAP) is regarded as a biochemical marker relatively specific for osteoclasts (27). The TRAP activity for all tissue sections containing the periapical regions of the experimental and control groups was assessed using a TRAP kit (Sigma) in accordance with the manufacturer's instructions.

Statistical analysis
Statistical analyses (one way ANOVA and Tukey–Kramer tests) were performed using JUMP 7.0 software (SAS, Cary, NC, USA). $P < 0.05$ was considered significant.

Results
Histological observation
For the unexposed pulp group (group I), the periapical area was normal and no obvious bony resorption could be observed. Mild inflammation and small areas of periapical alveolar bone resorption were observed for specimens at week 1 (group A) and week 2 (group B). The degree of inflammatory infiltration and alveolar bone resorption became more prominent for specimens observed at week 3 (group C) to week 8 (group H). Furthermore, giant cells were noted for specimens observed at week 1 (group A) and week 2 (group B)

Table 1 Oligoprimers used for real-time quantitative reverse transcriptase polymerase chain reaction in the current study

Oligoprimers	Sense	Antisense
RANKL	5′-ACCATCAATGCTGCCGACAT-3′	5′-CTTGGCCCAGCCTCGAT-3′
OPG	5′-ATATTGCCCCCAACGTTCAAC-3′	5′-AGAGGGCGCATAGTCAGTAGACA-3′
β-actin	5′-CTGCCCTGGCTCCTAGCA-3′	5′-TAGAGCCACCAATCCACACAGA-3′

OPG, osteoprotegrin; RANKL, receptor activator of NF-κB ligand.
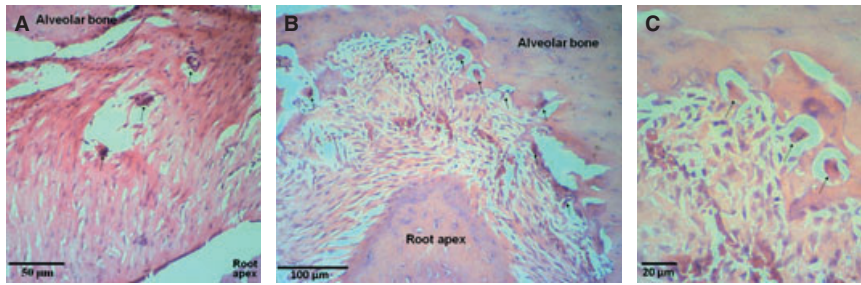
Figure 2 Some giant (osteoclastic-like) cells (arrows) were noted in representative lipopolysaccharide-induced rat periapical lesions at week 1 (group A) [A: hematoxylin & eosin (H–E) staining]; these became more prominent in representative rat periapical lesions observed at week 8 (group H) (B; C: H–E staining).

(Fig. 2A), and thereafter became more prominent for specimens observed at week 3 (group C) to week 8 (group H) (Fig. 2B,C).

Mean area of the induced rat periapical lesions on X-ray radiograph ($mAREA_{periapical}$)

Representative X-ray radiographs of the experimentally induced periapical lesions in the current experiment were depicted in Fig. 3, and the sequential mArea$_{periapical}$ was shown in Fig. 4.

The lowest mAREA$_{periapical}$ was observed at week 1 (group A) (0.32 ± 0.66 mm^2), whereas the highest mAREA$_{periapical}$ was noted at week 8 (group H) (1.51 ± 0.19 mm^2). Furthermore, the mAREA$_{periapical}$ significantly increased gradually from week 1 to week 3 (groups A–C), then remained more or less the same from week 3 to week 8 (groups C–H). Starting from week 3, the mAREA$_{periapical}$ observed each from week 3 to week 8 increased significantly as compared with the mAREA$_{periapical}$ observed from week 1 to week 2 respectively.

