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In situ expression of RANKL, RANK, osteoprotegerin and cytokines in osteoclasts of rat periodontal tissue

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Objectives: This study examined the *in situ* expression of receptor activator of nuclear factor-κB ligand (RANKL), receptor activator of nuclear factor-κB (RANK), osteoprotegerin, interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) in the osteoclasts of rat periodontal tissue.

Background: In periodontal disease, osteoclasts cause resorption of the alveolar bone. The function of osteoclasts is regulated by interaction with periodontal ligament cells (PDLs). Furthermore, various kinds of molecules such as RANKL, RANK, osteoprotegerin, IL-1β and TNFα are known to be related to the osteoclasts differentiation and function. It is therefore important to observe the expression of RANKL, RANK, osteoprotegerin and cytokines in osteoclasts and PDLs.

Methods: Four-week-old Wistar rats were used. Tooth movement was performed by the Waldo method, and the pathological bone resorption was induced. The demineralized maxillae and mandiblae were embedded with paraffin. *In situ* hybridization was performed to detect RANKL, RANK, osteoprotegerin, IL-1β, and TNFα mRNAs in osteoclasts and other cells using the specific RNA probes, respectively.

Results: Both RANKL and RANK were concomitantly expressed in some osteoclasts. RANKL was also positive in osteoblasts and PDLs. No IL-1β- and TNFα-positive osteoclast was noted. The positive signals of osteoprotegerin were detected in almost all osteoblasts, PDLs and odontoblasts. No osteoprotegerin-positive osteoclasts were observed. The number and the distribution pattern of RANKL- and RANK-expressing osteoclasts changed when orthodontic excessive force was applied to periodontal tissue. In addition, IL-1β and TNFα were shown to be expressed in osteoclasts under pathological status.

Conclusion: These findings suggest that an autocrine mechanism of RANKL–RANK exists in osteoclast, which is heightened in the pathological conditions. Furthermore, the autocrine mechanism of IL-1β and TNFα is also provided in osteoclast under pathological condition. These autocrine mechanisms therefore seem to regulate the osteoclast function in both physiological and pathological conditions.

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The active remodeling of alveolar bone in periodontal tissue is well known to occur in various kinds of conditions such as patients with a homeostatic status, inflammation, aging and orthodontic treatment. Osteoblasts and osteoclasts are the main cells that are responsible for the remodeling of alveolar bone. Osteoclasts are known to be cells that cause resorption of the alveolar bone in periodontitis, and therefore it seems to be important to elucidate the biological behavior of osteoclasts in both the physiological and pathological state. Recently, the receptor activator of nuclear factor-κB (RANK) ligand (RANKL) has been identified to be an osteoclast differentiation factor (1) and binds to RANK on the cell surface of osteoclastic cells. Osteoprotegerin is an inhibitory factor of osteoclastogenesis in competition with RANK (2, 3). Both RANKL and osteoprotegerin act as positive and negative regulators of osteoclastogenesis, respectively, and they are also thought to regulate bone remodeling concomitantly.

Interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) are other osteoclast activating factors. The intracellular signal transduction pathways of RANKL, IL-1β, and TNFα to activate osteoclasts are different from each other (4).

Periodontal ligament cells (PDLs) are thought to play some role in alveolar bone remodeling as well as osteoblasts and osteoclasts. Some investigators have reported that the fibroblasts derived from the PDLs are able to induce osteoclastogenesis *in vitro* (5, 6). The RANKL and osteoprotegerin expressions in osteoblasts and PDLs have already been reported (5, 7). The function of osteoclasts is regulated by interaction with these PDLs. It is therefore also important to observe the expression of RANKL, RANK, osteoprotegerin and cytokines in other cells that could be related to the biological function of osteoclasts. However, how these cells express various kinds of factors during alveolar bone remodeling *in vivo* is still unclear. Since alveolar bone remodeling is based on interactions with many kinds of cells, investigations *in vivo* are essential to understand the relationship of osteoclasts to other kinds of cells. The present study was therefore designed to clarify the changes in the distribution of osteoclasts expressing RANKL, RANK, osteoprotegerin, IL-1β and TNFα and their relationship with osteoblasts and PDLs in periodontal tissue during alveolar bone resorption.

