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Force-Induced Osteoclast Apoptosis In Vivo Is Accompanied by Elevation in Transforming Growth Factor β and Osteoprotegerin Expression

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ABSTRACT

The mechanism controlling the disappearance of osteoclasts from bone surfaces after bone resorption in vivo is largely unknown. This is because there is no suitable experimental system to trace the final fate of osteoclasts. Here, we used an experimental model of tooth movement in rats to show that preexisting osteoclasts disappeared from the bone surface through apoptosis during a force-induced rapid shift from bone resorption to formation. On the distal alveolar bone surface of the maxillary molar in growing rats, many mature osteoclasts were present. When light tensional force was applied to the bone surface through an orthodontic appliance, these preexisting osteoclasts gradually disappeared. One day after the application of force, about 24% of the osteoclasts exhibited apoptotic morphology and the proportion of apoptotic cells was increased to 41% by day 2, then decreased afterward. These changes were undetectable on the control distal alveolar bone surface, which is free from tensional force. As shown by in situ hybridization, a marked increase in transforming growth factor β1 (TGF-β1) and osteoprotegerin (OPG) messenger RNA (mRNA) was observed in the stretched cells on the tensioned distal bone surface, simultaneously with the loss of osteoclasts. Both of these factors are known to have a negative effect on osteoclast recruitment and survival. As early as 2 days after force application, some of these stretched cells were identified as cuboidal osteoblasts showing intense signals for both factors. Our data suggest there may be a sequential link in tensional force applied on the bone lining cells, up-regulation of TGF-β1/OPG, and disappearance of osteoclasts. (J Bone Miner Res 2000;15:1924–1934)

Key words: osteoclasts, apoptosis, transforming growth factor β, osteoprotegerin, in situ hybridization

INTRODUCTION

OSTEOCLASTS ARE bone resorbing cells of hematopoietic origin. Mature osteoclasts do not synthesize DNA and have no mitotic activity. To date, many researchers have noticed that isolated mature osteoclasts from bone are fragile and easily die in culture, predominantly by apoptosis, not necrosis.[1–4] This apoptotic death process is significantly promoted when serum in media is depleted or the cells are treated with various reagents, such as bisphosphonates.[5–13] In vivo, osteoclasts rapidly disappear from the bone surface in a reversal phase of bone remodeling, that is, a turnabout from bone resorption to bone formation. The disappearance of osteoclasts has been hypothesized with apoptosis as the

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ultimate fate of the cells.[2,14,15] However, the kinetics of disappearance of osteoclasts and the mechanism controlling the event are largely unknown.

In growing rats, the upper molars show physiological distal movement.[16,17] Many osteoclasts are present on the distal alveolar bone surface of the tooth and resorb the bone, whereas osteoblasts are aligned on the mesial alveolar bone surface where new bone formation proceeds. As we previously reported, the remodeling phase of the distal alveolar bone surface was changed from bone resorption to bone formation after the application of light tensional force.[18] In this study, we showed that preexisting osteoclasts on the distal alveolar bone surface disappeared with the morphological hallmarks of apoptosis. In response to mechanical force, cells of periodontal tissue alter expression of various genes.[19,20] Some changes, particularly in cytokines, which control bone remodeling, could be involved in the rapid disappearance of osteoclasts at the focal bone site. Studies on in vitro osteoclast culture have shown that interleukin-1 (IL-1), colony-stimulating factor 1 (CSF-1; also called macrophage-CSF [M-CSF]), and receptor activator of NF-κB ligand (RANKL; also called osteoprotegrin ligand [OPGL], tumor necrosis factor–related activation-induced cytokine [TRANCE], and osteoclast differentiation factor [ODF]) support osteoclast survival,[21] while transforming growth factor β (TGF-β) and OPG (also called osteoclastogenesis inhibitory factor [OCIF] and TNF receptor-like molecule 1 [TR1]) have been reported to inhibit their survival.[9,22–24] Here, we examined changes in expression of TGF-β and OPG by in situ hybridization and compared those to disappearance of osteoclasts from the bone surface during the force-induced bone remodeling transition period.

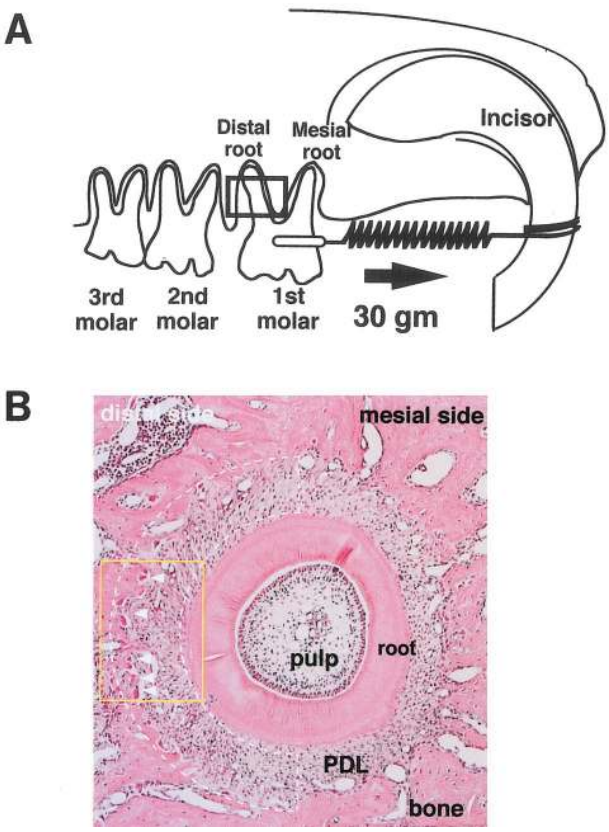


FIG. 1. The experimental tooth movement in rats. (A) Schematic representation of orthodontic mechanics to move the first maxillary molar to the mesial direction. A closed-coil spring was set between the maxillary incisor and the maxillary first molar with initial contractile force of 30 g. (B) Horizontal section of the distolingual root of the first maxillary molar in a control rat (H&E staining). On the distal alveolar bone surface, many osteoclasts, as recognized by large eosinophilic cells, were found (white arrowheads). Changes in numbers of osteoclasts in the distal half of the periodontal tissue (area enclosed by the dotted line in the figure) were counted in this study. Histological changes in the marked area with the solid yellow line were taken by photo and shown in Figs. 2 and 4.

