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RANKL, Osteopontin, and Osteoclast Homeostasis in a Hyper-Occlusion Mouse Model

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Abstract

The biological mechanisms that maintain the position of teeth in their sockets establish a dynamic equilibrium between bone resorption and apposition. In order to reveal some of the dynamics involved in the tissue responses toward occlusal forces on periodontal ligament and alveolar bone homeostasis, we have developed the first mouse model of hyper-occlusion. Swiss Webster mice were kept in hyper-occlusion for 0, 3, 6, and 9 d. Morphological and histological changes in the periodontium were assessed using micro CT and ground sections with fluorescent detection of vital dye labels. Sections were stained for tartrate-resistant acid phosphatase (TRAP), and expression of RANKL and OPN was analyzed by immunohistochemistry and real-time PCR. Traumatic occlusion resulted in enamel surface abrasion, inhibition of alveolar bone apposition, significant formation of osteoclasts at 3 d, 6 d, and 9 d, and upregulation of OPN and RANKL. Data from this study suggest that both OPN and RANKL contribute to the stimulation of bone resorption in the hyperocclusive state. In addition, we propose that the inhibition of alveolar bone apposition by occlusal forces is an important mechanism for the control of occlusal height that might work in synergy with RANKL induced bone resorption to maintain normal occlusion.

Keywords

Occlusal Trauma; RANKL; Osteopontin; Tooth Movement; Osteoclast

Introduction

Tooth sockets are intriguing biological environments in which continuous bone resorption and deposition maintain the occlusal position of teeth while a non-mineralized periodontal ligament of defined width provides a semi-resilient anchorage of tooth roots in the jaw apparatus. The dynamic structure of the tooth socket attachment apparatus is underscored by the rapid apposition of calcified tissues and distal drifting of the teeth (in rodents) that occurs as soon as occlusal forces are no longer in place (1). In contrast, the forces produced by normal occlusion have an inhibitory effect on unopposed eruption and physiologic drifting of teeth in mice (1–3). Both of these phenomena are superimposed over the normal bone turnover process mediated by osteoblasts and osteoclasts (4).

Bone modeling and remodeling are integral processes involving multiple feedback loops between all cells involved (4–7). During bone remodeling, receptor activator of nuclear factor κ B ligand (RANKL) expressed by osteoblasts coordinates local osteoclast development and resorption, which in turn stimulates bone synthesis by adjacent osteoblasts. Although osteoblasts are thought to be the major functional regulators of osteoclast activity, many other cell types also influence osteoclast activity, including periodontal ligament fibroblasts, which express RANKL in response to compressive forces *in vitro* and *in vivo* (8–11). Another important factor that mediates the osteoclast response to mechanical force is the extracellular matrix glycoprotein Osteopontin (OPN) (12). OPN contains an Arg-Gly-Asp (RGD) motif, essential for the binding of integrin receptors, and has been found to be important for osteoclast chemotaxis and attachment to bone (13–16). Prior research has established that orthodontic forces increase expression levels of both RANKL and OPN in the periodontal ligament and alveolar bone respectively (17–19).

To better understand the role of occlusal force and the influence of RANKL and OPN on periodontal ligament homeostasis we have developed an experimental mouse model of traumatic occlusion. This strategy results in an increase in occlusal force, which in turn induces a shift from the dynamic equilibrium state towards a resorptive state at periodontal ligament/alveolar bone interface of the root apex. We have used our traumatic hyper-occlusion model to precisely map the effects of occlusal forces on RANKL and OPN expression and osteoclast numbers over a period of nine days. The results from these studies were compared to previous findings using an un-opposed eruption model (20) to gain further insights into the balance of formative and resorptive tissue dynamics involved in the maintenance of dental occlusion.

Materials and Methods

Treatment of Animals

40 d old Swiss-Webster mice were purchased (Charles River; Wilmington, MA, USA) and separated into various experimental groups. Anesthesia was accomplished using Ketamine (100 mg/kg) and Xylazine (5 mg/kg). In order to create a hyperocclusive state, the three right maxillary molars were prepared to accept a stainless steel build-up by acid-etching tooth surfaces in 30% phosphoric acid for 30 s and rinsing thereafter. Stainless steel wire (.012" diameter) was cut into 5mm sections, folded in half and bonded to the occlusal surfaces of the right maxillary molars using a resin adhesive (Optibond Solo, Kerr, CA, USA) (Fig. 1). All animal experiments and procedures followed the guidelines of the University of Illinois at Chicago Animal Care Committee.