Materials and methods

Tissue preparation

Four-week-old Wistar rats (body weight 80–100 g, 15 males, Seac Yoshitomi Ltd, Fukuoka, Japan) were used in this study. All experimental procedures were performed according to the animal experimental guidelines of Kyushu University.

For *in situ* hybridization, each animal was perfused through the ascending aorta with diethylpyrocarbonate-treated 0.01 M phosphate-buffered saline containing 0.5% procaine hydrochloride and heparin (2000 U/300 ml) under sodium pentobarbital anesthesia (1 ml/kg, i.p.), followed by an immersion into fixative composed of 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 at 4°C. Both maxillae and mandiblae were immediately removed and then immersed in the same fixative for 4 h at 4°C. After the fixation, the specimens were demineralized in 5% EDTA in phosphate-buffered saline containing 4% sucrose for 2 weeks at 4°C. The demineralized specimens were dehydrated in ethanol, penetrated with xylene, and immersed in paraffin overnight using an automatic paraffin embedding machine. The paraffin sections measuring 4 μm-thickness were cut and mounted on aminopropyltriethoxysilane-coated slides. All tissue specimens were processed under sterile conditions.

Tooth movement procedure

Tooth movement was performed by the Waldo method (8). Briefly, a shortcut orthodontic elastic band (Rocky Mountain Morita, Tokyo, Japan) was inserted between the first and second molar of the maxilla under sodium pentobarbital anesthesia (1 ml/kg, i.p.). Since a previous report described that the appearance of rat osteoclasts reached a peak on the third day (9), tissue preparation was thus performed on the third day after tooth movement.

In situ hybridization

Preparation of RNA probes — Total RNAs were extracted from Wistar rat bone and spleen, and BALB/c mouse bone using the SV total RNA isolation system (Promega, Madison, WI, USA) and then a reverse-transcription polymerase chain reaction was performed using the SUPERSCRIPT™ One-Step RT-PCR with PLATINUM® Taq (Invitrogen, Carlsbad, CA, USA). The primer sets were designed to amplify RANKL, RANK, osteoprotegerin, TNFα, and IL-1β, respectively (Table 1). All selected regions in the mouse contain completely homologous sequences with those in the rat. The suitably digested PCR products were ligated into the pGEM-3Z Vector (Promega) to synthesize both anti-sense and sense probes. The ligated plasmids were then transformed into *Escherichia coli* DH5α competent cells (Takara, Tokyo, Japan) and therefore positive colonies were selected. The linearized plasmids were transcribed with T7 or SP6 polymerase and labeled with digoxigenin-UTP using the DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). All of the inserted DNA fragments were precisely confirmed by dideoxy sequencing (DYEnamic ET Terminator Cycle Sequencing Kit & sequencer; Amersham Biosciences Corp, Piscataway, NJ, USA).

Hybridization procedure — The hybridization procedure was performed as previously described by Yamaza *et al.* (10) with some slight modifications. Briefly, after deparaffinization and rehydration, the sections were fixed with 4% paraformaldehyde in diethylpyrocarbonate-treated phosphate-buffered saline for 10 min, incubated in 10 μg/ml proteinase K (Invitrogen) at 37°C for 30 min, and then acetylated with 0.25% (v/v) acetic anhydride at room temperature for 15 min. Following dehydration with ethanol, the

Table 1. Primers used for preparation of probes for *in situ* hybridization

Targeted gene	Primer sequence	Accession no.	Product size
RANKL	5′-CCC*AAGCTT*CAGATGGATCCT AACAGAATA-3′ 5′-GC*TCTAGA*AGTCTATGTCTTG AACTTTGAA-3′	AF187319	736
RANK	5′-CG*GGATCC*ATCATCTTCGGCG TTTACTACAGG-3′ 5′-G*GAATTC*CGTCCTAGAATCTC TGACTTCTGC-3′	AF019046	279
Osteoprotegerin	5′-CG*GGATCC*AGAGAGGATAAA ACGGAGACACAG-3′ 5′-G*GAATTC*GATTGAACCTGATT CCCTATCAT-3′	U94330	474
IL-1β	5-CCC*AAGCTT*GAACAACAAAAA TGCCTCGTGC-3′ 5′-GC*TCTAGA*GGTGAAGTCAACT ATGTCCCGACC-3′	M98820	402
TNFα	5′-AA*CTGCAG*TACTGAACTTCGG GGTGATTGGTCC-3′ 5′-G*GAATTC*CAGCCTTGTCCCTT GAAGAGAAC-3′	NM012675	295

