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Clastic cells are absent around the root surface in pulp-exposed periapical periodontitis lesions in mice

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Abstract

Introduction—Clastic cells, originating from the monocyte-macrophage lineage, resorb mineralized tissues. In periapical periodontitis, alveolar bone around the tooth apex becomes resorbed; however, the roots of the teeth are often left intact by yet unknown mechanisms. Here, we examined the status of clastic cells in a periapical periodontitis model in mice.

Methods—Periapical periodontitis was induced by performing pulp exposure on the maxillary first molar. The contralateral maxillary first molar was used as a control. The maxillae were harvested, fixed, and subjected to μ CT scanning and three-dimensional volumetric analysis. TRAP staining was performed and osteoclasts were quantified. Immunohistochemical staining was performed for RANKL, OPG, and F4/80, a marker for macrophages.

Results—At the apex of the tooth, pulp exposure resulted in periapical radiolucency with mineralized tissues at the surrounding bone surfaces but not on the root surfaces. Histologically, clastic cells were present on the bone surfaces but absent around the root surfaces. Expression of F4/80 and RANKL were not found at close proximity to the root surfaces, but OPG was globally expressed.

Conclusion—The absence of clastic cells around the root surface of pulp-exposed teeth, in part, is associated with the lack of macrophages and RANKL expression.

Keywords

Clastic cells; odontoclasts; osteoclasts; periapical periodontitis; bone resorption

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INTRODUCTION

Clastic cells, capable of resorbing mineralized tissues, are multinucleated cells differentiated from mononuclear precursors of monocyte-macrophage lineage (Arana-Chavez & Bradaschia-Correa, 2009). These mononuclear precursors respond to growth factors, such as M-CSF, which promotes proliferation and survival of the cells (Roodman 2006). Further activation of these cells are regulated by the balance between 1) receptor activator of NF- κ B ligand (RANKL), whose binding to its receptor RANK stimulates further differentiation of the multinucleated clastic cells, and 2) osteoprotegerin (OPG), a RANKL antagonist secreted by osteoblasts that prevents binding of RANKL to RANK (Tanaka et al. 2003). After differentiation and activation, these cells undergo highly polarized cytoplasmic organization demonstrated by a clear zone, ruffled border, and actin ring, and subsequently attach to and resorb mineralized matrices such as bone, calcified cartilages, dentin or cementum (Nakamura 1996).

Odontoclasts and cementoclasts are the clastic cells found near teeth where they resorb mineralized dental hard tissues such as dentin and cementum, respectively. Although odontoclasts and cementoclasts are viewed as the same cell type and have similar functional characteristics as osteoclasts in bone (Sahara et al 1994 & 1996, Oshiro 2001, Sasaki 2003, Hartsfield 2004), they differ in few notable ways such as being smaller in size, having less nuclei and underdeveloped clear zones (Hammarstrom and Lindskog 1985). Beside their phenotypes, osteoclasts and odontoclasts are suggested to function differentially, as Indomethacin administration caused suppression in alveolar resorption but enhancement of root resorption (Lasfargues and Saffar 1993). Nonetheless, the differences in clastic cells that are of tooth and bone origins are not fully understood.

In periapical periodontitis, the normal remodeling process that maintains alveolar bone in health is disturbed, causing an increase in clastic activity. As a result, bone structures around the root of the affected tooth are subjected to resorption, leading to a periapical radiolucent lesion (PARL). Yet, clinical observations suggest that the roots are often left intact by yet unknown mechanisms.

In this study, we hypothesized that clastic cells are absent around the root surface during periapical periodontitis and that the absence of clastic cells is due to unbalanced expression of RANKL/OPG. To test this hypothesis, we exposed the pulp of the teeth and induced experimental periapical periodontitis in mice.

MATERIALS AND METHODS

Animal

C57BL/6 mice (six-week-old female) were purchased from the Jackson Laboratory (Bar Harbor, ME) and kept in a pathogen-free vivarium in the University of California Los Angeles, Division of Laboratory Animal Medicine. All experimental protocols were approved by institutional guidelines from the Chancellor's Animal Research Committee (#2011-062).

The mouse model of periapical lesion

The pulp exposures in mice were performed as described previously (Song et al., 2016). Briefly, mice (n = 3) were anesthetized with ketamine/xylazine (100 and 5 mg/kg body weight, respectively) by intraperitoneal injection. A single pulp exposure was made on the left maxillary first molar using a high-speed ¼ round bur on a portable dental unit (Aseptico Inc., Woodinville, WA) under x10 magnification of an endodontic microscope (BM-LED stereo microscope, MEIJI Techno, Japan). Exposed teeth were left open to the oral environment without any coverage. The right maxillary first molar was used as a control without pulp exposure. Mice were sacrificed and maxillae were harvested at 3 weeks after pulp exposure (n = 3).

Human tissue samples

Discarded root tips with non-patient identifiers were obtained from the patients undergoing apicoectomy for routine dental procedures. All experimental protocols were exempted by institutional guidelines from our Institutional Review Board (#16-001932).