Mean relative mRNA expression of RANKL ($mrRANKL_{mRNA}$) and OPG ($mrOPG_{mRNA}$) for the induced rat periapical lesions

The sequential variation of mrRANKL$_{mRNA}$ and mrOPG$_{mRNA}$ for the current experiment was summarized respectively in Fig. 5A,B. The statistical analyses for mrRANKL$_{mRNA}$ and mrOPG$_{mRNA}$ among groups A–I were shown in Table 2.

The mrRANKL$_{mRNA}$ observed at week 1 (group A) and week 2 (group B) was 27% and 7%, respectively, which were both significantly lower than that of the unexposed pulp group (group I). Then, the mrRANKL$_{mRNA}$ observed at week 3 (group C) was elevated to 113%, which was slightly higher than the unexposed pulp group (group I) but significantly higher than the mrRANKL$_{mRNA}$ observed at week 1 (group A) and week 2 (group B). Subsequently, the mrRANKL$_{mRNA}$ observed at week 4 (group D), week 5 (group E) and week 6 (group F) was decreased similarly to 65%, 65% and 61%, respectively, which were all significantly lower than the mrRANKL$_{mRNA}$ observed at week 3 (group C) and that of the unexposed pulp group (group I) but significantly higher than the mrRANKL$_{mRNA}$ observed at week 1 (group A) and week 2 (group B). Finally, the mrRANKL$_{mRNA}$ observed at week 7 (group G) and week 8 (group H) was dramatically elevated to 185% and 208%, respectively, both of which were significantly higher than the mrRANKL$_{mRNA}$ observed from week 1 (group A) to week 6 (group F), as well as that for the unexposed pulp group (group I).
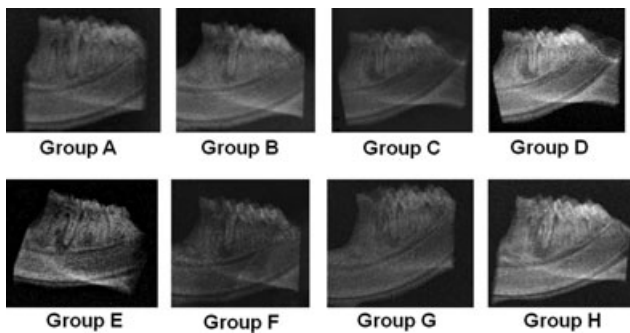
Figure 3 Representative X-ray radiographs of lipopolysaccharide-induced rat periapical lesions observed from week 1 to week 8 (groups A–H).
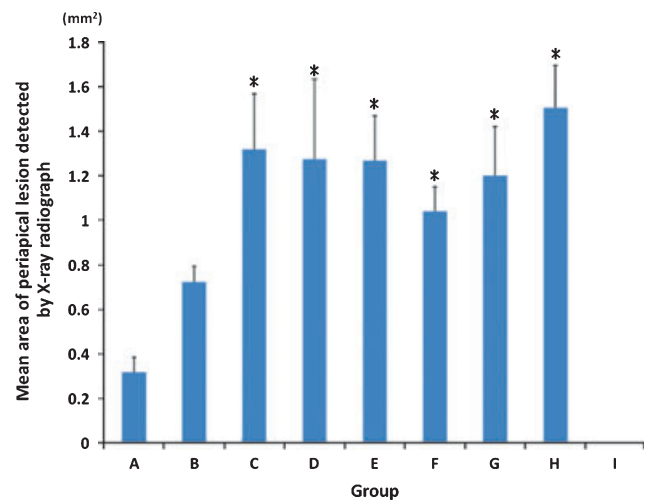
Figure 4 Sequential changes in the mean area of the lipopolysaccharide-induced rat periapical lesions detected by X-ray radiograph. *$P < 0.05$ as compared with groups A & B respectively.
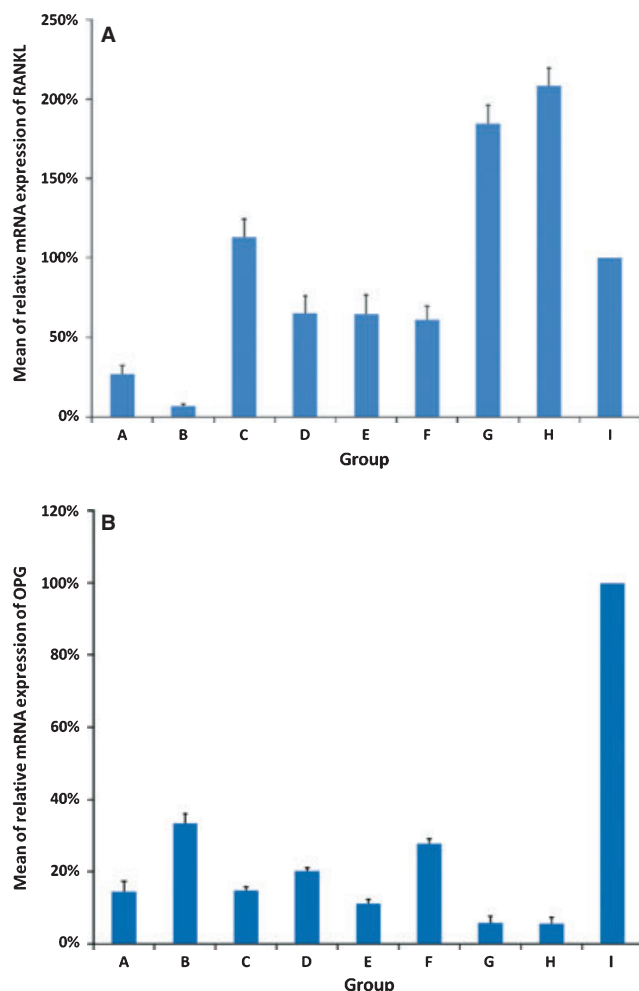
Figure 5 Sequential changes in the mean of the relative mRNA expression of receptor activator of NF-κB ligand (A) and osteoprotegrin (B) for the lipopolysaccharide-induced rat periapical lesions.

