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Osteocytes Play an Important Role in Experimental Periodontitis in Healthy and Diabetic Mice Through Expression of RANKL

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Abstract

Aim—Periodontitis results from bacteria induced inflammation. A key cytokine, RANKL is produced by a number of cell types. The cellular source of RANKL critical for periodontitis has not been established.

Methods—We induced periodontal bone loss by oral inoculation of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in both normoglycemic and streptozotocin induced type 1 diabetic mice. Experimental transgenic mice had osteocyte specific deletion of floxed RANKL mediated by DMP-1 driven Cre recombinase. Outcomes were assessed by micro-CT, histomorphometric analysis, immunofluorescent analysis of RANKL and TRAP staining for osteoclasts and osteoclast activity.

Results—Oral infection stimulated RANKL expression in osteocytes of wild type mice, which was increased by diabetes and blocked in transgenic mice. Infected wild type mice had significant bone loss and increased osteoclast numbers and activity, which were further enhanced by diabetes. No bone loss or increase in osteoclastogenesis or activity was detected in experimental transgenic mice that were normoglycemic or diabetic.

Conclusions—This study demonstrates for the first time the essential role of osteocytes in bacteria induced periodontal bone loss and in diabetes-enhanced periodontitis.

Keywords

Bone resorption; diabetic; genetic deletion; gingiva; immunofluorescence; infection; knockout; periodontal; PDL; RANKL; transgenic

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Introduction

Periodontitis is a biofilm-induced infectious disease that causes loss of bone around the teeth. Approximately 46% of US adults have moderate to severe periodontitis (Eke et al., 2015). The host response induces tissue destruction that is initiated by the expression of inflammatory mediators which induce recruitment of a number of leukocyte subsets. Prominent among these are neutrophils, macrophages and T- and B-lymphocytes. An important feature of periodontitis is the close proximity of the inflammatory infiltrate to the alveolar bone surrounding the teeth (Rowe and Bradley, 1981, Graves et al., 1998)

The inflammatory cytokines IL-1, TNF and IL-6 as well as prostaglandins have been shown to play an essential role in mediating periodontal bone loss (Graves et al., 2012). Many of these mediators induce bone loss by induction of receptor activator of nuclear factor kappa-B ligand (RANKL). B- and T-lymphocytes are thought to be primary sources of RANKL in periodontitis (Kawai et al., 2006, Liu et al., 2003). In contrast osteoblast lineage cells are an important source of RANKL in conditions characterized by unloading of mechanical forces, bone loss induced by ovariectomy or arthritis, whereas the loss of RANKL in T-lymphocytes does not confer such protection (Xiong et al., 2011, Nakashima et al., 2011, Fumoto et al., 2014). Moreover, we have shown that the induction of periodontal disease in mice stimulates RANKL production in bone-lining osteoblastic cells and osteocytes (Pacios et al., 2015, Nakashima et al., 2011). However, the specific role of osteocytes in contributing to bacteria-induced periodontal bone loss has not been investigated.

To investigate the role of osteocytes in periodontitis we examined mice with conditional deletion of floxed RANKL by Cre recombinase driven by a promoter element from the dentin matrix acidic phosphoprotein 1 (DMP1) gene. DMP1 is an extracellular matrix protein found in bone and dentin (George et al., 1993, D'Souza et al., 1997). Recent studies demonstrate that osteocytes express high levels of DMP1 in a lineage specific manner along with odontoblasts (Fen et al., 2002, Komori, 2014). Mice with DMP-1 driven Cre-recombinase and floxed RANKL have RANKL expression that is specifically blocked in osteocytes (Xiong et al., 2011). While this significantly reduces bone resorption associated with unloading in adult mice, during development they have normal cancellous bone formation and normal tooth eruption indicating that osteocyte-produced RANKL is most important in processes that occur after development. We show here that specific deletion of RANKL in osteocytes blocks bacteria-induced periodontal bone loss in both normoglycemic conditions and when periodontal disease is enhanced by diabetes. These data show for the first time that osteocytes play an essential role in periodontitis.

