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The upregulation of receptor activator NF-κB ligand expression by interleukin-1α and *Porphyromonas endodontalis* in human osteoblastic cells

S.-C. Chen[1,2], F.-M. Huang[3], S.-S. Lee[4], M.-Z. Li[1] & Y.-C. Chang[3,5]

[1]Institute of Medicine, Chung Shan Medical University; [2]Section of Metabolism & Endocrinology, Cheng Ching Hospital; [3]Oral Medicine Center, Chung Shan Medical University Hospital; [4]School of Public Health, Chung Shan Medical University; and [5]School of Dentistry, Chung Shan Medical University, Taichung, Taiwan

Abstract

Chen S-C, Huang F-M, Lee S-S, Li, M-Z, Chang Y-C. The upregulation of receptor activator NF-κB ligand expression by interleukin-1α and *Porphyromonas endodontalis* in human osteoblastic cells. *International Endodontic Journal*, **42**, 375–380, 2009.

Aim To investigate the receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) in osteoblastic cells stimulated with inflammatory mediators.

Methodology The expression of RANKL in human osteoblastic cell line U2OS stimulated by pro-inflammatory cytokine interleukin (IL)-1α and black-pigmented bacteria *Porphyromonas endodontalis* was investigated by Western blot and enzyme-linked immunosorbent assay (ELISA). The significance of the results obtained from control and treated groups was statistically analysed by the paired Student's *t*-test.

Results IL-1α was found to upregulate RANKL production in U2OS cells ($P < 0.05$). Investigations of the time dependence of RANKL expression in IL-1α-treated cells revealed a rapid accumulation of RANKL protein after 1 h of exposure; it remained elevated throughout the 24-h incubation period shown by Western blot and ELISA. In addition, *P. endodontalis* also increased RANKL expression in U2OS cells after 4-h incubation period demonstrated by Western blot and ELISA ($P < 0.05$).

Conclusions IL-1α and *P. endodontalis* may be involved in developing apical periodontitis through the stimulation of RANKL production.

Keywords: apical periodontitis, IL-1α, osteoblastic cell, *P. endodontalis*, RANKL.

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Introduction

Apical periodontitis is an inflammatory disorder of periradicular tissues caused by persistent microbial infection within the root canal system of the affected tooth (Nair 2006). Continuous flow of bacteria and their products through the apical foramen induces influx, activation and coordinated interaction of immune-inflammatory cells within the periapical area. The pathologic responses involve a complex array of immunologic mechanisms, some of which may act primarily to protect the pulp and periapical region, whereas others mediate periapical tissue destruction, particularly bone resorption (Stashenko *et al.* 1998).

A recently identified tumour necrosis factor (TNF) super family molecule, receptor activator of NF-κB ligand (RANKL), its receptor (RANK) and natural antagonist, osteoprotegerin, have been shown to be the key regulators of bone remodelling and are directly involved in the differentiation, activation and survival of osteoclasts and osteoclast precursors (Simonet *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998). Membrane-bound or soluble RANKL is primarily produced in osteoblastic lineages and activated T cells and

Correspondence: Dr Yu-Chao Chang DDS. MMS. PhD, Professor, School of Dentistry, Chung Shan Medical University, 110, Sec. 1, Chien-Kuo N. Rd., Taichung, Taiwan (Tel.: 886 4 22015111: fax: 886 4 24759065; e-mail: cyc@csmu.edu.tw).

stimulates osteoclast differentiation (Kong *et al.* 1999a,b, Suda *et al.* 1999).

Interleukin-1 (IL-1) plays a central role in the regulation of immunological and inflammatory reactions. Biological activity of IL-1 molecules seems to be directly relevant to bone resorption (Stashenko *et al.* 1987). IL-1α has been found to express in periapical lesions (Bando *et al.* 1993, Honma *et al.* 1998). IL-1α may play an important role in the pathogenesis of apical periodontitis.

Black-pigmented gram-negative anaerobic bacteria, such as *Prevotella* spp. and *Porphyromonas* spp. are relative common in infected root canals and endodontic abscesses (Bogen & Slots 1999, Jacinto *et al.* 2003). *Porphyromonas endodontalis* (*P. endodontalis*, formerly *Bacteroides endodontalis*) is a black-pigmented, anaerobic, rod-shaped bacterium, which is strongly associated with endodontic infection. This species, as well as other black-pigmented anaerobic rods, has been implicated in the aetiology of infected root canals and apical periodontitis (Siqueira *et al.* 2001, Fouad *et al.* 2002, Gomes *et al.* 2005, Tomazinho & Avila-Campos 2007). *Porphyromonas endodontalis* is capable of degrading several important proteins, such as immunoglobulins, complement factors and heptoglobulin (van Winkelhoff *et al.* 1992). The invasion of host tissue by *P. endodontalis* may induce periapical destruction.