MATERIALS AND METHODS

Experimental tooth movement

Twenty-eight male Wistar rats (5 weeks old) with an average body weight of 152.5 ± 6.3 g (mean ± SD) were used. They were fed ground laboratory chow (Oriental Yeast Co., Ltd., Tokyo, Japan) and given water ad libitum.

The right first maxillary molar was moved mesially as described by Hashimoto et al.[18] Briefly, an Elgiloy® closed-coil spring (0.007 in × 0.036 in; RMMC, Tokyo, Japan) was set between the incisors and the right maxillary first molar with an initial contractile force of 30 g. The spring edge of the molar side was fixed with a small clamp that was held by shallow cavities made on the buccal and labial surface of the molar, while the incisor side was fixed with a ligature wire that encircled the neck of the incisors. Figure 1A shows a schematic diagram of the orthodontic mechanics used in this study. The left maxillary first molar acted as a control tooth where only a clamp was set without fixing a spring. At time 0 (before orthodontic tooth movement) and every 24 h for up to 3 days after the application of force, four animals were killed under ether anesthesia. The animals were fixed with freshly made 4% paraformaldehyde (PFA) in Dulbecco's phosphate-buffered saline (PBS; pH 7.4) by cardiac perfusion.

Preparation of sections

Maxillas of the perfused rats were decalcified with 10% EDTA in diethylpyrocarbonate-treated H_2O (pH 7.4) at 4°C for 10 days. The samples were embedded in paraffin. Fifty serial horizontal sections (6-μm thickness) were obtained at the level of the first molar bifurcation to a depth of 300 μm by a microtome.

Sections were subjected to terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate–digoxigenin (dUTP-DIG) nick end labeling (TUNEL) using a peroxidase-based in situ apoptosis detection kit (ApopTag Plus; Oncor Co., Ltd. Gaithersburg, MD, U.S.A.),[25] followed by tartrate-resistant acid phosphatase (TRAPase) stainings[26] and counterstaining with methyl green.

For immunofluorescence staining of cathepsin K, sections were first incubated with 5% skim milk in PBS and sequentially treated with rabbit polyclonal anti-mouse cathepsin K immunoglobulin G (IgG)[27] and tetramethylrhodamine isothyocyanate (TRITC)-labeled goat anti-rabbit IgG (EY Laboratories, San Mateo, CA, U.S.A.). The sections were counterstained with 1 μg/ml of Hoechst 33258 in PBS and examined with an Axiophoto fluorescence microscope (Zeiss, Oberkochen, Germany). Immunohistochemistry for pro-osteocalcin was performed as described previously,[18] using rabbit polyclonal antibody against the propeptide of rat osteocalcin.

Identification of apoptotic, necrotic, and intact osteoclasts

Apoptotic osteoclasts were identified using the criteria defined by Hughes et al.,[6] that is, by the presence of chromatin condensation and/or nuclear fragmentation in osteoclasts showing concentration of TRAPase-positive cytoplasm and loss of adhesion to the underlying bone matrix. About half of the apoptotic osteoclasts, determined by nuclear morphology, were TUNEL positive. Necrotic osteoclasts were defined as the cells with nuclear and cytoplasmic swelling and pallor without nuclear disintegration.

The osteoclasts on the alveolar bone surface and in the periodontal ligament of the distal half area around the distolingual root were counted in each section. Figure 1B shows the area examined. Values from eight sections at 30-μm intervals in the examined area were averaged for each animal.

In situ hybridization

Rat TGF-β1 complementary DNA (cDNA) in pUC118 (obtained from Riken, Saitama, Japan) was digested with *Eco*RI/*Bgl* II. A 0.3-kilobase (kb) DNA fragment including bases 995-1317 in the published TGF-β1 cDNA sequence (Qian et al.,[28] GenBank accession number X52498) was subcloned into a pBluescript KS vector. A 0.4-kb DNA fragment of human OPG cDNA (bases 91–468 in the published cDNA sequence by Simonet et al.,[29] accession number U94332) was obtained by a reverse-transcription polymerase chain reaction (RT-PCR) from human thyroid total RNA and subcloned into a pBluescript KS vector.

Using each partial cDNA as a template, DIG-11-UTP–labeled single-stranded antisense and sense RNA probes were prepared with a DIG-RNA Labeling Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The specificity of these probes was confirmed by filter hybridizations. In situ hybridization was performed by the method of Miyazaki et al.[30] The hybridization signal was detected as blue indolyl precipitation, using an alkaline phosphatase–based DIG detection kit (Roche Diagnostics). Counterstaining was omitted.

Recombinant epitope-tagged OPG

A partial OPG cDNA encoding an amino acid sequence of Phe3-Tyr358 in mature human OPG was prepared by RT-PCR using human thyroid total RNA as a template with the following primer sets: sense primer, 5′-AACCA*GGATCC*GTTTCCTCCAAAGTACCTTCAT (*Bam*HI site is underlined); antisense primer, 5′-TCTGG*TCTAGA*TTGTACATTGTGAAGCTGTGA (*Xba* I site is underlined). A *Bam*HI/*Xba* I fragment of the RT-PCR product was ligated with pcDNA3.1 (−)/Myc-His B-derived *Xba* I/*Sma* I fragment, which included a series of sequences of myc-tag, His6-Tag, stop codon (TGA), and poly(A) attachment signal (Invitrogen, Carlsbad, CA, U.S.A.), and then was inserted in the *Bam*HI/*Bgl* II site of a baculovirus transfer vector pACGP67A (Pharmingen, San Diego, CA, U.S.A.). The *Bgl* II edge of the transfer vector was blunt-ended before the ligation. Sf9 insect cells were cotransfected with the OPG-containing transfer vector and BaculoGold DNA (modified baculovirus DNA; Pharmingen). The recombinant histidine-tagged OPG (OPG-myc) secreted in media was purified with Ni^{2+} nitrile-triacetic acid resin (Quiagen, Valencia, CA, U.S.A.). The recombinant protein with an apparent molecular weight of 55 kDa reacted well with anti-myc antibody (Invitrogen) and anti-OPG peptide antibody (IMG-103; Imgenex, San Diego, CA, U.S.A.) in immunoblotting, which was performed as described previously.[18]