The $mrOPG_{mRNA}$ observed from week 1 to week 6 (groups A–F) fluctuated within the range of 11–33% and then decreased significantly by week 7 (6%, group G) and week 8 (5%, group H). Worthy of note, with the exception of the $mrOPG_{mRNA}$ observed at week 2 (group B), the $mrOPG_{mRNA}$ observed from week 1 to week 8 (groups A–H) was significantly lower than the corresponding levels of $mrRANKL_{mRNA}$ observed from week 1 to week 8 (groups A–H). Furthermore, the $mrOPG_{mRNA}$ observed from week 1 to week 8 (groups A–H) was significantly lower than that of the unexposed pulp group (group I). The sequential change of the ratio of $mrRANKL_{mRNA}$ and $mrOPG_{mRNA}$ was as shown in Fig. 6.

Immunohistochemical observation
Only a few RANKL- and OPG-positive cells were noted in the tissue specimens of normal periapical areas (group I) and the specimens containing the periapical lesions observed at week 1 (group A) and week 2 (group B) (Fig. 7A,B). The amount of RANKL- and OPG-positive cells then increased in the tissue specimens

Table 2 Statistical analyses of the mean of relative mRNA expression of RANKL ($mrRANKL_{mRNA}$) and OPG ($mrOPG_{mRNA}$) for the induced rat periapical lesions among groups (gps) A–I