Restriction compatible ends are italicized and additional flanking bases are underlined.

sections were pre-hybridized with hybridization solution without a probe at 37°C for 1 h. They were hybridized in a moist chamber with hybridization solution containing 1–2 μg/ml DIG-labeled RNA probes overnight at 42°C. The hybridization solution comprised 50% formamide, 5 × standard saline citrate, 2% blocking reagent, 0.1% *N*-lauroyl-sarcosine and 0.02% sodium dodecyl sulfate. Next, the sections were washed twice in 2 × standard saline citrate containing 50% formamide for 30 min at 42°C, three times in RNase buffer consisted of 0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA for 10 min at 37°C, followed by incubation with 20 μg/ml RNase A (Roche) for 30 min at 37°C to avoid any non-specific binding of the probe. They were then washed in RNase buffer for 15 min at 37°C, twice in 2 × standard saline citrate containing 50% formamide for 20 min at 42°C, 2 × standard saline citrate for 15 min at 37°C, and 0.1 × standard saline citrate for 15 min at 37°C. They were then further washed in phosphate-buffered saline and Tween (phosphate-buffered saline + 0.1% polyoxyethylene sorbitan monolaurate; Tween 20) for 15 min at room temperature, and incubated with 10% normal goat serum for 1 h at room temperature. Finally, the sections were treated with alkaline phosphatase-conjugated anti-DIG antibodies, and visualized with BM Purple (Roche).

Results

In situ hybridization of RANKL, RANK and osteoprotegerin

The positive signals of RANKL were detected in some multinucleated cells which were located along the alveolar bone surface. RANKL was also detected in osteoblasts, odontoblasts and PDLs (Fig. 1A–D). RANKL-positive multinucleated cells were also positive for tartrate-resistant acid phosphatase TRAP staining and were thus verified to be osteoclasts. RANKL-positive osteoclasts were located on the distal side of the alveolar bone surface facing the mesial side of each tooth root of the lower first molar. Meanwhile, RANKL-positive osteoblasts were observed on the mesial side of the alveolar bone (Fig. 1E). RANKL-positive PDLs showed no specific distribution pattern. Osteoblasts and chondrocytes in the chondyle of humerus were strongly positive for RANKL (11, 12), thus confirming that the *in situ* hybridization method used in this study actually detected RANKL mRNA (Fig. 1F–H).

We also performed *in situ* hybridization of RANK, the receptor for binding to RANKL, to confirm whether both RANKL- and TRAP-positive osteoclasts express this receptor. The positive signals of RANK were detected in multinucleated osteoclasts and mononuclear mesenchymal cells. RANKL-positive multinucleated osteoclasts concomitantly expressed RANK in the serial sections (Fig. 2).

Since osteoprotegerin is one of the crucial factors for the regulation of bone remodeling together with RANKL and RANK (2, 13), we examined the expression pattern of osteoprotegerin in periodontal tissue. The positive signals of osteoprotegerin were detected in almost all osteoblasts, PDLs and odontoblasts (Fig. 3). However, no osteoprotegerin-positive osteoclasts were observed.

No hybridization signals were detected in the control sections using the sense probe of RANKL, RANK, and osteoprotegerin (Fig. 1I).

In situ expression of RANKL, RANK and osteoprotegerin during tooth movement

Previous reports have already described that an orthodontic excessive force for tooth movement caused pathological bone resorption and induced an increase in the number of osteoclasts in the alveolar bone. We used the Waldo method to examine the changes in the expression pattern of RANKL, RANK and osteoprotegerin in the osteoclasts and other cells under pathological condition caused by drastic tooth movement (8).