In vitro osteoclast formation and effect of OPG-myc on osteoclasts

Murine osteoclast-like cells were prepared on collagen gel plates by the coculture of murine calvaria–derived stromal cells and bone marrow cells as described by Jimi et al.[31] On day 6 of coculture, osteoclast-like cells together with stromal cells were harvested with 0.1% collagenase treatment (Worthington Biochemical, Freehold, NJ, U.S.A.) and resuspended in fresh α-minimum essential medium (α-MEM; Gibco, Gaithersburg, MD, U.S.A.) containing 10% fetal calf serum (FCS). The cells were placed onto dentine disks set in wells of a 96-well plate. After 2 h of preincubation, the dentine disks were washed briefly and transferred to 48-well plates. The cells were cultured for 48 h in 400 μl of α-MEM/10% FCS in the presence or absence of various amounts of OPG-myc, recombinant human RANKL (rh-RANKL; PeproTechEC, London, U.K.; reviewed in Refs. 32–35) or with both factors.

After incubation, attached and nonattached cells were fixed with 4% PFA/PBS for 1 h and stained for TRAPase activity followed by nuclear staining with Hoechst 33258 (1 μg/ml in PBS). The nonadherent cells were centrifuged and trapped onto nitrocellulose membranes set in a 96-well vacuum device (ATTO, Tokyo, Japan). Cells were examined under a fluorescence microscope. Apoptotic cells were detected according to the criteria described above. Some cell preparations were subjected to a single-cell gel electrophoresis assay (Comet assay; Trevigen, Gaithersburg, MD, U.S.A.) to detect DNA fragmentation.[36] Briefly, cells were fixed with methanol, embedded in 0.4% low-temperature melting agarose, and put on a glass slide. The immobilized cells in the agarose were subjected to electrophoresis at 1 V/cm for 5 minutes, and DNA was stained with SYBR® Green. Fragmented nuclear DNA migrated from the cells after the electrophoresis and exhibited a cometlike pattern.

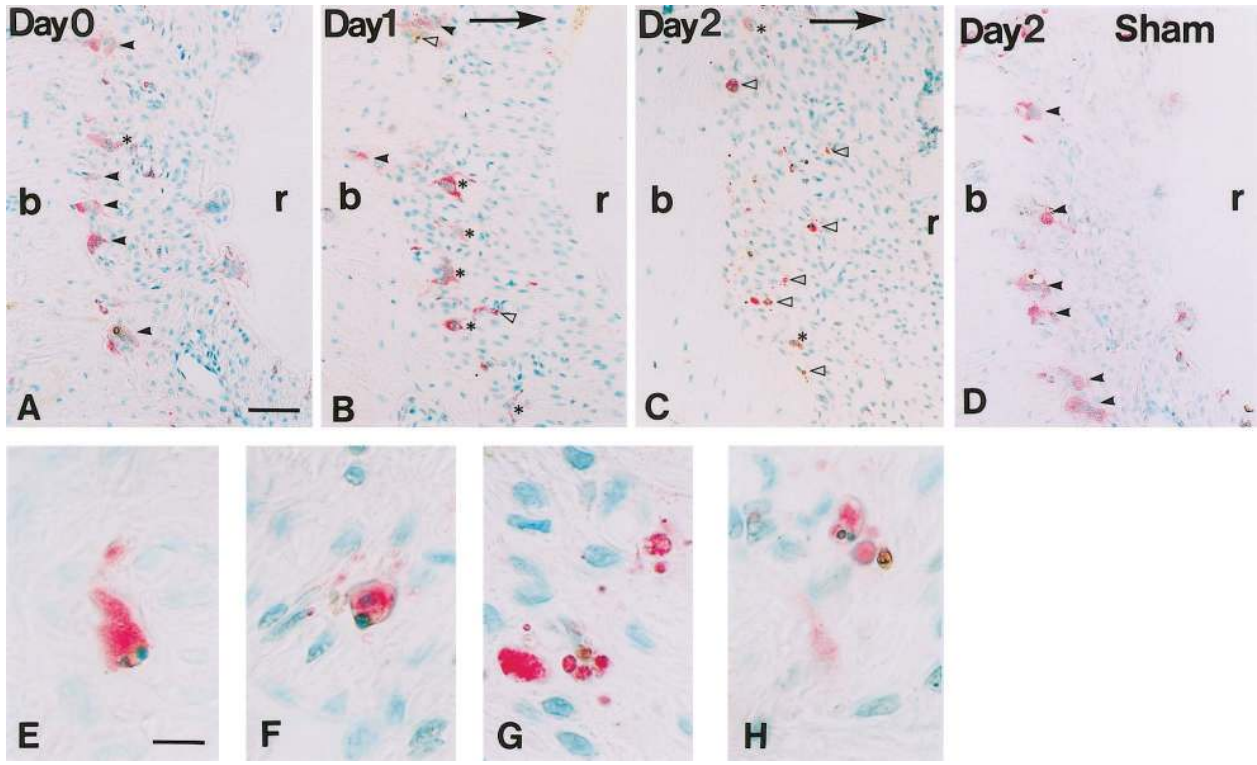


FIG. 2. Changes in osteoclasts at the distal alveolar bone surface after tensional force application. Sections were double stained with TUNEL (positive in brown) and TRAPase (positive in red), and then counter stained with methyl green. (A–C) Changes observed from day 0 to day 2, as viewed by low power magnification. Intact TRAPase-positive multinuclear cells (closed arrowheads) disappeared from the bone surface after force application. Concomitantly with disappearance, some osteoclasts separated from the bone surface (*) and some showed typical apoptotic morphology, with cell shrinkage and nuclear condensation (open arrowheads). Large arrows in B and C show the direction of force applied. (D) Distal alveolar bone surface of the left maxillary first molar on which only a clamp was set (sham operated, day 2). Without tensional force application, osteoclast apoptosis was not induced. (E–H) High power magnification of typical apoptotic osteoclasts appeared on day 2. b, alveolar bone; r, dental root. Bar = 50 μm in A–D. Bar = 10 μm in E–H.

