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Local delivery of osteoprotegerin inhibits mechanically mediated bone modeling in orthodontic tooth movement

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Abstract

Introduction—The RANKL-OPG axis is a key regulator of osteoclastogenesis and bone turnover activity. Its contribution to bone resorption under altered mechanical states, however, has not been fully elucidated. Here we examined the role of OPG in regulating mechanically induced bone modeling in a rat model of orthodontic tooth movement.

Methods—The maxillary first molars of male Sprague-Dawley rats were moved mesially using a calibrated nickel–titanium spring attached to the maxillary incisor teeth. Two different doses (0.5 mg/kg, 5.0 mg/kg) of a recombinant fusion protein (OPG-Fc), were injected twice weekly mesial to the first molars. Tooth movement was measured using stone casts that were scanned and magnified. Changes in bone quantity were measured using micro-computed tomography and histomorphometric analysis was used to quantify osteoclasts and volumetric parameters. Finally, circulating levels of TRAP-5b (a bone resorption marker) was measured using enzyme-linked immunosorbent assay.

Results—The 5.0 mg/kg OPG-Fc dose showed a potent reduction in mesial molar movement and osteoclast numbers compared to controls ($p < 0.01$). The molar movement was inhibited by 45.7%, 70.6%, and 78.7% compared to controls at days 7, 14, and 21 respectively, with the high dose of OPG. The 0.5 mg dose also significantly ($p < 0.05$) inhibited molar movement at days 7 (43.8%) and 14 (31.8%). While incisor retraction was also decreased by OPG-Fc, the ratio of incisor to molar tooth movement was markedly better in the high-dose OPG group (5.2:1, $p < 0.001$) compared to the control group (2.3:1) and the low-dose OPG group (2.0:1).

Conclusions—Local delivery of OPG-Fc inhibits osteoclastogenesis and tooth movement at targeted dental sites.

Keywords

RANKL inhibitor; OPG; Osteoclast; Micro-computed tomography; Tooth movement

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Introduction

Bone deposition and resorption result from the interaction between bone-forming osteoblasts and bone-resorbing osteoclasts. Cells of the osteoblast lineage are not only involved in bone formation, but also regulate osteoclast formation, activation, and survival. This regulation is indirectly mediated by receptor activator of nuclear factor κ B ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily. RANKL is produced by osteoblast lineage cells, periodontal ligament (PDL) cells, and by T lymphocytes. RANKL binds to a receptor called RANK, which is located on the surface of osteoclasts and osteoclast precursors. The binding of RANKL to RANK induces osteoclastogenesis, activates mature osteoclasts, mediates their attachment to bone, and promotes their survival. The activity of RANKL is controlled by a soluble decoy receptor called osteoprotegerin (OPG), which binds to RANK and inhibits osteoclast formation, activation, and survival [1].

While the contribution of RANKL–OPG in bone turnover mediated via systemic and local biologic agents has been well studied [2,3], less is known of the contribution of these proteins to mechanically induced bone turnover. Several in vitro studies provide important insights into the potential role of mechanically regulated OPG and RANKL in modulating bone turnover in altered strain environments. Compressive mechanical loading of isolated PDL cells leads to a substantial upregulation of RANKL with little change or slight increase in OPG expression which in turn increases osteoclastogenesis when loaded cells are co-cultured with peripheral bone mononuclear cells [4,5]. Also, conditioned media from osteoblasts subjected to microgravity show an increase in the RANKL/OPG ratio, which is accompanied by increased osteoclastogenesis and bone resorption in mouse bone marrow cultures when compared to cells grown at 1 g. In contrast, dynamic tensile loading upregulates OPG mRNA and concentration of OPG in conditioned medium, while having little effect on the levels of RANKL in human PDL cells [6], and conditioned media of PDL cells subjected to cyclical tensile force inhibit osteoclastogenesis [7]. Similarly, human osteoblasts demonstrate increased OPG expression but also a decrease in soluble RANKL when subjected to cyclic tensile strain [8]. Moreover, osteoblasts cultured on artificial substrates and subjected to bending also demonstrate increased levels of OPG relative to RANKL [9]. Finally, ST-2 murine bone marrow stromal cells exposed to oscillating fluid flow show a maximal reduction in RANKL/OPG immediately after the end of flow with a significant increase in OPG and decrease in RANKL [10]. RAW 264.7 monocytes co-cultured with ST-2 cells and subjected to fluid flow showed a decrease in osteoclast formation when compared with control cells. Together, these studies demonstrate that different types of mechanical strains by differentially regulating OPG and RANKL result in important differences in the net osteolytic responses. Specifically, the findings suggest that while microgravity and cyclic compressive forces likely contribute to a net increase in osteolytic activity by enhancing the RANKL/OPG ratio, the reverse is true for cyclic tensile strain and oscillating fluid flow. While these in vitro studies may not precisely represent the complex nature of mechanical strains experienced by loaded bone in vivo, including that in the PDL during orthodontic tooth movement, they indicate that cells are capable of perceiving and responding differently to diverse strain histories.