Three days after the treatment, many inflammatory cells and multinucleated osteoclasts were observed in the periodontal tissue between the first and second molar where the elastic band for orthodontic purpose had been inserted. An inflammatory reaction is known to increase the osteoclast activity. Many inflammatory cells appeared in the periodontal tissue corresponding to the site where the elastic band was inserted, and therefore we did not examine this area

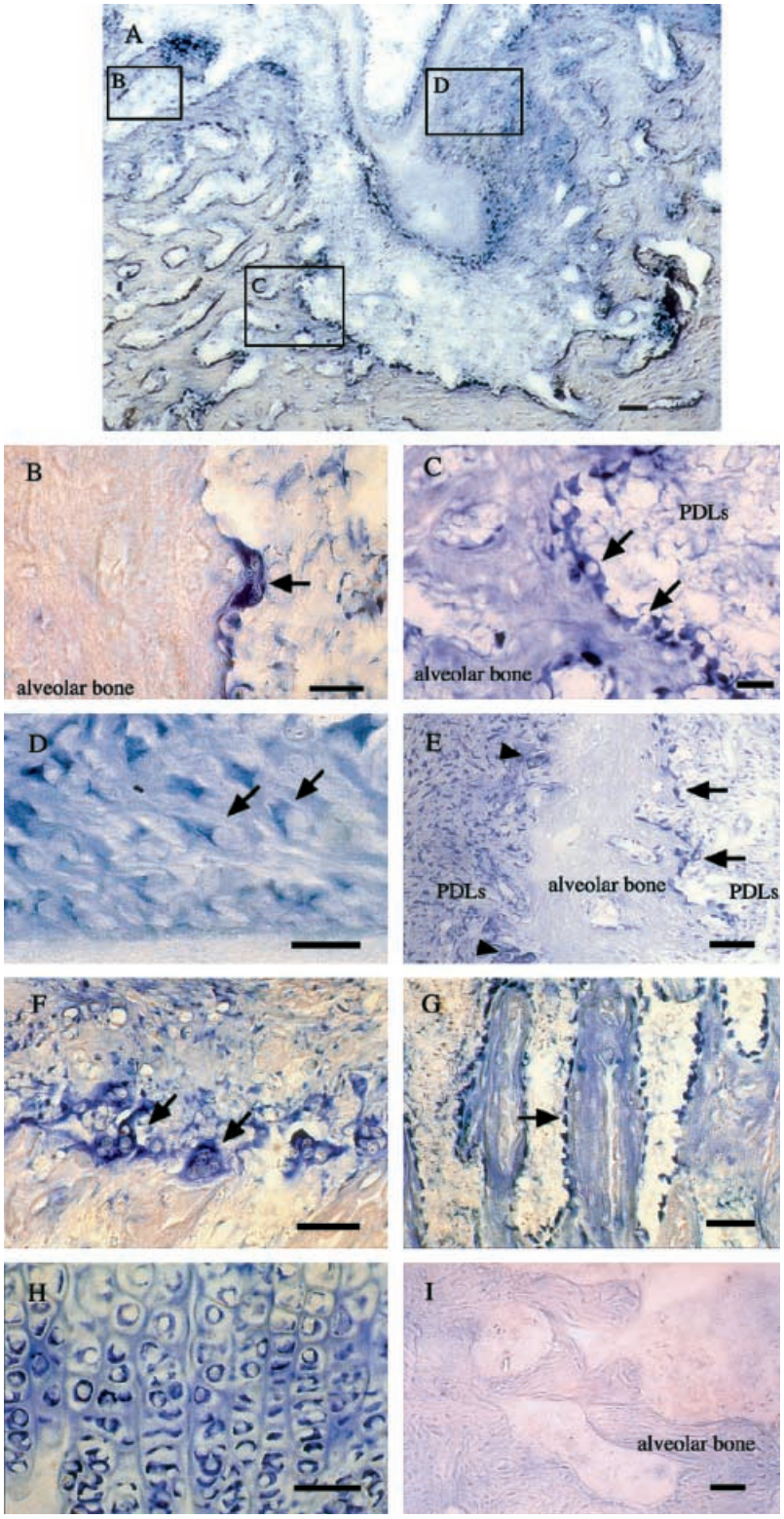

Fig. 1. (A) Low magnification of the *in situ* expression of RANKL mRNA in rat periodontal tissue. Positive signals appeared as a blue color which was observed in the cytoplasm. (B)–(D) A higher magnification of the areas encircled by a square in (A). Some osteoclasts (B, arrow), osteoblasts (C, arrows), and PDLs (D, arrows) clearly expressed RANKL mRNA. (E) The distribution of RANKL mRNA positive-osteoclasts and -osteoblasts in the alveolar bone in physiological condition. RANKL-positive osteoclasts were observed on the distal