Tissue Processing

Groups of 10 mice were maintained in hyper-occlusion for periods of 9 d, 6 d, 3 d and 0 d (control), after which they were sacrificed by CO₂. All 0 d mice (controls) were sacrificed immediately after treatment. Experiments were timed so that all mice of varying treatment lengths were the same age and sacrificed together on the same day. Mandibles were collected and immersed in 4% paraformaldehyde for 24 h followed by decalcification for 2 wk with 5% EDTA and 1% paraformaldehyde. Specimens were dehydrated, embedded in paraffin, and cut in 6µm sagittal sections down the long axis of the molar teeth to be used for TRAP staining or immunohistochemistry.

Tartrate resistant acid phosphatase staining and osteoclast counting

Paraffin sections were deparaffinized, rehydrated and incubated in acetate buffered solution containing naphthol AS-MX phosphate, Fast Garnet GBC salt, and tartrate solution (.67 mol/L) (Sigma, St Louis, MO, USA) for 60 min. Sections were counterstained using Villanueva osteochrome bone stain and hematoxylin. Sections on slides were numbered and an equal

number of sections from each mouse were selected using a random number table. Sections were photographed and TRAP positive cells in the periodontal ligament space were counted. Three areas of similar area were defined to aid in the counting process that could consistently be identified. The furcation area was defined area coronal to the furcal arch, the apex area was defined as the area of periodontal ligament apical to the border of cellular and acellular cementum (apical third), and the remaining area was designated as the interradicular root surface (intermediate third) (Fig. 3F). Osteoclast counts after 3 d, 6 d, and 9 d of treatment were compared to the control and analyzed for significance using the Mann-Whitney U test. Six mice were used at each time point.

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated and treated with 6% peroxide and methanol followed by a brief incubation in 10 mM Sodium Citrate Buffer with .05% tween 20 at pH 6.0 for antigen retrieval. Sections were incubated with monoclonal antibody for RANKL (12A380) at a concentration of 5µg/ml (Alexis Biochemicals, San Diego, CA, USA) or Osteopontin (SC-21742) at a dilution of 1:200 (Santa Cruz, CA, USA), washed three times in PBS and visualized using a Histomouse Broad Spectrum AEC kit (Zymed, San Francisco, CA, USA) and Hematoxylin as a counterstain. The following controls were used to determine the specificity of the antibodies: (i) tissue controls - the specificity of the antibody was evaluated in tissues with known immunoreactivity (ii) antibody controls by using a dilution series, (iii) controls with pre-absorbed antibody to exclude unspecific binding, (iv) omission of primary antibody as a systemic control.

Vital-dye Labeling and von Kossa

A control group (10 mice) and a treatment group (10 mice) were injected intraperitoneally with tetracycline hydrochloride (50 mg/kg in .9% sodium chloride) at 0 d, alizarin red (25 mg/kg in .9% sodium chloride) at 3 d, and calcein blue (30mg/kg in .9% sodium chloride) at 6 d. Due to their ability to chelate calcium, vital dyes are incorporated into mineralizing hard tissue. Their fluorescent properties are particularly advantageous as they allow for the visualization of mineralization over a period of several days. Treatment and control groups were sacrificed at 9 d by CO₂ gas, and mandibles were stored in formalin overnight for fixation. Mandibles were then dehydrated and infiltrated with resin (Technovit 2000, Exakt Technologies, Oklahoma City, OK, USA) and then prepared into undecalcified ground sections for fluorescent microscopy or von Kossa's staining. Ground sections were treated with 5 % silver nitrate as previously described in von Kossa's staining method (21).

Morphometry Preparation and MicroCT

Three mice treated for 9 d were sacrificed along with three control mice. Mice were skeletonized by removing all soft tissue. The mandibles were photographed and studied for morphological differences. Resin embedded mandibles were imaged by synchrotron micro CT (Argonne National Labs, Argonne, IL) at 0 d (control) and 9 d. Image slices were reconstructed into 3D volume images using ImageJ software (NIH, Bethesda, MD, USA) and the Volume Viewer/3D slicer plug-in (Internationale Medieninformatik, Berlin, Germany). Midroot Sagittal slices and 3D reconstructions were examined for changes in PDL and bone morphometry.