Surprisingly, there have been relatively few studies addressing the mechanisms of tissue destruction in apical periodontitis. The subsequent reactions leading to periapical injury after the induction of IL-1α and *P. endodontalis* remains to be elucidated. Menezes *et al.* (2006) have shown that the presence of RANKL in periapical cyst and granulomas may involve the development of periapical lesions. Moreover, Sabeti *et al.* (2005) have reported that RANKL may play a role in apical periodontitis-induced bone resorption. However, little information is available about the presence of RANKL and its role in apical periodontitis and associated bone resorption. Osteoblasts are considered as cells primarily concerned with providing physical barriers and structural components in periapical tissues. These cells may be important in the recruitment of immune cells and contribute to the inflammation (Yang *et al.* 2003). In this study, the expression of RANKL in human osteoblastic cell line U2OS cells stimulated by pro-inflammatory cytokine IL-1α and *P. endodontalis* was investigated by the Western blot and enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Chemicals and materials

IL-1α was purchased from Sigma Chemical Co. (St Louis, MO, USA). All culture materials were obtained from GIBCO (Grand Island, NY, USA). IL-1α was directly dissolved in the culture medium. The final concentration of IL-1α used in this study was 20 ng mL^{-1}.

Bacterial strain and preparation of supernatant

Porphyromonas endodontalis (ATCC 27067) were grown under anerobic conditions and harvested at the end of the logarithmic phase of growth as described previously (Yang *et al.* 2003, Huang *et al.* 2005). Briefly, they were maintained in brain–heart infusion broth pre-reduced anaerobically, sterilized and supplemented with 5 mg L^{-1} haemin and 0.5 mg L^{-1} menadione. The density of the inoculum, prepared in brain–heart infusion broth, was adjusted to a turbidity of 2 McFarland standard (6×10^8 colony-forming units mL^{-1}). After centrifugation, supernatants were filter-sterilized using a 0.2-μm filter and stored at −80 °C until used. The supernatants of *P. endodontalis* were directly diluted in culture medium and the final dilution was 1 : 50 according to a previous study (Chang *et al.* 2003).

Cell culture

U2OS cells (American Tissue Type Collection HTB 96) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg mL^{-1} of streptomycin, 100 mg mL^{-1} of penicillin at 37 °C in humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min and aliquots of separated cells were subcultured. The cells were subcultured at 1 : 4 splits every third day.

Treatments

Confluent cells were trypsinized, counted and plated at a concentration of 5×10^4 cells in 60-mm-culture dish and allowed for 48 h to achieve confluence. Prior to treatment, the cells were washed with serum-free DMEM and immediately exposed for the indicated incubation times to 20 ng mL^{-1} IL-1α and supernatants of *P. endodontalis* (1 : 50). After different periods

of time (0, 1, 2, 4, 8 and 24 h), cell lysates and the conditioned medium samples were collected for Western blot and ELISA respectively.

Western blot

Cell extracts were solubilized with SDS-solubilization buffer (5 mmol L^{-1} EDTA, 1 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} Tris–HCl, pH 7.5 and 0.5% Trition X-100, 2 mmol L^{-1} phenylmethysulfonyl fluoride and 1 mmol L^{-1} *N*-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12 000 *g* at 4 °C and the protein concentrations determined with Bradford reagent using bovine serum albumin (BSA) as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10% SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% BSA for 2 h, rinsed and then incubated with primary antibodies anti-RANKL (Chemicon International Inc., Temecula, CA, USA) and diluted 1 : 1000 in PBS containing 0.05% Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1 : 2000 in the same buffer, washed again as described above and treated with 1 : 2000 streptavidin-peroxidase solution for 30 min. After a new series of washing steps, the reactions were developed using diaminobenzidine (Zymed, South San Francisco, CA, USA). All steps were done at room temperature. As a loading control *β*-actin (Santa Cruz Biotechnology, CA, USA) was included. The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000; AlphaInnotech, San Liandro, CA, USA). Each densitometric value, expressed as the mean ± SD, was obtained from three independent experiments.

Enzyme-linked immunosorbent assay

Levels of RANKL antigen were determined by ELISA (R&D systems, Minneapolis, MN, USA). Briefly, 20 μL of conditioned media were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and RANKL levels were determined with a calibration curve using human RANKL as a standard. The amounts of RANKL were expressed as pg L^{-1} protein. Each value was expressed as the mean ± SD.

Statistical analysis

Triplicate separate experiments were performed throughout this study. The significance of the results obtained from control and treated groups was statistically analysed by the paired Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

IL-1α was found to upregulate RANKL production in U2OS cells ($P < 0.05$). Investigations of the time dependence of RANKL expression in IL-1α-treated cells revealed a rapid accumulation of the RANKL protein after 1 h of exposure that remained elevated throughout the 24-h incubation period (Fig. 1a). The quantitative measurement by the AlphaImager 2000 was shown in Fig. 1b. The levels of the RANKL increased about 2.1-, 2.4-, 2.7-, 2.8- and 3.7-fold after exposure to IL-1α for 1, 2, 4, 8 and 24 h respectively.