side of alveolar bone (arrowheads). Meanwhile RANKL-positive osteoblasts (arrows) were observed on the mesial side. (F)–(H) The signals of RANKL mRNA were detected in osteoclasts (F, arrows), osteoblasts (G, arrows) and chondrocytes (H). (I) No signals were observed in cells with a sense probe. A, E–I: bar = 200 μm; B, C, D: Bar = 100 μm.

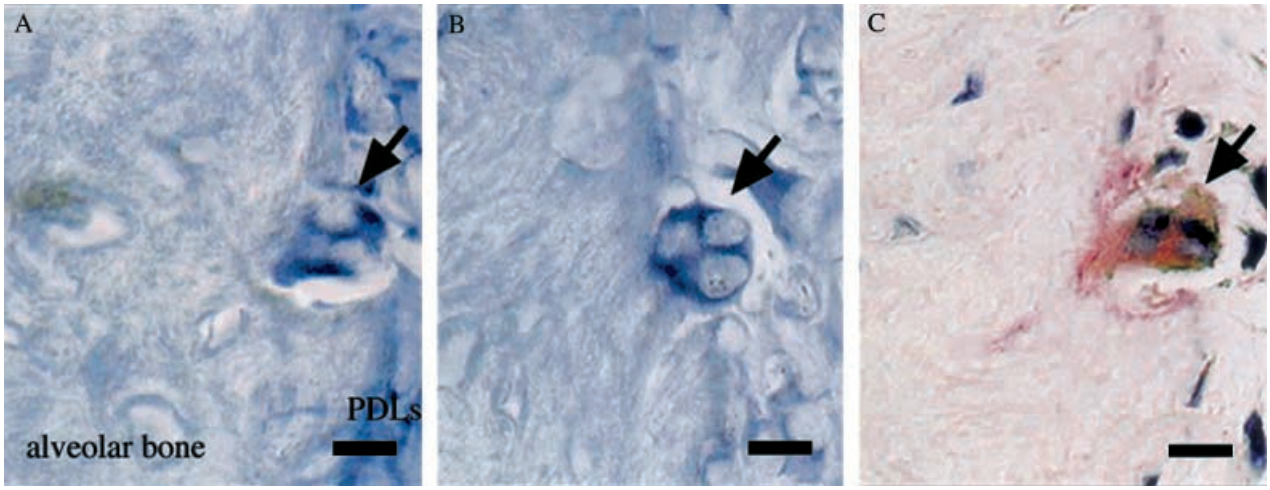

Fig. 2. RANKL and RANK mRNA expressions in osteoclasts in serial sections. Both RANKL (A) and RANK (B) mRNA were concomitantly expressed in the same osteoclast (arrows). This osteoclast was also positive for TRAP staining (C). Bar = 50 μm.

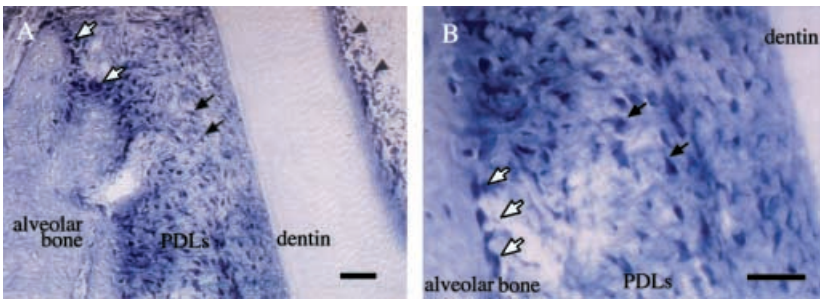

Fig. 3. Osteoprotegerin mRNA expression in rat alveolar bone tissue. Osteoblasts (white arrows), PDLs (black arrows), and odontoblasts (arrowheads) expressed osteoprotegerin mRNA under physiological conditions. No positive signals were detected in the osteoclasts or osteocytes. A higher magnification of each osteoblasts and fibroblasts were showed in (B). Bar = 200 μm.