Materials and Methods

All methods were carried out in accordance with the approved guidelines of the Institutional Animal Care and Use Committee at the University of Pennsylvania. Experimental DMP1-Cre.RANKL^{f/f} mice backcrossed onto a C57/B6 background were obtained from Dr. Jerry Feng of the Baylor College of Dentistry in Dallas, Texas and Dr. Charles O'Brien, University of Arkansas for Medical Sciences, Little Rock, AR. They were obtained by

breeding DMP-1.Cre-recombinase transgenic mice originally described in ref (Feng et al., 2006) with floxed RANKL transgenic mice originally described in (Xiong et al., 2011). They express Cre recombinase under a control element of the *Dmp1* promoter so that expression is restricted to osteocytes and odontoblasts (Lu et al., 2007). DMP1-Cre mice were crossed with ROSA26 reporter mice which express beta-galactosidase when a floxed stop signal is removed by Cre recombinase and were obtained from Jackson Laboratories (Bar Harbor, Maine). Control C57/B6 mice were obtained from Jackson laboratories. Experiments were initiated when the mice were 8–10 weeks old. Type 1 diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (40–50 mg/kg) for 5 consecutive days. Control animals were treated with citrate buffer alone adjusted pH 4.5. Peripheral blood glucose was monitored with a glucometer. Mice were considered to be hyperglycemic when their glucose values exceeded 225 mg/dl. Mice were maintained in the diabetic (hyperglycemic) state for approximately three weeks prior to initiating experiments.

Inoculation of bacteria and tissue harvest

Mice were pretreated with trimethoprim (1.6 mg/ml) and sulfadimethoxine (8 mg/ml) added to the drinking water which was changed every other day and given for 7 consecutive days to reduce the endogenous oral bacteria. *Porphyromonas gingivalis* (Pg) strain (ATCC 33277) and *Fusobacterium nucleatum* (Fn) strain (ATCC 25586) were inoculated in 100 µl of sterile methylcellulose so that 2×10^9 colony forming units of each bacterium were applied in 200 µl of 2% methylcellulose. Oral inoculation with *Porphyromonas gingivalis*-*Fusobacterium nucleatum* bacteria (Pg-Fn) is an effective model for studying periodontitis (Polak et al., 2009) and a dose which we have previously published and found to be effective in mice with a C57/B6 background (Pacios et al., 2015). Bacterial inoculation was performed three times per week over a 2-week period. Mice were euthanized at six weeks after the last bacterial inoculation by decapitation under anesthesia. Specimens were fixed in 4% paraformaldehyde at 4°C for 24 hours, transferred to PBS, taken for microCT analysis and subsequently decalcified in 10% EDTA (Fisher Scientific; Hampton, New Hampshire) for 4–5 weeks. The specimens were embedded in paraffin and sectioned in sagittal sections of 4-µm thickness that included the region of the molar teeth.

Analysis of Bone

Specimens were examined with a MicroCT scanner (viva CT40 SCANCO Medical, Brüttisellen, Switzerland) and analyzed using OssiriX MD imaging software (Pixmeo; Geneva, Switzerland). The bone area between the first and second molar was measured sagittal slices between the distobuccal root of the first molar and the mesiobuccal root of the second molar. The micro CT slice that gave the most bone loss interproximally was examined for bone area coronally from the cement-enamel junctions (CEJs) of the first and second molars and apically 700 µm from the CEJ. The lateral boundaries were defined by the cemental surface of each tooth and presented as the percent total area occupied by bone. The bone height was analyzed by measuring the crest to cemento-enamel junction (CEJ) distance in the same sagittal slice. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and histomorphometric analysis was performed using Nikon NIS-elements image analysis software (Nikon, Tokyo, Japan) at 200X magnification following the same approach described for micro-CT. Osteoclasts were counted as multi-

nucleated tartrate resistant acid phosphatase (TRAP) positive cells lining the coronal 0.35mm of alveolar bone adjacent to the second molar as we have previously described (Wu et al., 2016). We focused on the bone adjacent to the second molar because of the “distal drift” of teeth noted in the mouse (Gilmore and Glickman, 1959). The number of osteoclasts and the corresponding length of bone was measured using image analysis (NIS Elements, Nikon) and the data are presented as the number of osteoclasts per mm bone length. The percent eroded bone surface was measured in TRAP stained sections. The same bone surface examined for osteoclasts was assessed by image analysis for the percent of the bone length that had resorption lacunae as we have described (Xiao et al., 2015).