Statistical analysis

The statistical differences among groups were evaluated using one-way analysis of variance (ANOVA). Fisher's protected least significant difference test was used to identify differences between the groups when ANOVA indicated that a significant difference existed.

RESULTS

Change in number of apoptotic osteoclasts

Before application of force, many TRAPase-positive multinuclear osteoclasts were observed on the distal alveolar bone surface of the first maxillary molar root (Figs. 1B and 2A). Only 1.4% of them were classified as apoptotic cells at this stage (Table 1, day 0). The number of intact osteoclasts significantly diminished within 3 days of tensional force application. On day 1, preexistent osteoclasts showed a tendency to detach from the bone surface and a significant number of osteoclasts (23.6%) exhibited apoptotic morphology (Fig. 2B and Table 1). On day 2, the proportion of apoptotic osteoclasts increased to 41.1% but had decreased by day 3 (Fig. 2C and Table 1). When more apoptotic osteoclasts were observed, a greater number of osteoclasts disappeared during the next 24 h. This probably indicates that most of the apoptotic osteoclasts are scavenged within 24 h. The apoptotic osteoclasts lost bone adhesion and showed cytoplasmic contraction. Some of them showed membrane fragmentation (Figs. 1E–1H). The rapid apoptotic disappearance of osteoclasts was not observed on the distal alveolar bone surface of the left molar to which a clamp was attached without a spring (Fig. 2D).

To further confirm the identity of cell types susceptible to apoptosis in this model, we stained the sections with anti-cathepsin K antibody. Cathepsin K is a cysteine proteinase abundantly expressed in osteoclasts but not in osteoblasts or fibroblasts.[27,37–39] As shown in Figs. 3A and 3B, many cathepsin K–positive multinuclear cells were observed on the distal alveolar bone surface in the control animals. Two days after application of the force, apoptotic cells with small condensed nuclei showed apparent immunoreactivity of cathepsin K (Figs. 3C and 3D). Thus, under the experimental

TABLE 1. ENUMERATION OF OSTEOCLASTS IN THE DISTAL PERIODONTAL AREA AFTER FORCE APPLICATION ONTO THE MAXILLARY FIRST MOLAR

	Day 0	*Day 1*	*Day 2*	*Day 3*
Total number of osteoclasts in the area	14.4 ± 1.2	11.2 ± 0.7*	9.6 ± 0.8*	2.7 ± 0.4*,†,‡
Intact bone-associated osteoclasts	13.5 ± 1.0	4.7 ± 0.8*	2.1 ± 0.6*,†	0.8 ± 0.2*,†
Intact non–bone-associated osteoclasts	0.7 ± 0.2	3.9 ± 0.2*	3.7 ± 0.2*	1.1 ± 0.2†,‡
Apoptotic osteoclasts[a]	0.2 ± 0.1	2.6 ± 0.1*	3.9 ± 0.2*,†	0.9 ± 0.4†,‡
Percent of apoptotic osteoclasts in the area	1.4 ± 0.9	23.6 ± 2.2*	41.1 ± 1.8*,†	28.8 ± 7.5*

The TRAPase-positive multinuclear cells (osteoclasts) localized in the distal periodontal tissue of the distopalatal root of the maxillary first molar (the marked area in Fig. 1B) were counted. Values from eight sections at 30-μm intervals were averaged for each animal and the values in the table represent the mean ± SEM from four individual animals in each group. Apoptotic osteoclasts were identified by the criteria defined by Hughes et al.[6]

[a] Most of the apoptotic osteoclasts were away from the bone surface.

* Significantly different from day 0, $p < 0.05$; † significantly different from day 1, $p < 0.05$; ‡ significantly different from day 2, $p < 0.05$.

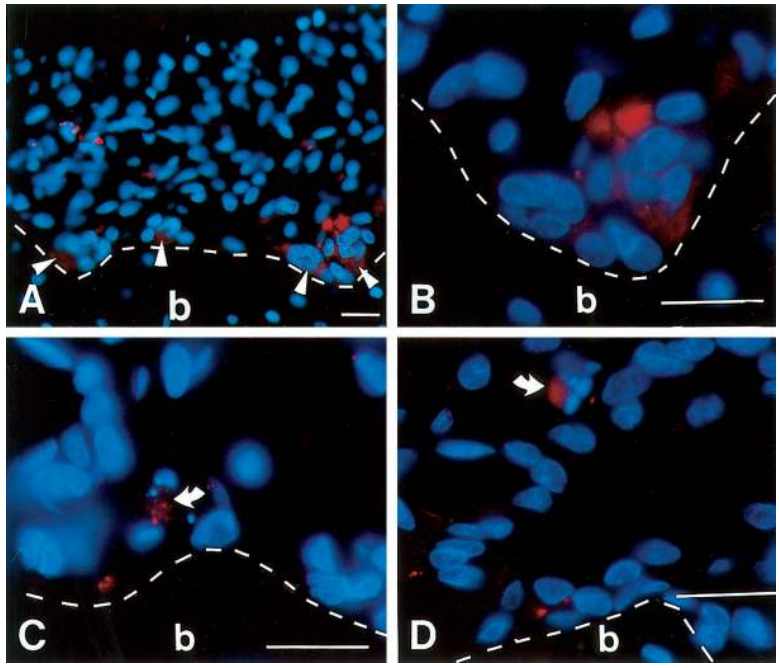


FIG. 3. Immunofluorescence staining of cathepsin K, followed by nuclear staining with Hoechst 33258. (A) Many cathepsin K–positive multinuclear cells (indicated in red TRITC fluorescence, arrowheads) were assembled on the distal alveolar bone surface before force application. (B) High power magnification of the left bottom area in A. (C and D) High power magnification of the apoptotic cells (arrows) appeared on day 2. The apoptotic cells, showing condensed cell nuclei, were positive for cathepsin K immunoreactivity. b, bone. Bar = 20 μm.

conditions employed, osteoclasts were most susceptible to apoptosis, whereas the other cell types in the periodontal tissue were morphologically intact.