(Fig. 4A–B). The number of RANKL-positive osteoclasts increased on the mesial side of alveolar bone surfaces directed toward each root of the first molar where active bone resorption occurred. The number of osteoclasts also increased on the distal side of the alveolar bone surfaces in each root of the second molar. Interestingly, most of the osteoclasts expressed RANKL mRNA after the tooth movement (Figs 4C and D). These RANKL-positive osteoclasts also concomitantly expressed RANK mRNA as seen under physiological conditions.

The distribution pattern of RANKL-positive osteoblasts also changed, and they were observed on the distal bone surface in each first molar root, although no numerical change was found. The number of RANKL-positive PDLs also increased. However, no specific distribution pattern of these cells was recognized (data not shown).

No change in the number of osteoprotegerin-positive osteoblasts, odontoblasts or PDLs was observed after the treatment.

In situ hybridization of IL-1β and TNFα

It was recently reported that IL-1β and TNFα play important roles in the pathogenesis of inflammatory osteolysis *in vitro* (14, 15). Therefore, it is reasonable to assume that these cytokines appear to induce RANKL. We performed *in situ* hybridization for IL-1β and TNFα in osteoclasts, osteoblasts and PDLs in the alveolar bone to examine the relationship of these cytokines to the RANKL expression.

Under physiological conditions, the positive signals of IL-1β and TNFα were detected in some osteoblasts. PDLs were also positive for IL-1β and TNFα. Neither any IL-1β- nor TNFα-positive osteoclasts were noted (data not shown).

Under pathological conditions, IL-1β and TNFα were clearly detected in some osteoclasts. PDLs were also positive for these cytokines, and were mainly localized at areas of active bone resorption, and were also closely associated with osteoclasts (Fig. 5).

Discussion

This study showed that osteoclasts expressed RANKL mRNA by means of the *in situ* hybridization method. The expression of RANKL mRNA in osteoclasts has already been reported (11, 16). Previous studies showed that RANKL was expressed in cell membrane of ruffled border and the clear zone cytoplasm by immunoelectron microscopic technique (17, 18). Our present study and previous reports may thus indicate that osteoclasts are a potent producer of RANKL. Since osteoclasts express RANK, the receptor of RANKL, on their cell surface (19), it is reasonable to assume that osteoclasts have a capability of the RANKL–RANK autocrine mechanism. This hypothesis was also described by Myers *et al.* (20). However, the functional roles of RANKL–RANK

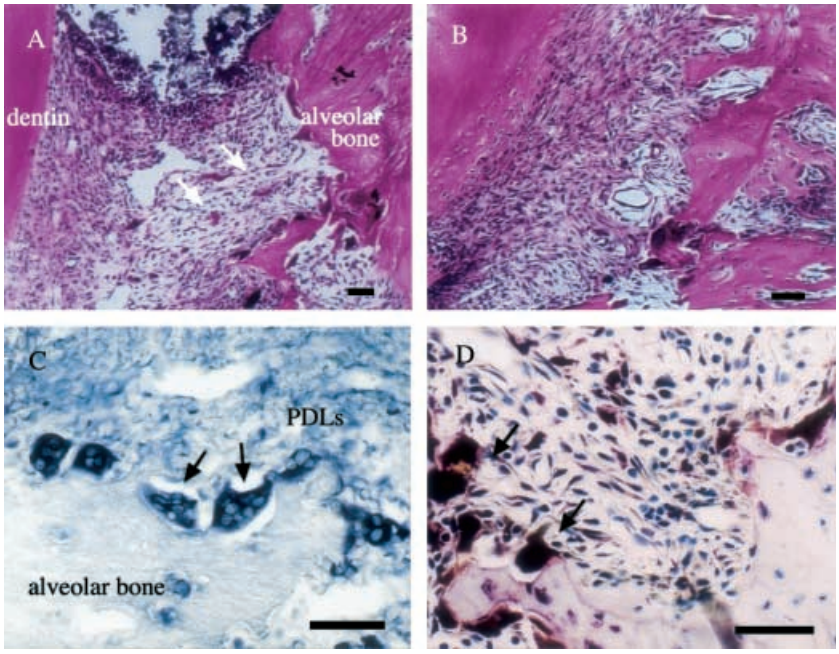

Fig. 4. RANKL mRNA expression in the osteoclasts after experimental tooth movement. Many inflammatory cells (arrows) appeared in the periodontal tissue corresponding to the site where the elastic band was inserted (A, hematoxylin and eosin staining). The examined areas showed no inflammatory reaction (B, hematoxylin and eosin staining). Experimental tooth movement caused an increase in the number of RANKL-positive (C) and TRAP-positive osteoclasts (D) (arrows). Bar = 200 μm.