Immunofluorescence

RANKL was examined by immunofluorescence following antigen retrieval with proteinase K, blocking of nonspecific sites with donkey serum in blocking buffer and incubation with primary antibody specific for RANKL (sc-7628) from Santa Cruz Biotechnology (Santa Cruz, California) at 2ug/ml or normal goat IgG control at 2 ug/ml. Specimens were then incubated with biotinylated secondary antibody (Jackson Immuno Research; West Grove, Pennsylvania), avidin biotin complex (ABC Vector Elite; Vector Labs) followed by tyramide signal amplification. Specimens were mounted in mounting media containing DAPI. Mean fluorescence intensity was measured with capture time set so that the highest group had a maximum fluorescence intensity of 3000 to ensure that measurements were made within a linear range of detection as we have previously described (Xu et al., 2015). Six to 7 images were captured of the alveolar bone between the first and second molar, the connective tissue above the bone crest and sub-epithelial connective tissue. Images were captured using a fluorescence microscope (Eclipse 90i; Nikon) and analyzed with Nikon AR image analysis software (Nikon). Beta-galactosidase was assessed by immunofluorescence using the same approach except that antigen retrieval was performed by immersing slides in citric acid (pH 6.0, 10 mM) at 95° C for 15–20 minutes. Primary antibody specific for beta-galactosidase was purchased from Bioss Antibodies (Woburn, Massachusetts) and compared to matched control rabbit IgG. Sections were counterstained with DAPI.

Statistical analysis

Statistical analyses were performed using GraphPad Prism6 Software (La Jolla, California). The Kruskal-Wallis non-parametric analysis of variance test was used to establish differences between infected and non-infected mice within each wild-type or transgenic group and the Kolmogorov-Smirnov non-parametric T-test was used to establish differences between WT and transgenic (RANKL ablated) mice for each parameter. The significance level was set at $P < 0.05$ and each group had 5–7 mice with the mouse set as the unit of measurement.

Results

Tissue specificity of Dmp1-Cre mice

Beta-galactosidase immunofluorescence analysis of ROSA26 reporter mice (DMP1Cre⁺Rosa) revealed the tissue specificity of Cre-recombinase expression in DMP1 mice (Figure 1). β -galactosidase was detected in the vast majority of osteocytes as determined by the high

percentage of osteocytes that were immunopositive for β -galactosidase in immunofluorescent images compared to the total number of osteocytes in DAPI images. In addition, an occasional bone lining cell was β -galactosidase positive. The result confirms published reports that DMP-1 drives high levels of Cre-recombinase expression in osteocytes (Figure 1, bottom row) (Feng et al., 2006). The control group of wild-type mice showed no expression and no detection was noted in sections from DMP1-Cre⁺Rosa mice incubated with control IgG (Figure 1).

Analysis of bone loss

In the normoglycemic (NG) group, oral infection induced periodontal bone loss in the wild-type mice while little to no bone loss was detected in the experimental (DMP1-Cre⁺.RANKL^{f/f}) group (Figure 2A). In histomorphometric analysis of normoglycemic mice (NG) there was a 20% reduction in the percent bone area in infected WT mice compared to non-infected WT mice ($P < 0.05$) and no bone loss in infected experimental mice compared to the matched non-infected group ($P > 0.05$, Figure 2B). In the diabetic (DB) mice oral infection induced a 42% loss of bone in the WT group compared to the non-infected control ($P < 0.05$). The infected experimental (DMP1-Cre⁺.RANKL^{f/f}) mice experienced no significant bone loss compared to the non-infected experimental mice ($P > 0.05$, Figure 2B). The difference between infected experimental and infected control mice was significant for both WT and diabetic groups ($P < 0.05$). Similar results were obtained by micro CT analysis. Oral infection induced approximately 20% bone loss in the normoglycemic WT mice and almost 40% in the diabetic WT mice ($P < 0.05$, Figure 2C) and each was significantly greater in the WT mice compared to matched experimental mice with osteocyte-deletion of RANKL ($P < 0.05$). Infection of experimental (DMP1-Cre⁺.RANKL^{f/f}) diabetic or normoglycemic mice did not induce bone loss ($P > 0.05$, Figure 2C). When distance from CEJ to bone height was measured infection induced a significant increase in normoglycemic (NG) and diabetic (DB) WT. The CEJ to bone distance was significantly greater in the WT compared to experimental mice with osteocyte-ablation of RANKL for both infected groups ($P < 0.05$, Figure 2D).