Comparisons	P values	
	$mrRANKL_{mRNA}$	$mrOPG_{mRNA}$
Group A[1]		
Gp A vs. Gp B	S	S
Gp A vs. Gp C	S	NS
Gp A vs. Gp D	S	NS
Gp A vs. Gp E	S	NS
Gp A vs. Gp F	S	NS
Gp A vs. Gp G	S	S
Gp A vs. Gp H	S	S
Gp A vs. Gp I	S	S
Group B		
Gp B vs. Gp C	S	S
Gp B vs. Gp D	S	NS
Gp B vs. Gp E	S	S
Gp B vs. Gp F	S	NS
Gp B vs. Gp G	S	S
Gp B vs. Gp H	S	S
Gp B vs. Gp I	S	S
Group C		
Gp C vs. Gp D	S	NS
Gp C vs. Gp E	S	NS
Gp C vs. Gp F	S	NS
Gp C vs. Gp G	S	S
Gp C vs. Gp H	S	S
Gp C vs. Gp I	NS	S
Group D		
Gp D vs. Gp E	NS	NS
Gp D vs. Gp F	NS	NS
Gp D vs. Gp G	S	NS
Gp D vs. Gp H	S	NS
Gp D vs. Gp I	NS	S
Group E		
Gp E vs. Gp F	NS	S
Gp E vs. Gp G	S	NS
Gp E vs. Gp H	S	NS
Gp E vs. Gp I	NS	S
Group F		
Gp F vs. Gp G	S	S
Gp F vs. Gp H	S	S
Gp F vs. Gp I	NS	S
Group G		
Gp G vs. Gp H	S	NS
Gp G vs. Gp I	S	S
Group H		
Gp H vs. Gp I	S	S

OPG, osteoprotegrin; RANKL, receptor activator of NF-κB ligand.
[1]$mrRANKL_{mRNA}$/$mrOPG_{mRNA}$ of one group (e.g. Group A) was respectively compared each with other groups (e.g. Groups B–I) using one-way ANOVA and Tukey–Kramer tests.
S, statistical significance ($P < 0.05$) (shaded areas); NS, non-statistical significance ($P > 0.05$) (non-shaded areas).

containing the periapical lesions from week 3 (group C) to week 8 (group H) (Fig. 7C,D). Omission of primary antisera in control sections disclosed negative findings for RANKL and OPG activities in all sections, while the positive control sections showed positive reactions for RANKL and OPG activities.

Enzyme histochemical observation
Only a few osteoclast-like cells were found in the tissue sections of normal periapical areas (group I) as well as in the tissue sections containing the periapical lesions
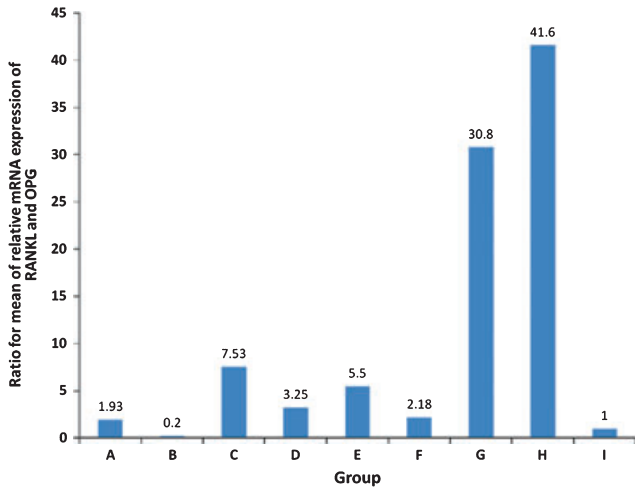
Figure 6 Sequential changes in the ratio of the mean of the relative mRNA expression of receptor activator of NF-κB ligand and osteoprotegrin for the lipopolysaccharide-induced rat periapical lesions.
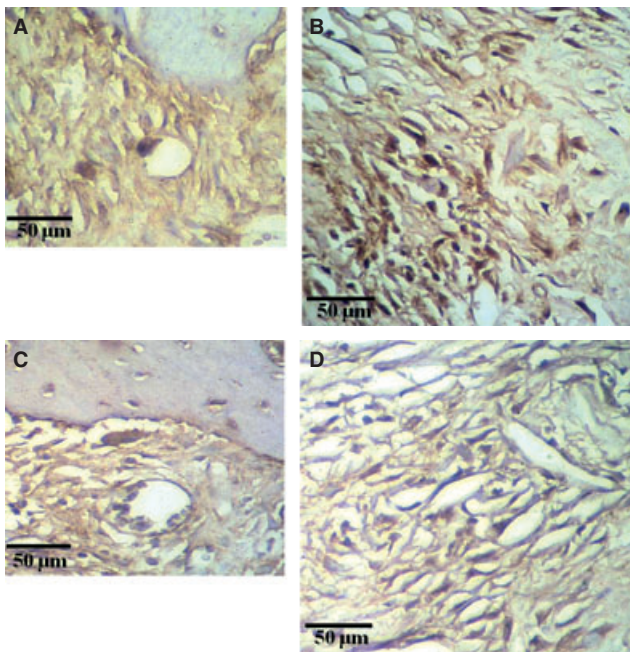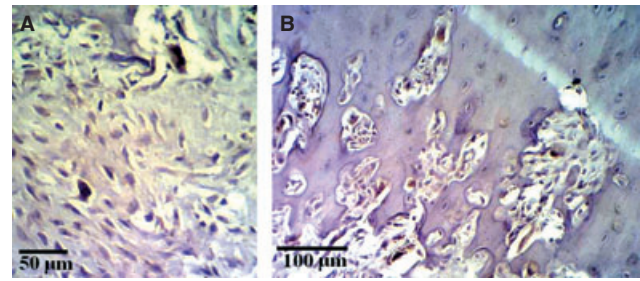