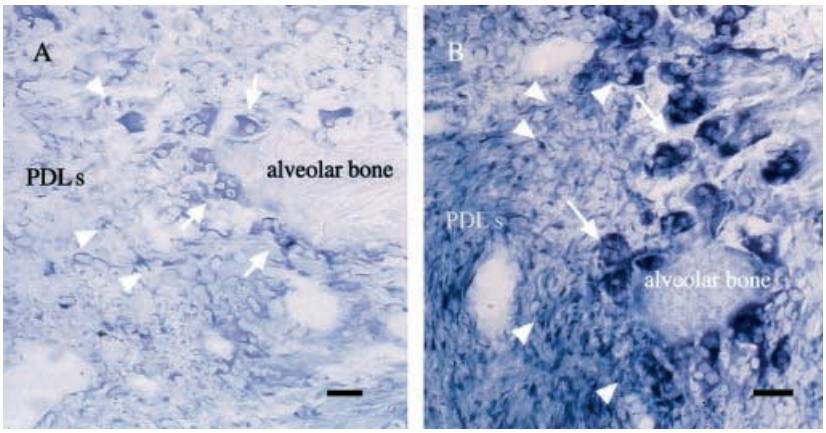

Fig. 5. In situ expressions of IL-1β and TNFα after tooth movement. IL-1β (A) and TNFα mRNA (B) were detected in the osteoclasts (arrows) after tooth movement. PDLs expressing cytokines (arrowheads) were closely associated with osteoclasts. Bar = 250 μm.

autocrine activity in osteoclasts are unclear at present. Fuller *et al.* reported that RANKL induced drastic change in osteoclast motility and spreading, and increased osteoclast survival through inhibition of apoptosis of mature osteoclasts (21). It is well known that the number of osteoblasts decreases with aging. Decrease of the number of PDLs caused by apoptosis is also reported. Meanwhile, no remarkable change in the number of osteoclasts through the life has ever been reported. Both osteoblasts and PDLs were positive for RANKL mRNA, as a main source of this protein, and therefore the osteoclastic function theoretically decreases as the number of osteoblasts and PDLs decreases. This paradoxical phenomenon might be explained by the RANKL–RANK autocrine mechanism in the osteoclast. The autocrine secretion of RANKL by osteoclasts thus seems to maintain the function of osteoclasts in the physiological conditions. Ikeda *et al.* has reported that RANKL consisted of three different isoforms (22). RANKL 3 in these isoforms did not have any transmembrane domain and therefore was considered to be a soluble form. RANKL 3 released from osteoclasts may be responsible for the RANKL–RANK autocrine mechanism.

Osteoclasts under physiological conditions, expressed no mRNA signals of IL-1β and TNFα. Itoh *et al.* also reported that mature osteoclasts did not express IL-1β and TNFα *in vitro* (23). Meanwhile, these cytokines were expressed in the osteoclasts under pathological conditions. A previous report described that osteoclasts produced IL-1β and TNFα by themselves, and these cytokines were considered to act as an important mechanism for the autocrine regulation of proliferation and the differentiation in osteoclasts (24–26). In addition, the transduction pathway of these cytokines to activate osteoclasts has been shown to be independent of the RANKL–RANK pathway (15). It therefore seems likely that IL-1β and TNFα expressed in osteoclasts under the pathological conditions act as an autocrine regulation of osteoclast proliferation and differentiation in order to adapt to the drastic changes in the environment induced by excessive orthodontic force.