We applied light force to avoid periodontal tissue inflammation. As expected, no apparent infiltration of inflammatory cells such as neutrophils and lymphocytes was found in the section, after hematoxylin and eosin (H&E) staining (data not shown).

Changes in expression of TGF-β1 and OPG

Next, we examined expression of messenger RNAs (mRNAs) for TGF-β1 and OPG, which have been reported to inhibit osteoclast survival in vitro,[9,23–24] by in situ hybridization. Before application of force, the TGF-β1 mRNA signal was weakly found in the distal alveolar bone surface lining cells and in the periodontal ligament cells (Fig. 4A). Osteoclasts also showed a weak TGF-β1 mRNA signal, consistent with previous reports.[40,41] The expression of TGF-β1 mRNA was elevated greatly in stretched bone lining cells 1 day after force application (Fig. 4B). The signals became more intense on day 2 and were confined to cuboidal cells aligned on the tensioned bone surface (Fig. 4C).

As with TGF-β1, the expression of OPG mRNA was enhanced in the bone lining cells after force application. On day 0, OPG mRNA was found in the periodontal ligament cells and in some bone lining cells but not in osteoclasts (Fig. 4D). On day 1, the signal was apparent in the stretched cells on the bone surface and became more intense in the cuboidal cells appearing on day 2 (Figs. 4E and 4F). In serial sections, these cuboidal cells showed a positive immunoreactivity for pro-osteocalcin, indicating they were mature osteoblasts (Fig. 4G). Thus, expression of TGF-β1