Osteoclast numbers

Osteoclasts were counted in TRAP stained sections (Figure 3A). In the normoglycemic group oral infection stimulated a 4-fold increase in osteoclasts in the WT normoglycemic mice and a 7-fold increase in diabetic WT mice compared to non-infected controls ($P < 0.05$). The infected experimental (DMP1-Cre⁺.RANKL^{f/f}) mice had no increase in osteoclasts ($P > 0.05$, Figure 3B). The number of osteoclasts in infected WT mice was greater than the number in matched experimental transgenic mice for both normoglycemic and diabetic animals ($P < 0.05$, Figure 3B), consistent with the greater bone loss in WT groups (Figure 2). Very similar results were obtained when osteoclast eroded surface was measured, indicating that bacterial infection increased osteoclast activity in normoglycemic and diabetic groups of WT mice but not in animals with RANKL deletion in osteocytes (Figure 3C).

RANKL Immunofluorescence

Induction of experimental periodontitis stimulated RANKL expression in WT mice but not experimental (DMP1-Cre⁺.RANKL^{f/f}) mice (Figure 4A). The level of RANKL expression in

bone was 2-fold higher in the infected WT compared to transgenic mice ($P<0.05$, Figure 4). RANKL levels were not elevated in bone by infection in the experimental transgenic groups ($P<0.05$, Figure 4B). RANKL expression was also examined in the gingiva. Infection stimulated a ~50% increase in RANKL expression in the gingiva of wild type mice compared to uninfected animals. However, there was no difference between WT and (DMP1-Cre⁺.RANKL^{f/f}) animals (Figure 4C).

Discussion

We found that osteocytes play a critical role in infection induced periodontal bone loss based on findings that osteocyte-lineage deletion of RANKL blocks bacteria induced periodontal bone loss and osteoclastogenesis. Deletion of RANKL in osteocytes had the same impact on halting periodontal bone loss in diabetic animals as it did in their normoglycemic counterparts.

By use of a ROSA26 reporter mouse we found that most osteocytes and occasional bone lining cells expressed Cre recombinase under control of the DMP1 promoter. This is consistent with other reports that DMP1 is expressed in a lineage specific manner in odontoblasts and osteocytes (Lu et al., 2007, Kamel-ElSayed et al., 2015). Our results indicate that osteocytes are a critical source of RANKL in bacteria induced periodontitis. The occasional detection of DMP1 positive cells on the bone surface is consistent with the presence of surface osteocytes that are distinct from osteoblasts or immature mesenchymal cells that line bone surfaces (Kamel-ElSayed et al., 2015). Osteocytes have dendritic processes that connect them to each other. These dendritic processes can reach the bone surface and come into direct contact with osteoblasts and osteoclasts. It is likely that the cellular dendritic processes play a role in releasing RANKL at the bone surface which leads to formation of osteoclasts and bone resorption in periodontitis.

Inflammatory cells mediate bacteria-induced osteoclastogenesis and bone resorption (Xiao et al., 2016, Hajishengallis et al., 2015). It has been previously reported that in animal models and human specimens a major source of RANKL associated with periodontitis is lymphocytes (Kawai et al., 2006, Lin et al., 2010, Kanzaki et al., 2016). To our knowledge the expression of RANKL by osteocytes in humans has not been examined. We have previously shown that the introduction of periodontal pathogens to the oral cavity of mice induces NF- κ B and RANKL expression in osteoblastic bone lining cells and osteocytes (Pacios et al., 2015). Although we saw statistical differences in bone resorption and osteoclast numbers and activity between WT and experimental mice we cannot rule out the possibility that leukocyte sources of RANKL may also play a role in periodontal bone loss. It is clear though, that osteocytes, which have not been previously thought of as a key producer of RANKL in periodontal bone loss, should be considered as an important source of RANKL in periodontitis as it is in other bone pathologies such as osteoporosis, rheumatoid arthritis and fatigue-induced microdamage (Xiong et al., 2011, O'Brien et al., 2012, Kennedy et al., 2012).