Real-time quantitative PCR

Root apices of mice treated for 0 d (control), 3 d, 6 d, and 9 d (5 each) were removed and stored in liquid nitrogen. TRIZOL LS Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract mRNA according to the manufacturer's instructions. RNAs underwent RT-PCR using random hexamer primers. Real-time quantitative PCR was conducted using the SuperScript III

Platinum two-step qPCR kit (Invitrogen, Carlsbad, CA, USA) with DLUX fluorogenic primers. Primer sequences for RANKL and OPN were designed and synthesized by Invitrogen (FAM labeled LUX Designer primers, Invitrogen, Carlsbad, CA, USA). Samples were normalized using ribosome 18s RNA (JOE labeled LUX primer set, Invitrogen, Carlsbad, CA, USA). Reaction condition were as follows; 2 min at 50 °C (one cycle), 10 min at 95 °C (one cycle), and 15 s at 95 °C and 1 min at 60 °C (40 cycles). PCR products were continuously monitored with an ABI PRISM 7900 detection system (Applied Biosystems, Foster City, USA). Relative expression levels were calculated using the 2^{-Delta Delta Ct} method (22) and values were graphed as the mean expression level $\pm$ standard deviation. Statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by a Tukey-Kramer pairwise test of significance with $\alpha=.05$.

Results

Morphological changes

Skeletonized mandibles and micro-CT 3D reconstructions from control and treatment groups were analyzed for morphological differences between control molars and molars subjected to hyper-occlusion for 9 d. Significant levels (0.223 ± 0.015 mm) of abrasion were detected on the occlusal surfaces of the molar teeth subjected to traumatic occlusion compared to the control group (Fig. 2A–C). In control mice, the physiological eruption of molar teeth was documented by the presence of three distinct layers of dye (Fig. 2D). Distinct vital dye layers were absent in the experimental hyper-occlusion group indicative of a disruption of the continuous deposition of alveolar bone and cementum at the root apex (Fig 2E). Hyper-occlusion also affected the morphology of the dense cortical alveolar bone proper immediately bordering the periodontal ligament (Fig. 2F,G). Visualization of mineralized phosphates by von Kossa's stain revealed a decrease in the thickness of the dense alveolar bone proper bordering the periodontal ligament as a result of traumatic occlusion when compared to the controls (Fig. 2F,G). Analysis of Micro CT sections revealed a narrowing of the periodontal ligament as well as the presence of direct bone resorption, cementum resorption, and undermining resorption in tooth furcations under the pressures of traumatic occlusion (Fig. 2H,I).

TRAP positive cell counts

TRAP stainings were performed in order to correlate the extensive periodontal tissue remodeling in molars subjected to hyperocclusion with osteoclast number and biology. In this study, tooth sections were examined at the furcation, in the alveolar bone along the interradicular areas, and at the apex, revealing extensive cementum and root resorption in all three areas (Fig. 3). At 3 d, 6 d, and 9 d, there was a significant increase in TRAP positive cells at root apices ($P < 0.003$). Significant osteoclast increases were also measured at 6 d and 9 d in the furcation area ($P < 0.004$), and at interradicular bone surfaces ($p < 0.003$) when compared to the controls (Fig. 3E).

RANKL expression

In order to test to what extent the important osteoclastogenic factor RANKL might be involved in the remodeling of tooth anchorage tissues during hyper-occlusion, immunohistochemical and quantitative real-time RT-PCR assays were conducted. RANKL staining in the PDL of control animals was weak, with isolated areas of specific expression adjacent to blood vessels. In contrast, immunostained sections of mice in hyper-occlusion exhibited dramatically high levels of RANKL expression at 6 d and 9 d. Especially high RANKL expression was seen at 6 d in the periodontal ligament adjacent to blood vessels and at root apices (Fig. 4B, D). Quantitative real-time RT-PCR confirmed our immunohistochemical analysis, demonstrating a 2-fold increased RANKL expression in periodontal tissues vs. the control on 6 d and 9 d of the experiment (Fig. 4).

Osteopontin expression

A third molecule that was of particular interest to document the molecular response to hyper-occlusion was the mechanosensitive and osteoclastogenic protein osteopontin (OPN). Hyper-occlusion led to increased OPN staining on the surfaces of alveolar bone surrounding root apices at 6 d (Fig 5B) and 9 d (not shown). In contrast, OPN was not visualized on the surface of alveolar bone immediately apical to the apex in the control groups (Fig 5A). Additionally, osteopontin mRNA expression was found to be 2-fold upregulated at 6 d of the hyper-occlusion study as determined by quantitative real-time PCR.

Discussion

In the present study we have developed a build-up model of traumatic hyper-occlusion to investigate the morphological changes and molecular mechanisms of bone homeostasis in the dentition. This is the first mouse model of hyper-occlusion, allowing for extensive comparisons with other mouse models related to tooth movement and molecular responses to trauma. As a first step we conducted morphometric studies using skeletonized mandibles, micro-CT, and ground sections for the purpose of establishing and validating the model. The effects of traumatic occlusion were immediately visible including severe attrition of teeth subjected to traumatic antagonists. Not only was there extensive occlusal wear on the build-up side, but there was also increased cuspal wear on the side opposite of the buildup. The fixated build-up resulted in traumatic occlusion and extensive tissue remodeling and changes in gene expression in the opposed periodontal apparatus, which form the basis of the present study.