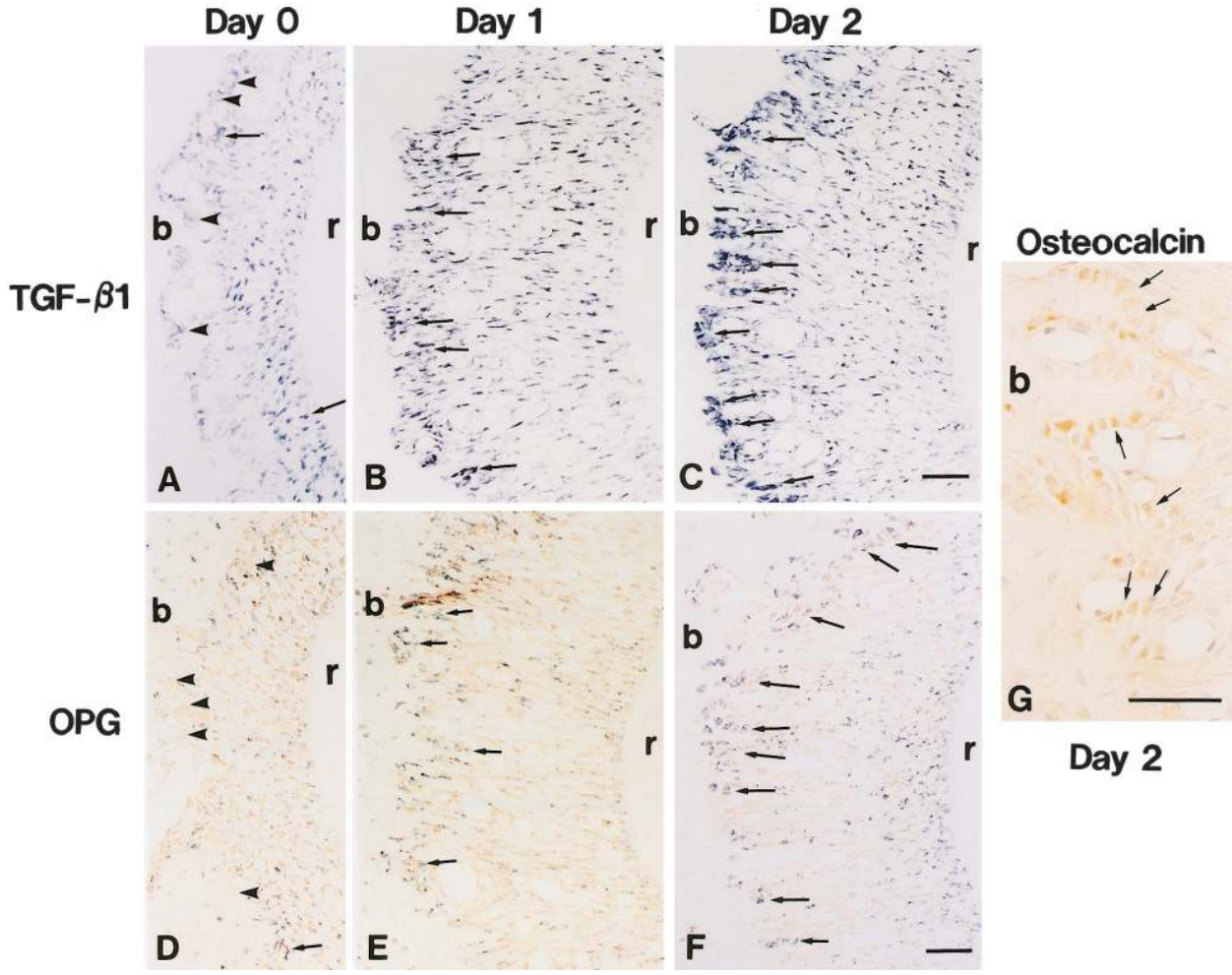


FIG. 4. Changes in expression of mRNAs for TGF-β1 and OPG at the distal alveolar bone surface after tensional force application. Sections were stained with DIG-UTP–labeled antisense-RNA probes for (A–C) TGF-β1 and (D–F) OPG, respectively. The positive hybridization signals were shown in blue as the bromo-chloro-indolyl precipitation. After force application, intensity of the TGF-β1 signal significantly increased in cells on the bone surface (arrows). Expression of TGF-β1 in the fibroblastic periodontal cells near the tooth root (r) was not significantly altered, compared with bone surface cells. Similar to TGF-β1, expression of OPG was enhanced in cells on the bone surface by tensional force application (arrows). (G) Immunohistochemical staining with antiosteocalcin propeptide antibody. Pro-osteocalcin (in brown) was confined to cuboidal cells appearing on the distal alveolar bone surface after 2 days of tensional force application (arrows). Closed arrowheads indicate intact osteoclasts. b, bone; r, tooth root. Bar = 50 μm.

and OPG was well correlated with differentiation of the bone lining cells to osteoblasts at the tensioned alveolar bone surface. Supporting this notion, expression of TGF-β1 and OPG mRNA was observed in pro-osteocalcin–positive osteoblasts on the mesial alveolar bone surface in a normal rat (5 weeks old, before force application; Figs. 5A–5C). In contrast to the distal alveolar bone surface, no osteoclasts were found in the mesial site. Moreover, we could not find osteoclasts localized adjacent to cuboidal osteoblastic cells with intense OPG mRNA signals in jaw and calvarial bone sections (data not shown). Authenticity of the hybridization signals was tested using sense-RNA probes for TGF-β1 and OPG mRNA, respectively (Figs. 5D and 5E). No apparent labeling was obtained in either case.

OPG promoted apoptosis in mouse osteoclast-like cells

Previously, Akatsu et al.[24] have documented that osteoclast-like cells developed by a murine marrow cell culture system disappeared from culture plates when OPG was added. However, they did not actually count the apoptotic osteoclasts in the media. Loss of attachment happens in the apoptotic cell death process but also could be observed in the necrotic death process, especially when a high dose of apoptosis-inducing reagents were used. In this study, we attempted to obtain evidence that is more direct in proving whether or not OPG can induce only apoptosis in osteoclasts by morphological examination of cells on solid support and in media, respectively. The OPG used in this