The number of both RANKL and cytokines expressing osteclasts increased when excessive orthodontic force was applied to periodontal tissue. The localization of osteoclasts also changed in comparison to the physiological condition. These findings indicate that osteoclasts in the periodontal tissue actually reacted against excessive orthodontic force, and the active resorption of alveolar bone occurred on the distal side for urgent adaptation (Fig. 6). The autocrine secretion of RANKL, IL-1β and TNFα in the osteoclasts may thus be heightened and also may play an important role in the active resorption of alveolar bone

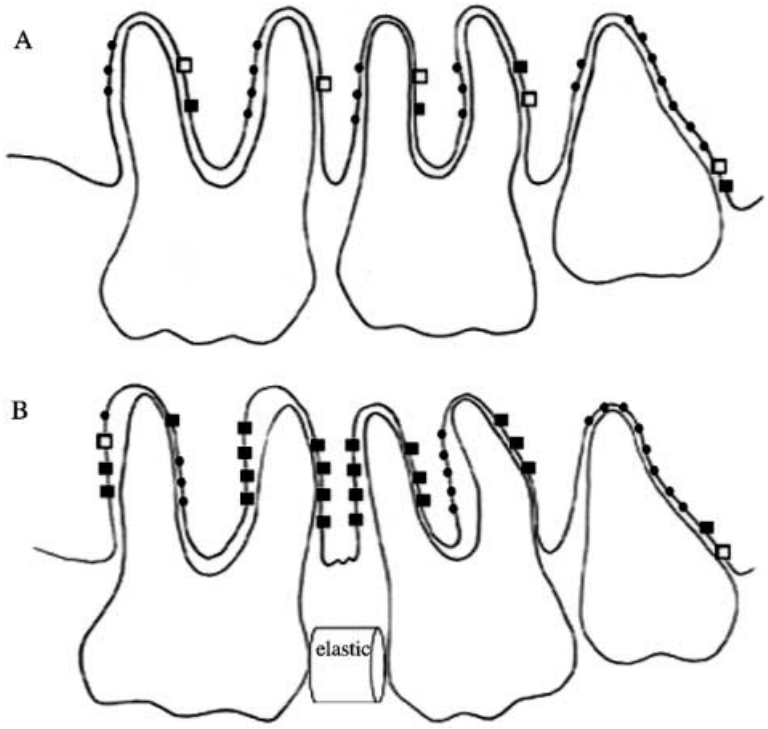

Fig. 6. The change in the distribution pattern of RANKL mRNA-positive osteoclasts and osteoblasts before (A) and after (B) tooth movement. The black boxes and white boxes symbolize the RANKL-positive and -negative osteoclasts, respectively. The circles symbolize RANKL-positive osteoblasts. (A) RANKL mRNA-positive osteoclasts localized on the distal side of alveolar bone facing the mesial side of each tooth in physiological condition. Meanwhile, the osteoblasts located on the opposite side. (B) Experimental tooth movement caused an increase in the number of osteoclasts. The first molar received a reversal of the orthodontic force compared to physiological conditions, and therefore the distribution of osteoclasts changed to the mesial surface of the alveolar bone.

under pathological conditions. However, the RANKL expression in osteoclasts was seen under both physiological and pathological conditions, whereas, in contrast, IL-1β and TNFα expression was only observed under the pathological conditions. These findings may indicate that the osteoclastic activating autocrine pathway by IL-1β and TNFα is therefore different from the RANKL–RANK autocrine mechanism.

In this study, PDLs clearly expressed RANKL and osteoprotegerin under the physiological conditions. However, the distribution of these cells changed during tooth movement, and was localized at areas of active bone resorption in association with the osteoclasts. These PDLs also expressed IL-1β and TNFα under pathological conditions, as well as in a physiological environment. Previous studies described that PDLs synthesized both RANKL and osteoprotegerin, and also participated in the regulatory mechanism of the osteoclastic function (5, 27). Therefore, PDLs expressing RANKL, IL-1β and TNFα may control the osteoclast function in areas of bone resorption.

In conclusion, we demonstrated that osteoclasts in the periodontal tissue expressed RANKL, IL-1β and TNFα under both physiological and pathological conditions, and these factors seem to regulate the osteoclast function by some autocrine mechanisms. However, further examinations are required to confirm the presence of an autocrine osteoclast activating mechanism in the osteoclast itself.

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