Our results are also consistent with RANKL playing a prominent role in stimulating periodontal bone loss enhanced by diabetic conditions (Wu et al., 2015). We found that the

diabetic group had a consistently high level of bone loss, consistent with earlier studies in animals (Kang et al., 2012, Pacios et al., 2012) and humans. Diabetes can enhance bone loss through the upregulation of the inflammatory response which regulates osteoclastogenesis and causes uncoupling of bone formation following resorption in part due to increased apoptosis of osteoblast lineage cells (Pacios et al., 2012, Pacios et al., 2013). This is consistent with findings that diabetes up-regulates and prolongs inflammation that increases RANKL expression (Naguib et al., 2004, Ribeiro et al., 2011, Xiao et al., 2017). Diabetes-enhanced inflammation also alters the microbiome to render it more pathogenic and capable of inducing higher RANKL expression (Xiao et al., 2017). We found that RANKL produced by osteocytes was highest in the diabetic impacted group then matched normoglycemic animals, which coincided with increased osteoclast numbers and osteoclast activity reflected by an increased eroded bone surface.

The findings of this study shed light on the cellular regulation of bone resorption and the role of osteocytes in the disease process. We demonstrate for the first time the important role of osteocytes in periodontal bone loss through the expression of RANKL. This finding may contribute to future studies that target osteocytes to greater understanding how they contribute to inflammatory bone loss under both normoglycemic and diabetic conditions.

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Clinical Relevance

Scientific Rationale

It is well known that periodontitis involves bone loss that is stimulated by inflammation. RANKL is critical cytokine in this process although its key cellular source in periodontitis is unknown.

Findings

We show for the first time through lineage specific deletion of RANKL the surprising role of osteocytes in bacteria induced periodontal bone loss under both normoglycemic conditions and in diabetes-enhanced periodontitis.

Implications

The results provide new insight into the etiology of periodontal bone loss by describing how inflammation affects bone cells, particularly osteocytes to induce periodontal bone resorption through RANKL production.

Fig.1.

DMP1Cre.ROSA26 experimental transgenic mice demonstrate Cre recombinase expression in osteocytes. Immunofluorescence was carried out with specific antibody to beta-galactosidase or control IgG. Histologic sections were obtained from transgenic ROSA26 reporter mice that express Cre recombinase or wildtype mice. Top three rows, original magnification 200 X; bottom row, original magnification 400 X. Arrows point to beta-galactosidase positive cells.

Fig.2.

Bacteria induced periodontitis is blocked in mice with osteocyte specific deletion of RANKL. **(A)** Representative hematoxylin and eosin (HE) stained sagittal sections from the interproximal region between the 1st and 2nd molars (original magnification 200x). The white line traces the alveolar bone. **(B)** Histomorphometric analysis of bone area between 1st and 2nd molars. Values are expressed as the bone area to total interproximal tissue area as described in Materials and Methods. **(C)** Micro-CT analyses of bone area between 1st and 2nd molars. Values are expressed as the bone area to total interproximal tissue area as described in Materials and Methods. **(D)** Micro-CT analyses of the distance from bone crest

to CEJ. Data represent mean $\pm$ SEM of 5–7 animals per group. * indicates $p < 0.05$ compared to uninfected baseline control; + indicates $p < 0.05$ compared to matched experimental group.

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Fig.3.

Bacteria induced osteoclastogenesis and osteoclast activity is blocked in mice with osteocyte specific deletion of RANKL. (A) Representative TRAP stained sections mice from the interproximal region of periodontal tissue of an infected WT mouse (original magnification 200x). Arrows point to osteoclasts on the alveolar bone adjacent to the mesial surface of the 2nd molar. (B) Osteoclasts were counted in TRAP stained sections. (C) Eroded bone surface was measured as the percent bone surface with resorption lacunae in TRAP-stained sections. Data represent mean $\pm$ SEM of 5–7 animals per group. * indicates $p < 0.05$ compared to uninfected baseline control; + indicates $p < 0.05$ compared to matched experimental group.

Fig.4.

Bacteria induce RANKL expression in osteocytes of wild type mice but not experimental mice. **(A)** The RANKL expression in osteocytes in bone was detected by immunofluorescence with a RANKL specific antibody. Data are expressed as the mean fluorescence intensity **(B)** RANKL expression was measured in gingiva by immunofluorescence with a RANKL specific antibody. Data represent mean $\pm$ SEM of 5–7 animals per group. * indicates $p < 0.05$ compared to uninfected baseline control; + indicates $p < 0.05$ compared to matched experimental group.