In an earlier study we used vital dyes to demonstrate that the absence of occlusal force on mice molar teeth leads to the rapid apposition of calcified tissues and eruption of the teeth (1). The images from our previous vital dye experiments reveal that mechanical forces generated during normal occlusion has an inhibitory effect on the eruption of the teeth but does not altogether prevent eruption of the teeth (1,20). This finding was confirmed in our control groups which also featured evenly spaced fluorescent layers in the alveolar bone and cementum of control teeth. In contrast to normal occlusion, experimental hyper-occlusion resulted in a net blockage of the deposition of bone and cementum and an activation of resorption as shown by the absence of distinct dye layers after 9 d of treatment. The presence of dye layers and small numbers of osteoclasts in the control groups, compared to the absence of dye layers and increased osteoclast numbers in treatment groups, led us to conclude that the normal position of the root apex in the alveolar bone socket is maintained as a sublime equilibrium between continuous growth and continuous remodeling. In contrast, our hyperocclusion studies indicate that the effect of occlusal trauma is manifested as an increase in the resorptive component of this equilibrium, effectively off-setting the effect of continuous growth and eruption.

Osteopontin was strongly expressed in the cellular cementum and the alveolar bone/periodontal ligament interface of mouse molars. Osteopontin is a multifunctional protein involved in bone turnover, osteoclastogenesis, and osteoblast function (23). The role of osteopontin in mineralized tissue homeostasis depends greatly on its final posttranslational modification state (24). For example, OPN is expressed in cementum where it is thought to be involved in the stabilization of mineralization rather than in the remodeling by osteoclasts. In bone, osteopontin is found localized at cement lines and sites of resorption (24). Studies in force-induced bone resorption and orthodontic tooth movement using osteopontin KO mice have demonstrated that osteopontin also plays an important role in the transmission and tissue-related effects of mechanical force on bone remodeling (18,25).

Our studies demonstrated strong osteopontin expression on the apical alveolar bone surface as a result of hyper-occlusion while osteopontin was absent in the control. The complete lack of OPN immunolabeling on the apical bone surface of our control tissues was somewhat a surprise

finding, indicating that there was little or no bone resorption in this area despite the constant stimulation by occlusal mechanical force. Together with our vital dye labeling results showing continuous apposition of mineralized tissues at the root apex, the lack of osteopontin on the apical alveolar bone surface leads us to believe that the position of the root apex in the alveolar bone socket in physiologically occluding teeth is maintained by an inhibition of bone apposition rather than by the stimulation of bone resorption. In contrast, increased levels of osteopontin in the hyper-occlusive state may facilitate osteoclast activation and cementum/alveolar bone surface resorption in tandem with an inhibition of eruption as a response to mechanical trauma.

As a potent activator of osteoclasts, RANKL may play a role in the force mediated homeostasis of the periodontal tissues. Our immunohistochemical and quantitative real-time RT-PCR data have demonstrated that hyper-occlusion resulted in an increased expression of RANKL in periodontal tissues. Though RANKL staining was much greater under traumatic occlusion, areas of RANKL expression were still detected in the PDL of control mice by immunohistochemistry. RANKL expression in control mice was less specific and intense compared to the treatment groups where there was a strong and specific localization of RANKL to the areas of the root apices, alveolar crest and the furcation (data not shown). High levels of RANKL in the periodontal ligament of traumatized periodontia might be related to osteoclast activation and bone/cementum resorption in adjacent areas. In contrast, low levels of RANKL expression in non-traumatic occlusal states may be related to the continuous re-modeling of periodontal tissues.

In summary, our mouse model provided a predictable and repeatable system for the study of the effects of force on bone remodeling. Traumatic occlusion resulted in an induction of RANKL and OPN expression, both of which may contribute to an increased activation and migration of osteoclasts. In addition, the data from our study provide evidence that the maintenance of a physiological occlusal state is achieved primarily by the effect of occlusal forces on the inhibition of bone apposition rather than by an equilibrium between continuous apical tissue deposition off-set by occlusal force-stimulated bone resorption.

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Fig. 1. Molar build-up technique for obtaining experimental traumatic occlusion. A hyper-occlusion situation was created by bonding a U-shaped .012 diameter stainless-steel wire (BU) to the occlusal surfaces of the maxillary right-side molars: (M) indicates the left side maxillary first molar for orientation.