Tissue preparation

The harvested maxillae were fixed with 4% paraformaldehyde in PBS, pH 7.4, at 4° overnight and stored in 70% ethanol solution. Fixed tissues were subjected to µCT scanning, after which maxillae were decalcified with 5% EDTA and 4% sucrose in PBS, pH 7.4 for 2 weeks at 4°C. The decalcification solution was changed daily for 3 weeks, and decalcified tissues were sent to the UCLA Translational Procurement Core Laboratory (TPCL) for paraffin embedding.

µCT Scan and Three-Dimensional Volumetric Analysis

µCT scanning (Scanco µCT 40; Scanco Medical, Brüttisellen, Switzerland) of maxillae was performed using a voxel size of 20 µm³ and a 0.5 mm Aluminum filter at 55 kVp and 145 µA with an integration time of 200 ms using a cylindrical tube (FOV/Diameter: 20.48 mm). Resolution was set to medium (1024 × 1024 × 148 pixels). Two-dimensional images were reconstructed using µCT v6.1 software (Scanco Medical, Brüttisellen, Switzerland) and three-dimensional images obtained by the CTvol software (Bruker microCT, Kontich, Belgium).

Tartrate-resistant acid phosphatase (TRAP) histochemical staining

TRAP staining and quantification were performed as described previously (Williams et al., 2014). The sectioned slides (n = 3, every 5 cuts) were incubated with TRAP solution (Sigma-Aldrich), and osteoclasts were identified by the presence of multiple nuclei (n>5). Osteoclasts number was measured using ImageJ software version 1.48 (NIH) on digital pictures taken through Olympus microscope (model DP72; Olympus) at 100× magnification.

Immunohistochemical staining

Immunohistochemistry staining was performed with anti-F4/80 (MCA497GA, ABD Serotec, Minneapolis, MN), anti-RANKL (ab45039, Abcam, Cambridge, MA) and anti-OPG (ab73400) on 4-µm-thick sections according to the manufacturer's protocol. The

samples were counterstained with hematoxylin, and photos were taken under a microscope (model DP72; Olympus, Tokyo, Japan).

Statistical Analysis

The outcome measurements were expressed as means $\pm$ standard deviation. For comparison between the two groups, a Student *t* test was used. All statistical tests were performed using the SPSS 23 software (IBM Corp, Somers, NY), with P values < 0.05 considered significant. All experiments were performed at least twice with *n* = 3 unless otherwise indicated.

RESULTS

Pulp exposure leads to periapical radiolucency and bone resorption at the apex

To induce periapical periodontitis in mice, we made a single pulp exposure on the first maxillary molars in mice. After 3 weeks, we sacrificed the mice, harvested the maxillae, and performed μ CT scan to examine the resulting PARLs. As expected, significant bone resorption was observed around the root tips (Fig. 1A and 1B). Quantification of bone around the root tips revealed a significant increase in PARL sizes in all roots (Fig. 1C–1E). Histological examination revealed increased infiltration of inflammatory cells and bone resorption around the apex of the root without notable resorption at the root surface (Fig. 1F). These data suggest that pulp exposure leads to increased bone resorption around the root tip but not the root itself.

Clastic cells are absent around the root surface

To examine the status of the clastic cells at the root tip area, we stained for TRAP, a marker for matured clastic cells. As expected, teeth without pulp exposures did not show any cells that are positive to TRAP staining (Fig. 2A, top panels). On the other hand, teeth with pulp exposures revealed increased TRAP-positive clastic cells on bone surfaces around the root tips but not on the root surfaces (Fig 2A, bottom panels). When TRAP+ cells were counted, significant numbers of TRAP-positive cells were found on the bone surfaces but not on the root surfaces (Fig. 2B), suggesting that increased bone resorption, but not root resorption, is due to increased numbers of clastic cells on the bone surface.

Expression of F4/80, RANKL, and OPG around the periapical periodontitis

Clastic cells originate from monocyte-lineage macrophages upon RANKL stimulation, which can be antagonized by OPG (Xu & Teitelbaum, 2013) Therefore, we examined the expression of F4/80, a marker for macrophages as well as RANKL and OPG. Immunohistochemical (IHC) staining for F4/80 showed heavy staining around the bone surface; interestingly, no staining was observed immediately adjacent to the root surface (Fig. 3A and B). Similarly, RANKL staining was evident around the bone surface but not on the root surface (Fig. 3C and D). On the other hand, OPG expression was ubiquitously present around both bone and root surface. These data suggest that the absence of clastic cells around the root surface is associated with the absence of macrophages and RANKL.

Clastic cells are absent around the root tip in patients

To examine whether the absence of clastic cells on the root surface is also seen at the clinical level, we obtained root tips that were subjected to apicoectomy due to failed root canal therapy and presence of PARLs. When we examined sections for the presence of TRAP-positive cells, we found no clastic cells around the root tips (Fig. 4), suggesting that unresorbed root tips at the PARLs are largely due to the absence of clastic cells.