In addition, the results of Western blot were confirmed by ELISA. Similar pattern was seen by Western blot. The production of RANKL was enhanced by the IL-1α as compared with control ($P < 0.05$). As shown in Fig. 2, the amounts of RANKL were about

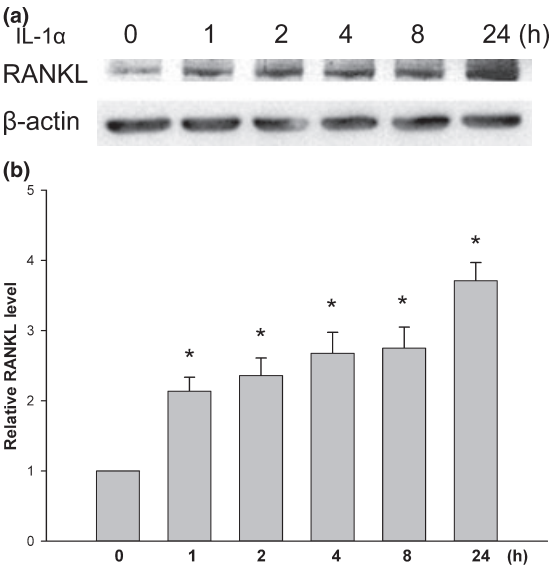

Figure 1 (a) Kinetics of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) expression in U2OS cells exposed to IL-1α for 0, 1, 2, 4, 8 and 24 h respectively. *β*-actin was used as a loading control. (b) Levels of RANKL treated with IL-1α were measured by densitometric gel imaging. *Significant difference from control values with $P < 0.05$.

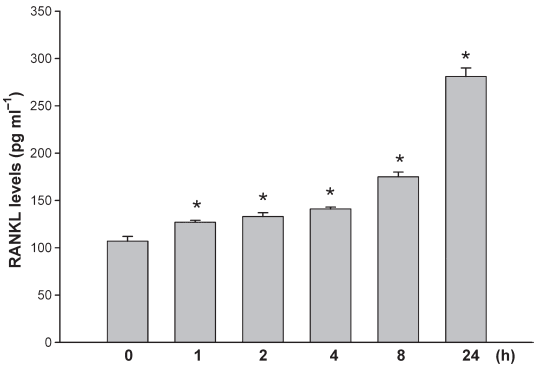

Figure 2 Expression the protein levels receptor activator of nuclear factor-kappa B (NF-κB) ligand of conditioned medium from U2OS cells treated with the IL-1α for 0, 1, 2, 4, 8 and 24 h respectively. Each value was expressed as the mean ± SD of optical density from triplicate independent experiments. *Significant difference from control values with $P < 0.05$.

107, 127, 135, 141, 175 and 281 pg mL^{-1} after exposure to IL-1α for 0, 1, 2, 4, 8 and 24 h respectively.

Porphyromonas endodontalis was also found to increase RANKL production in U2OS cells ($P < 0.05$). The kinetics of this response showed that RANKL was significantly increased in cell lysates after 4-h post *P. endodontalis* challenge and remained elevation throughout the 24-h incubation period (Fig. 3a). The quantitative measurement by the AlphaImager 2000 is shown in Fig. 3b. RANKL levels increased about 1.7-, 2.3- and 3.1-fold after exposure to *P. endodontalis* for 4, 8 and 24 h respectively.

The results from ELISA demonstrated that the production of RANKL was enhanced by *P. endodontalis* after the 4 h treatment period as compared with control ($P < 0.05$). As shown in Fig. 4, the amounts of RANKL were about 108, 110, 119, 137, 161 and 208 pg mL^{-1} after exposure to *endodontalis* for 0, 1, 2, 4, 8 and 24 h respectively.

Discussion

Diagnosis and treatment of inflammatory bone resorption induced by apical periodontitis present a clinical challenge. This process involves a local inflammatory reaction. However, the exact mechanism and sequence of events associated with the activation of osteoclastic activity to produce bone resorption-induced apical periodontitis has not been fully elucidated. It was suggested that a novel secreted glycoprotein ligand known as RANKL may play a critical role in the