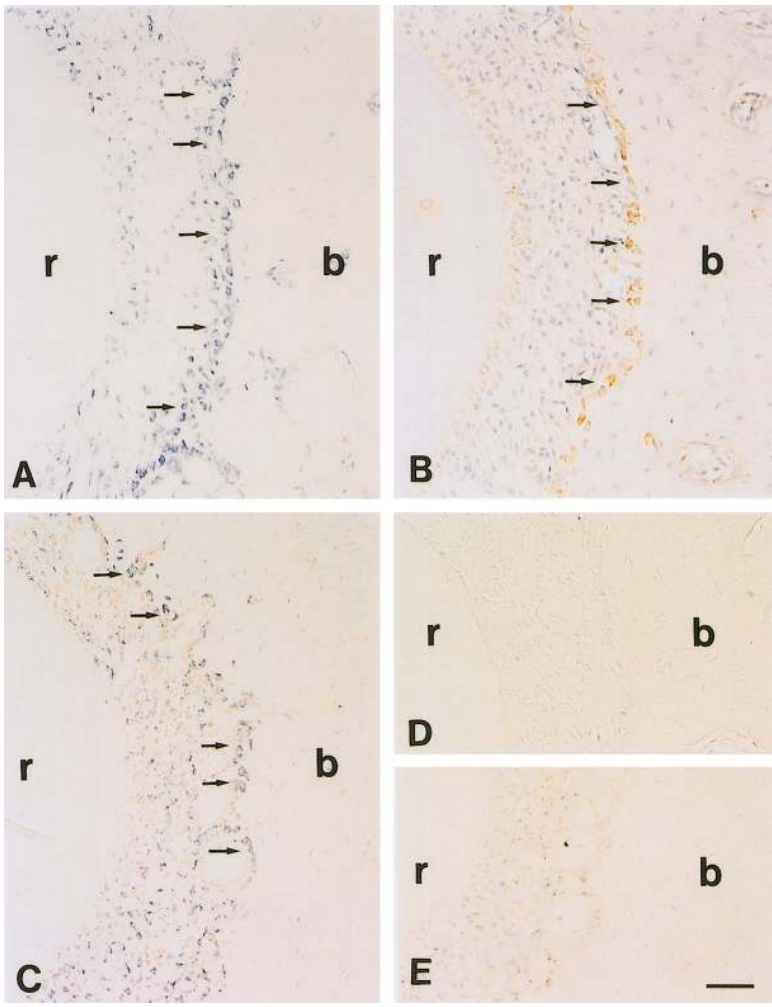


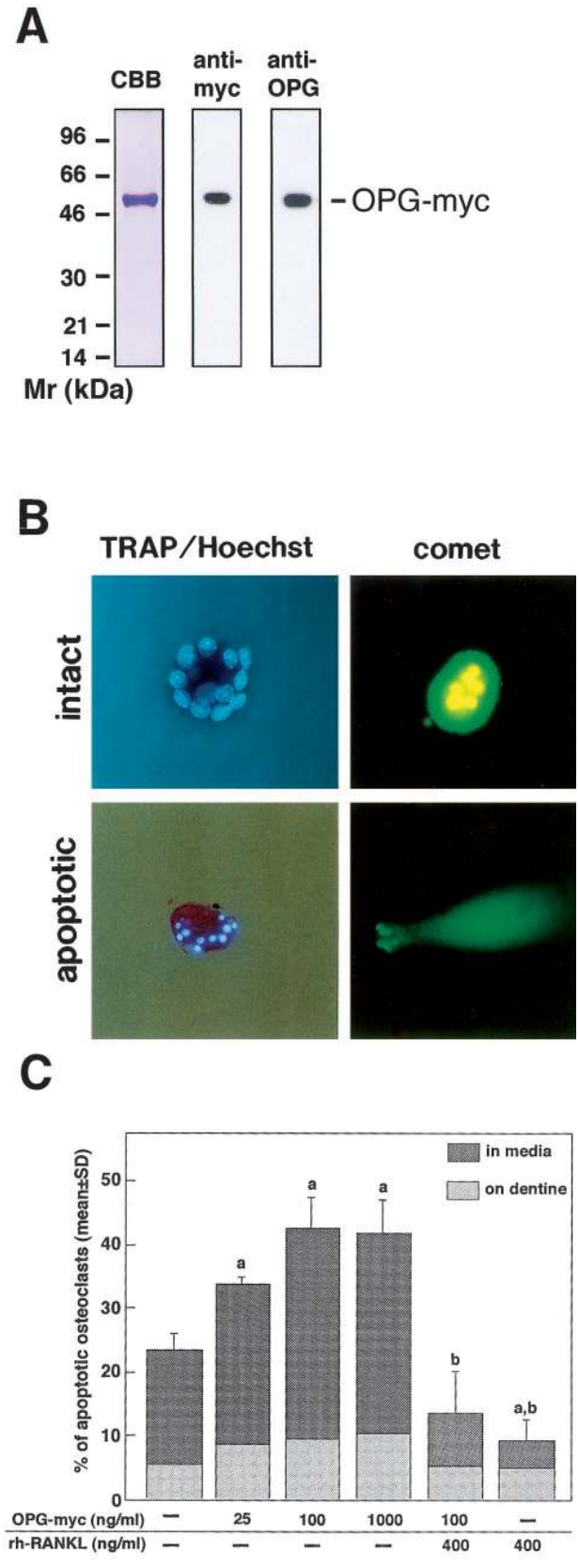
FIG. 5. Expression of TGF-β1, osteocalcin, and OPG at the mesial periodontal tissue in a control rat. Strong in situ hybridization signals for (A) TGF-β1 and (C) OPG were confined to cuboidal cells lining the mesial alveolar bone surface. Distribution of these cells overlapped osteocalcin-producing cells (positive for pro-osteocalcin, shown in B). Thus, osteoblasts were the major cells positive for TGF-β1 and OPG in this section. Arrows indicate positive staining. The validity of the in situ hybridization was tested by using sense-RNA probes for (D) TGF-β1 (D) and (E) OPG, respectively. Bar = 50 μm.

study (OPG-myc, Fig. 6A) showed complete inhibition of osteoclast-like cell formation in a murine coculture system at a dose higher than 100 ng/ml (data not shown).

Murine osteoclast-like cells together with stromal cells were cultured on dentine disks for 48 h in α-MEM/10% FCS in the presence or absence of OPG-myc. During the 48-h culture, 23% of osteoclast-like cells spontaneously fell into apoptosis under the culture conditions we employed, one-third of which were found on the dentine disks and the rest of them were recovered in the culture media (Fig. 6C). Picnotic nuclei of the apoptotic osteoclast-like cell contained fragmented DNA, as revealed by Comet assay (Fig. 6B). By adding OPG-myc to the culture media (100 ng/ml), apoptosis in osteoclast-like cells was significantly enhanced to 42% (Fig. 6C). Ten times more OPG-myc had no further effect. Even at this high concentration, the population of necrotic osteoclasts, which exhibited nuclear swelling and faint TRAPase activity, was less than 5% (data not shown). Most of the apoptotic cells induced by OPG-myc lost substrate attachment and were released into the culture media. The effect of OPG-myc at 100 ng/ml was abrogated completely by adding 400 ng/ml of rh-RANKL (about eight times more molar excess than OPG-myc) to the culture media. Spontaneous osteoclast-like cell apoptosis was protected by adding rh-RANKL to the media.

DISCUSSION

This report showed for the first time that osteoclasts on a bone surface disappeared through apoptosis during force-induced reversal from bone resorption to formation. These apoptotic osteoclasts were scavenged rapidly, presumably by tissue macrophages.[14,38] Because the bone remodeling shift from resorption to formation occurred on the entire distal alveolar bone surface sequentially, we could follow the precise kinetics of apoptotic disappearance of osteoclasts. What kinds of factors are involved in commitment of osteoclast apoptosis in this model? A direct mechanical effect on osteoclasts was of course a principal candidate. Fibroblastic cells in the periodontal tissue showed a stretched form after force application, as shown in Fig. 2B or 2C. These fibroblasts were associated with the periodontal ligament collagen fibers, which conveyed the orthodontic force to the bone surface. However, osteoclasts were free from such fibrous connections, and do not seem to be pulled



out mechanically from the bone surface by the fibers. Therefore, rather than such a direct mechanical effect on osteoclasts, we thought some biological responses evoked in cells around the bone surface controlled induction of apoptosis in osteoclasts.

As we previously reported, cells lining the distal alveolar bone surface had differentiated into mature osteoblasts as early as 2 days after tensional force application.[18] During this osteoblastic differentiation, we showed a significant induction of mRNAs for TGF-β1 and OPG. An increase or decrease in signals demonstrated by in situ hybridization was indicative but not literally quantitative; therefore, our data should be qualified further with a quantitative method such as Northern blotting. However, at this time, we were unsuccessful in this approach because of the difficulty in taking a sample from this very small area (<1 mm^3) of the distal periodontal hard tissue.