Figure 7 Representative photomicrograph of receptor activator of NF-κB ligand- and osteoprotegrin-positive cells in the lipopolysaccharide-induced rat periapical tissue specimens observed at week 1 (group A) (A and B) and week 8 (group H) (C and D).

observed at week 1 (group A) and week 2 (group B) (Fig. 8A). The amount of osteoclast-like cells increased in the tissue sections observed from week 3 (group C) to week 8 (group H) (Fig. 8B).

Discussion

As mentioned earlier, most previous studies on RANKL and OPG expression in experimentally induced rat periapical lesions have only reported short-term data

Figure 8 Representative photomicrograph of tartrate-resistant acid phosphatase in the lipopolysaccharide-induced rat periapical tissue specimens observed at week 1 (group A) (A) and week 8 (group H) (B).

(observed from week 1 to 4) (11–13), only one study (12), to our knowledge, providing related data for week 8, but still lacking relevant information for weeks 5–7. In this study, we documented the long-term sequential expression of RANKL and OPG from week 1 to week 8 every week in tissue samples obtained from the LPS-induced rat periapical lesions.

In the current study, low protein expressions of RANKL and OPG were found using immunochemistry in periapical tissue specimens observed at week 1 and week 2, and the amount became more prominent in specimens observed at week 3–8. Nevertheless, immunochemistry would not provide the most specific quantitative changes of RANKL and OPG.

Worthy of note, by using the more specific and advanced method of real-time QRT-PCR, delicate quantitative alterations in RANKL and OPG mRNA were successfully documented for LPS-induced periapical rat lesions in the present study. We found that the $mrRANKL_{mRNA}$ observed at week 1 (group A) and week 2 (group B) was significantly lower than in the unexposed pulp group (group I). This is perhaps due to the fact that, at the commencement of the experiment, a potential unspecified mechanism has been triggered intending to inhibit the production of RANKL so as to suppress the growth of the experimentally induced periapical lesions. However, with the progress of the experiment (observed at week 3, group C), this mechanism is retarded, as reflected by a rebound of the $mrRANKL_{mRNA}$, approximating the normal value of unexposed pulp group (group I). Therefore, the findings in the initial period of our study were consistent with those of previous studies (11–13). Then, on further observation, the $mrRANKL_{mRNA}$ again declined but became almost constant at the 4th week (group D), 5th week (group E) and 6th week (group F), which may imply a partial recovery of the potential mechanism of the animals.