Limited in vivo data also point to the role of RANKL and OPG in modulating mechanically mediated bone turnover. This includes the effects of the administration of recombinant human OPG in reversing the decrease in bone mineral content (BMC), bone mineral density (BMD), and bone strength back to normal levels in limbs of immobilized rats [11]. It has also been demonstrated that excess OPG actually impairs bone remodeling in a situation such as callus repair following fracture indicating that the ideal ratio of OPG to RANKL is dependent on the mechanical model being employed [12]. Finally, local OPG gene transfer to sites significantly diminishes while RANKL gene transfer significantly enhances orthodontic tooth movement

possibly by respectively inhibiting or enhancing RANKL-mediated osteoclastogenesis [13, 14]. Orthodontic tooth movement is a well-utilized in vivo model for determining the contributions of various exogenous and endogenous agents to mechanically mediated bone modeling [15–19]. It results from the application of forces to teeth that cause bone resorption under pressure and bone deposition under tension. The entire process is based on bone turnover in which the bone surrounding the roots undergoes degradation and active reparative mechanisms as a response to orthodontic forces.

Recently, a fusion protein OPG-Fc and other RANKL inhibitors have been shown to reduce bone resorption systemically and preserve bone in a variety of clinical and preclinical disease settings. Examples include primary osteoporosis, Paget's disease, rheumatoid arthritis, hypercalcemia of malignancy, osteolytic metastases, postmenopausal bone loss, and periodontal disease [1,20–27]. While the potential benefits of these RANKL inhibitors in systemic conditions resulting in significant bone loss are evident, there are also possible uses for local RANKL inhibition such as during mechano-modulation of bone modeling during orthodontic tooth movement, where it is often necessary to minimize unnecessary movement of teeth. The stability of anchor teeth, which tend to inadvertently move during treatment, is a critical shortcoming in orthodontics. In order to combat this undesirable movement, orthodontists have developed several mechanical methods of improving anchorage. However, these have substantial limitations including the need for compliance, discomfort, cost or lack of efficacy [28,29]. Given these disadvantages, a pharmacological approach aimed at utilizing the known biological mechanisms underlying tooth movement may provide an effective, non-compliant, non-visible means of anchorage. If the resorptive process of modeling during tooth movement can be inhibited, tooth movement may be inhibited as well. Accordingly, if OPG is involved in mechano-modulation of bone modeling, its local inhibition of RANKL may provide a novel pharmacological approach for preventing unneeded tooth movement that is highly desirable for preserving orthodontic anchorage.

In this study we tested the hypothesis that local delivery of OPG-Fc will inhibit mechanically induced bone modeling in a rat model of orthodontic tooth movement at the site of OPG-Fc delivery. The specific objectives of this study were to: (1) assess the magnitude of movement of molar and incisor teeth at sites closest and distant, respectively, to the administration of OPG-Fc; (2) quantify the bone resorptive activity by histomorphometric assessment of osteoclasts; (3) determine the effects of OPG-Fc on bone density through micro-CT analysis; and (4) assay for serum levels of a bone resorption maker, TRAP-5b.