Fig. 2.

Effects of traumatic occlusion on crown and alveolar bone morphology. (A–C). Molar tooth abrasion as a result of traumatic occlusion. Significant abrasion (arrow) of the occlusal surfaces occurred as a consequence of 9 d of traumatic occlusion in the experimental group (B) compared to the balanced occlusal profile of the control group (A). (C) Micro-CT 3D reconstruction illustrates the effects of abrasion (ABR) and an almost leveled occlusal surface in a row of mouse molars (M₁, M₂, M₃) after being exposed to hyper-occlusion for 9 d. (D–E) Ground sections of mice subjected to vital dye labeling of mineralization fronts (arrows) reveal continuous cementum and alveolar bone deposition as evidence of constant molar eruption in control teeth (D), while the mice in hyper-occlusion showed no new cementum and/or bone apposition as evidenced by an absence of dye layers (E): alveolar bone (AB), cementum (CEM), and periodontal ligament (PDL). (F–G) Visualization of periapical

mineralized tissues by von Kossa's stain. Note the decrease in the thickness of the dark brown stained alveolar bone proper (ABP) in molars subjected to traumatic occlusion (G) compared to control teeth (F). (H–I). Micro CT sections revealed areas of bone and cementum resorption (arrows) in tooth furcations during traumatic occlusion (I) as compared to the control (H): alveolar bone (AB) periodontal ligament (PDL), dentin (DEN), island of alveolar bone (*) undermined by resorption. Bars: 1mm A,B; 500µm C; 150 µm D,E,F,G; 250 µm H,I;

Fig. 3.

Effect of traumatic hyper-occlusion on the number of tartrate-resistant acid phosphatase (TRAP) positive cells. There was a significant increase in TRAP-positive cells at 3 d, 6 d, and 9 d in our hyper-occlusion model compared to the controls. (E) In our model, the number of TRAP positive cells was significantly increased at 6 d and 9 d at the apex ($p < 0.003$), in the furcation ($p < 0.004$), and on inter-radicular surfaces ($p < 0.003$) when compared to the controls. Plates A-D display examples of bone remodeling and increases in the number of TRAP positive cells. (A-B) Significant numbers of TRAP positive cells (arrow) were detected in the inter-radicular region of the alveolar bone (AB) as a result of traumatic occlusion (B) compared to the control (A). (C) Traumatic occlusion also resulted in apical root resorption as evidenced

by the presence of odontoclasts (arrow). (D) Bone and cementum (CEM) resorption as a result of traumatic occlusion were also prominent in the first molar furcation area. Note the increase in small TRAP-positive cells adjacent to the capillary (BV). Cementoclast (*) Osteoclast (arrow). Bars: 250µm A,B,C; 100µm D; (F) Defined areas for osteoclast counting Furcation (3) Interradicular area (2) Apex (1).

Fig. 4.

Increased RANKL expression as a result of traumatic occlusion. (A–B) Compared to the control (A), tissues subjected to 6 d of traumatic occlusion contained numerous distinct areas of high RANKL expression in the periodontal ligament (PDL) and in the dental papilla (B). (C–D) Significant upregulation of RANKL expression in the apical PDL during traumatic occlusion (D) vs. the control (C): root apex (AP) alveolar bone (AB). (E) Hyper-occlusion resulted in a gradual increase in the expression of the essential osteoclast inducing signal RANKL: RANKL gene expression was significantly unregulated at 6 d and 9 d compared to 0 d as evidenced by quantitative real-time PCR. * Significantly different from the values in the 0 d (control) group ($P < 0.05$) Bar: 100 μ m A,B,C,D.

Fig. 5. Effect of hyperocclusion on osteopontin (OPN) expression in the periodontal ligament and at the root apex. (A) In controls, OPN was detected at the alveolar bone/PDL interface on the mesial and distal aspect but not apical. (B) Hyper-occlusion resulted in the deposition of OPN on the surface of alveolar bone surrounding root apices. There was also significantly increased OPN immunoreactivity in the periodontal ligament between the root apex (AP) and the adjacent alveolar bone (AB). (C) In our quantitative real-time PCR time-course analysis, osteopontin mRNA expression was significantly upregulated at 6 d compared to 0 d and 3 d, and subsequently reduced at 9 d. These data suggest a delayed response of osteopontin gene expression toward the traumatic signals and a subsequent adaptation of the tissue response. * Significantly different from the values in the 0 d (control) group ($P < 0.05$) Bar: 75 μ m A,B.