Nonetheless, in the final period of observation [7th week (group G) and 8th week (group H)], we speculate that the potential mechanism had almost completely deteriorated, as indicated by a more or less two-fold increase of the $mrRANKL_{mRNA}$ as compared with the level observed in the unexposed pulp group (group I). This finding is also supported by the highest number of

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giant (osteoclast-like) cells as well as the greatest mean areas of the rat periapical lesions measured on X-ray radiographs at week 7 (group G) and week 8 (group H). This interesting finding differs from the results of Kawashima et al.,(12) who reported a more or less same level observed at week 8 as compared with week 4. This discrepancy may be due to the fact that LPS, with the capacity to upgrade the periapical infection, was used in the present study. On the other hand, the sequential change of the ratio of $mrRANKL_{mRNA}$ and $mrOPG_{mRNA}$ was principally similar to the alteration in $mrRANKL_{mRNA}$ described earlier—an initial increase in the $mrRANKL_{mRNA}/mrOPG_{mRNA}$ ratio was observed at the 3rd week, which then decreased at the 4th–6th weeks but drastically increased at the 7th and 8th weeks. Consequently, the current study is, to our knowledge, the first to report on the long-term alterations of mRNA expression of RANKL and OPG in LPS-induced periapical lesions in a rat model.

In the present study, not only alterations in RANKL and OPG expression, but also the expression of giant (osteoclast-like) cells histologically and enzymes histochemically, as well as the mean areas of the periapical lesions radiographically, were documented during long-term observation of LPS-induced rat periapical lesions. We found that the trend of RANKL and OPG in the current study has been compatible with the results of histological, and enzyme histochemical observations and imaging analyses: The later the observation, the greater the bony destruction and the higher the level of giant (osteoclast-like) cells. Furthermore, the protein expression of RANKL was consistent with TRAP activity. Hence, supported by the histological, enzyme histochemical and imaging examinations, our results, in line with previous animal (11–13) and human (28–30) reports, indicated that RANKL and OPG, as well as the RANKL/OPG ratio, played a central role in the initiation of periapical bone destruction.

Finally, two critical aspects for our study need further attention. First, as the effect of LPS is concentration dependent, a nearly equal quantity of LPS would be expected to be available to periapical tissue of each rat in the current study. In contrast to most *in vitro* studies in which a fixed concentration of LPS would easily be measured and applied to the culture system (20, 21, 23), for *in vivo* study, this objective may not easily be attained, especially for small animals such as rat. We claimed that this goal would potentially be attained in the current study, provided the paper point is saturated with a fixed amount of LPS, and additionally, the paper point is ascertained to be inserted correctly into the root canal orifices; otherwise, there may be a caution of variable concentrations of LPS to be available to the periapical tissue of each rat.

Second, as reviewed by Suzuki et al. (31), more than 90% of the RNA transcription studies appeared in high-impact journals employed only one housekeeping gene. Furthermore, to our knowledge, most analyses on the role of RANKL and OPG in the rat periapical lesion development using real time QRT-PCR also used only one housekeeping gene [either glyceraldehydes 3-phosphate dehydrogenase (GAPDH)/β-actin] (11–13, 22). However, some authors have claimed that it is important to use more than one housekeeping genes to obtain the most reliable results in RNA transcription studies (32, 33). In the current study, only β-actin was employed as the reference gene. Hence, it is worthwhile for future experiments to use more than one housekeeping gene for normalization, not only to further confirm but also to acquire the most reliable data of the current study.

In conclusion, using LPS to produce periapical infection in a Wistar rat model, the present study demonstrates the long-term sequential expression of RANKL and OPG every week from week 1 to week 8. The long-term findings of a high RANKL level and RANKL/OPG ratio further extend the potential application of the Wistar rat model for future experimental trials using RANKL inhibitor (34, 35) to evaluate the treatment outcome for LPS-induced rat periapical lesions by monitoring the change in RANKL expression or the RANKL/OPG ratio in the samples.

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