A significant increase in signals for TGF-β1 mRNA was observed in stretched cells on the bone surface as early as 24 h after force application. In the promoter region of TGF-β1 gene, there is a putative stress responsive element,[42,43] and indeed, expression of TGF-β1 in human osteoblast-like periodontal ligament cells was shown to be increased by application of cyclic strain in vitro.[44] There-

FIG. 6. OPG promotes apoptosis in osteoclasts. (A) Purity and authenticity of myc-tagged OPG (OPG-myc) used in this study. The OPG-myc was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and stained with Coomassie brilliant blue. OPG-myc reacted well with anti-myc monoclonal antibody (anti-myc) and rabbit polyclonal anti-OPG antibody (anti-OPG), during immunoblotting. (B) Discrimination of intact and apoptotic murine osteoclast-like cells developed in in vitro mixed cell cultures. The osteoclast-like cells were identified as multinuclear TRAPase-positive cells. Intact cells contain large spherical nuclei and show no migration of nuclear DNA from the cells in the Comet assay, whereas apoptotic cells possess small picnotic nuclei and exhibit a cometlike DNA migration pattern in the Comet assay. (C) In vitro–developed murine osteoclast-like cells together with stromal cells were replated onto dentine disks and cultured for 48 h in α-MEM/10% FCS in the presence of various amounts of OPG-myc or rh-RANKL. Among the total osteoclast-like cells, apoptotic cells in the culture media and on dentine disks were counted separately after TRAP/Hoechst staining (as shown in B). By adding OPG, the rate of apoptosis increased from about 20% (spontaneous apoptosis in the culture) to 40%. The effect of OPG was abrogated completely in the presence of a molar excess of rh-RANKL. The rh-RANKL alone also rescued osteoclasts from apoptosis in these culture conditions. This experiment was repeated three times and representative data are shown. Bars represent the mean $\pm$ SD; $n = 4$; a, significantly different from the control culture (no OPG/rh-RANKL added), $p < 0.01$; b, significantly different from the treatment with OPG (100 ng/ml), $p < 0.01$.

fore, the applied orthodontic force may activate transcription of TGF-β1 gene in those stretched cells. Consistent with our results, such a rapid increase in TGF-β1 in crevicular gingival fluid was reported in human orthodontic tooth movement.[45] TGF-β can recruit osteoblast precursors to the site of bone formation and has effects on osteoblasts to differentiate and enhance production of bone matrix proteins.[46] Preosteoblasts and matrix-secreting osteoblasts are known to produce latent TGF-β1.[40,41] During tooth movement, latent TGF-β could be activated in the periodontal tissue by plasmin, which is formed in the cascade of plasminogen activator produced by stretched periodontal ligament cells.[47,48] In contrast to its promotion of bone formation, in general, TGF-β inhibits osteoclastic bone resorption. TGF-β inhibits recruitment of osteoclast precursor cells and also directly suppresses bone resorption activity of osteoclasts.[49,50] We considered that TGF-β1 expressed on the tensioned bone surface promoted osteoblastogenesis and new bone formation while it inhibited further osteoclastogenesis at the site. To date, three independent research groups have shown that TGF-β1 can promote apoptosis in osteoclasts.[9,22,24] A possible involvement of TGF-β1 in promotion of osteoclast apoptosis at the stretched bone surface cannot be excluded but may be limited, because its effective concentration has been shown to be restricted within a narrow range (peak at 10 ng/ml).[9,22] Moreover, the proapoptotic effect of TGF-β1 could be mediated by other factor(s) produced by osteoblast/stromal cells under control of TGF-β1. Murakami et al.[23] and Takai et al.[51] reported that expression of OPG was increased rapidly in an osteoblastic cell line and bone marrow–derived stromal cells by TGF-β1 treatment in a dose-dependent manner. Our data showed a time course of OPG mRNA expression up-regulation that was very similar to that observed for TGF-β1 (Fig. 5). We speculate that because of the force application, locally expressed TGF-β1 was involved in up-regulation of OPG in cells on the tensioned alveolar bone surface.

Our in vitro experiments, as consistent with Akatsu et al.,[24] showed OPG potently promoted apoptosis in osteoclast-like cells generated by an in vitro coculture system. The effect was clearly reproducible, and there seemed to be no critical narrow window in its effective concentration, differing from that reported for TGF-β1. About 80% of the apoptotic cells lost substrate attachment and were released into the culture media when 100 ng/ml OPG was applied. This in vitro situation resembled that observed in situ where apoptotic osteoclasts separated from the bone surface. Even at the highest concentration used, no indication was observed of increased necrotic osteoclasts. The effect of OPG might be exerted by binding and masking the survival signal from RANKL that is expressed in stromal cells in the mixed cell osteoclast culture, because addition of excess amounts of rh-RANKL completely abrogated the proapoptotic effect of OPG.

In the experimental tooth movement model, our data showed a negative correlation in apoptotic disappearance of osteoclasts and an increase of OPG, which was well correlated with the expression profile of TGF-β1. Based on these observations together with consideration for the potent proapoptotic effect of OPG in vitro, our data may suggest a possible link of the following sequence: tensional force applied on bone lining cells, expression of TGF-β1/OPG, and disappearance of osteoclasts in this model. Of course, we cannot rule out involvement of other factors, such as free radicals produced in response to mechanical stress,[52] in induction of osteoclast apoptosis at the reversal site. Further studies are necessary. Hughes and Boyce[3] have observed many apoptotic osteoclasts on the calvarial bone surface where IL-1 was injected. IL-1 caused local bone resorption, which was ceased abruptly and followed by new bone formation. Apoptotic osteoclasts were observed mostly at the reversal sites from bone resorption to bone formation a few days after cessation of IL-1 injection. In this context, we will examine changes in expression of cytokines that support osteoclastogenesis and survival, such as IL-1, RANKL, and CSF-1,[21] in our experimental model. Besides up-regulation of proapoptotic cytokines, reciprocal loss of osteoclast survival factors from the area surrounding osteoclasts also may promote osteoclast apoptosis.

In conclusion, we showed that the experimental tooth movement model could be used as a tool to trace the final fate of osteoclasts in vivo. This experimental system allowed us to examine kinetics of apoptotic disappearance of osteoclasts. Our data suggest there may be a link between disappearance of osteoclasts and up-regulation of TGF-β1 and OPG expression, but we cannot rule out roles of other factors, which we will test in the future. We consider that use of this experimental tooth movement model in combination with a pharmacologic approach and/or gene manipulated animals can provide valuable insights in understanding the mechanism controlling the disappearance of osteoclasts from the bone surface after cessation of their role in vivo.

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