DISCUSSION

It has long been known that mineralized dental tissues (e.g., dentin and cementum) are highly resistant to resorption when compared to other tissues (Hammarstrom & Lindskog, 1985). However, the underlying mechanisms are poorly understood. Here, we experimentally confirmed this observation by using the pulp-exposure-induced periapical periodontitis model in mice. We further demonstrated that clastic cells are absent on root surfaces whereas they are enriched on the surrounding bone surfaces. We also showed that the reduced numbers of clastic cells on the root surface is associated with the absence of macrophages as well as RANKL expression immediately adjacent to the root surface, suggesting that the root surface confers an environment that suppresses the formation of clastic cells.

One critical step in the resorption process is attachment of clastic cells onto the mineralized tissues (Soyas et al., 2012). Clastic cells bind to mineralized tissues via $\alpha 5 \beta 3$ integrin which interacts with RGD-rich ligands such as vitronectin (Ross & Teitelbaum, 2005). Indeed, mice lacking $\beta 3$ integrins in osteoclasts exhibited osteoclast dysfunctions and increased trabecular bone formation (McHugh et al., 2000). As such, previous studies suggested that resorption resistance of root surfaces is thought to be due to the lack of an arginine-glycine-aspartic (RGD) amino acid domain on the uncalcified cementoid layer that covers the root surface (Hargreaves, 2011, Trope, 2002).

However, a recent study suggested an unexpected role of an RGD-containing protein in root resorption. Bone sialoprotein (BSP) is an extracellular matrix protein that belongs to the Small Intetrin Binding Ligand N-linked Glycoprotein (SIBLING) family, and plays important roles in cell attachment and migration through the integrin-binding RGD domain (Alam et al., 2014). Foster et al. demonstrated that BSP null mice exhibited increased root resorption as demonstrated by scanning electron microscopy (SEM) and histology (Foster et al., 2013). Interestingly, BSP over-expression in transgenic mice also exhibited increased bone and root resorption, presumably due to an enhanced osteoclast differentiation (Valverde et al., 2008). Yet, another RGD-containing protein, DMP-1, has no effect on root resorption as demonstrated by Dmp1 null mice (Ye et al., 2008). Such results indicate that an RGD-containing protein may play a more complicated role than was previously suggested.

It is also initially suggested that inherent resistance to resorption on the root surface is due to the periodontal ligament (PDL) that covers the root surface and serves as a barrier between the cementum and alveolar bone (Lasfargues & Saffar, 1993). However, PDL cells are often involved in periapical periodontitis and are known to be involved with clastic activity. For example, PDL fibroblasts (PDLFs), spindle-shaped connective tissue cells found in the PDL,

were found to increase expression of RANKL and other osteoclastogenesis-stimulating molecules *in vitro* when incubated with bacteria (Sokos et al., 2014). RANKL is highly expressed in PDL cells and supports the osteoclast differentiation process *in vitro* despite the fact that these cells also express high levels of OPG (Uchiyama et al., 2009, Sokos et al., 2015). More studies are required to clearly define the role of PDL and PLDFs in odontoclasts.

In summary, our study showed that the lack of clastic cells on the root surface is associated with absence of RANKL expression at the root surface (Fig. 3). Previous studies showed that atypical root resorption is accompanied by high expression of RANKL at the root surface (Foster et al., 2013). Because our *in vivo* analysis showed high expression of OPG but not RANKL at the root surface (Fig. 3), it is tempting to speculate that there is an active intrinsic mechanism around the root surface that prevents secretion of RANKL and inhibits osteoclast differentiation.

External root resorption is frequently observed in a physiologically normal setting such as the shedding of deciduous teeth. However, root resorption also occurs in many pathological circumstances, including orthodontic tooth movement, idiopathic disease processes, or accidental trauma such as the avulsion of teeth (Hartsfield et al., 2004, Wise & King, 2008). Precise understanding as to how external root resorption occurs would allow for prevention and treatment of these unwanted events.

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Figure 1. Pulp exposure leads to periapical radiolucency and bone resorption at the apex
 (A) μ CT image of the first maxillary molar in mice. (B) D-dimensional image of the cross section. Quantification of bone loss around the mesial (M) root (C), distopalatal root (D), and distal root (E). (F) H&E staining of the tooth. Bar indicates 100 μ m.

Figure 2. Clastic cells are absent around the root surface
(A) TRAP staining of the root at lower (right) and higher (left) magnification. (B) Quantification of TRAP-positive cells at the root surface and the bone surface.

Figure 3. Expression of F4/80, RANKL, and OPG around the periapical periodontitis
Immunohistochemical (IHC) staining was performed on tissue sections using anti-F4/80 (A), anti-RANKL (B), and anti-OPG (C) antibodies. Bar indicates 100 μ m.

Figure 4. Clastic cells are not found around the root tip in patients

Root tips were obtained from two patients undergoing apicoectomy surgery, and periapical (PA) x-ray images, H&E staining, and TRAP staining were obtained.