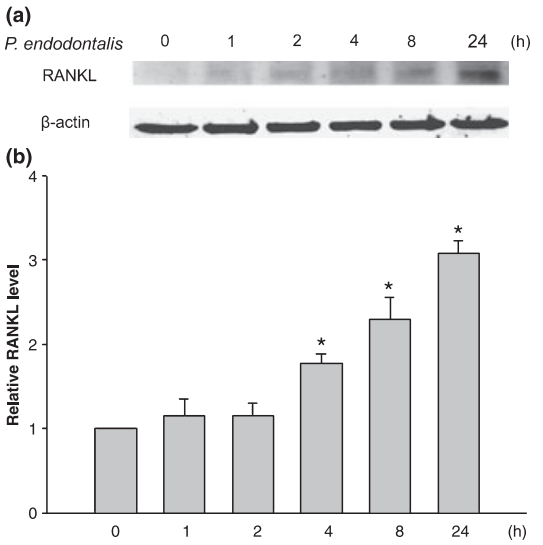

Figure 3 (a) Kinetics of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) expression in U2OS cells exposed to *Porphyromonas endodontalis* for 0, 1, 2, 4, 8 and 24 h respectively. β-actin was included to monitor equal protein loading. (b) Levels of RANKL treated with *P. endodontalis* were measured by densitometric gel imaging. *Significant difference from control values with $P < 0.05$.

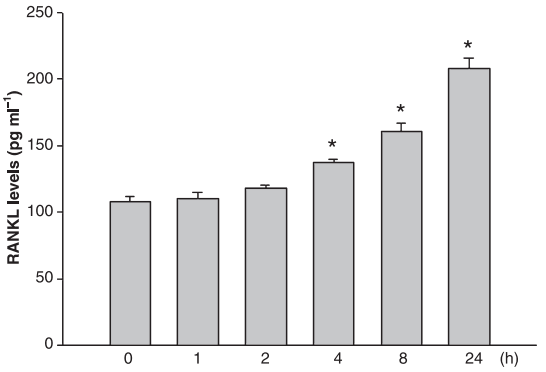

Figure 4 Expression the protein levels receptor activator of nuclear factor-kappa B (NF-κB) ligand of conditioned medium from U2OS cells treated with the *Porphyromonas endodontalis* for 0, 1, 2, 4, 8 and 24 h respectively. Each value was expressed as the mean ± SD of optical density from triplicate independent experiments. *Significant difference from control values with $P < 0.05$.

development of osteoclasts that result in bone resorption (Fuller *et al.* 1998). Recently, RANKL was found to be expressed in apical periodontitis (Sabeti *et al.* 2005). However, there is little information about the RANKL expression in inflammatory cytokine and endodontopathic bacteria-induced apical periodontitis.

Studies have reported that RANKL is expressed in periodontal ligament cells, osteoblasts, osteoclasts, stromal cells, T lymphocytes, endothelial cells and epithelial cells (Boyle *et al.* 2003). In this study, it was noted that RANKL was expressed intracellularly by Western blot and extracellularly by ELISA in an osteoblastic cell line U2OS cell. Similar results were reported by Mancini *et al.* (2007) who reported that U2OS cells can express RANKL mRNA and protein *in vitro*. Consistently, RANKL was found to be expressed in specimens from apical periodontitis (Sabeti *et al.* 2005). Thus, osteoblasts might be one of the cells that can express RANKL within apical periodontitis lesions.

It is accepted that IL-1 has multiple biological activities, such as the acceleration of bone resorption. IL-1α has been found to play an important role in the pathogenesis of periapical lesions (Bando *et al.* 1993, Honma *et al.* 1998). In this study, it was found that the expression of RANKL was upregulated by the IL-1α in U2OS cells. These results were in agreement with those of the previous studies, which IL-1α is known to modulate RANKL expression (Fukushima *et al.* 2005, Wei *et al.* 2005). Taken together, these findings suggest that IL-1α may be involved in developing apical periodontitis through the upregulation of RANKL production.

Bacteria and their products are the primary cause of pulpal necrosis and periapical lesions. How bacteria stimulate bone pathosis is still unclear. In this study, it was found initially that the expression of RANKL was upregulated by *P. endodontalis* in U2OS cells. Consistently, similar results were found evaluating the effects of several components of black-pigmented bacteria *P. gingivalis* on mRNA expression of RANKL (Okahashi *et al.* 2004, Kobayashi-Sakamoto *et al.* 2004). Taken together, *P. endodontalis* might promote cellular processes that stimulate the degradation of bone *via* RANKL expression.

In interpreting the results presented here, it is interesting to consider that IL-1α and *P. endodontalis* may lead to the upregulation of RANKL production in U2OS cells. The presence of excessive amounts of RANKL derived from U2OS cells may influence the inflammatory response by virtue of its ability to activate immune cells. Therefore, the capacity of U2OS cells to produce RANKL in response to IL-1α and *P. endodontalis* suggests that human osteoblastic cells additionally contributes to the orchestration of immuno-participant cells in the host defence network of apical periodontitis. Further studies seem necessary to identify not just the *in situ* localization of RANKL in apical periodontitis but also using a functional assay.

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