Materials and methods

Animals

A total of 39 male Sprague-Dawley rats (approximate weight 250–300 g) were utilized in this study. Thirty animals divided into groups of ten were subject to orthodontic forces in addition to volumetrically equivalent injections of 5.0 mg/kg human OPG-Fc (AMGEN, Inc., Thousand Oaks, CA), 0.5 mg/kg OPG-Fc, or phosphate-buffered saline (PBS) vehicle. Three animals received no appliances or injections and were sacrificed at baseline; three animals were vehicle-injected with no appliances, and three animals received high-dose OPG and no appliances. All injections were administered into the palatal mucosa adjacent to the mesial surface of the first molar teeth using 33-gauge microneedles (Hamilton Company, Reno, NV). The animals were evaluated at baseline, 3,7,10,14,17, and 21 days after appliance placement. All procedures were approved by the University of Michigan Committee of Use and Care of Animals. For baseline procedures including appliance placement, the animals were anaesthetized as described below.

Appliance placement

We utilized a previously established rat tooth movement model and appliance design (Fig. 1A) [30]. Animals were first placed under general anesthesia with ketamine (87 mg/kg) and xylazine (13 mg/kg) for initial appliance placement. A closed coil nickel-titanium spring (Sentalloy®, GAC, Ctr. Islip, NY) calibrated by an Instron Universal Testing Machine (Model 5565, Norwood, MA) providing a force of 54 ± 2 g at 4 mm activation was connected between the maxillary first molar and maxillary central incisor teeth with 0.010-in. steel ligatures. Previous studies have demonstrated that a 40–60 g level of force stimulated substantial molar tooth movement in rats [23,30–34]. A nickel–titanium spring was used to provide a relatively constant force level over the course of the experiment.

For placement of springs, grooves were prepared along the distolingual line angles of the maxillary first molars to allow easier ligature placement as well as prevent the ligatures from slipping over the contact areas once the teeth started to move [30]. In addition, grooves were prepared on the facial, mesial, and distal surfaces of the maxillary central incisors to prevent the ligatures from dislodging from the teeth due to their lingual curvature and eruption pattern. After the ligatures were tied and cut, composite resin (Transbond XT Light Cure Adhesive Paste, 3M Unitek, Monrovia, CA) was placed over the wire to prevent slipping as well as pulpal irritation due to exposed dentin. Finally, the mandibular incisors were reduced to prevent appliance breakage.

Measurement of tooth movement

Tooth movement was measured as previously described, with minor modifications [14,19]. At each time point including baseline, polyvinylsiloxane (Dimension Garant 2 L Quick, 3M/ESPE, St. Paul, MN) impressions were taken of the maxillary arches. Following fabrication of precise stone models (Jade Stone, Whip Mix Corp., Louisville, KY), the occlusal tooth surfaces were scanned (Epson Expression 1680, Epson America, Inc., Long Beach, CA) adjacent to a 100 mm ruler and then magnified 100× using calibrated imaging software (Adobe Photoshop 7.0.1, Adobe Systems, Inc., San Jose, CA). Using Adobe Photoshop's measuring tool, a single masked, calibrated examiner ($ICC > 0.972$; $0.954 \leq 95\%$ confidence interval ≤ 0.983) made measurements from the distobuccal groove of the maxillary first molar to the most distal surface of the maxillary third molar at each time point. The amount of tooth movement was calculated as the difference between the pre-treatment, intra- and post-treatment measurements. In addition to molar movement, the amount of incisor retraction was also measured relative to the distal surface of the third molar teeth. Incisor position was measured from the mesiodistal center of each central incisor at the facial gingival margin. The amount of tooth movement was calculated as the difference between the measurements made during and at the end of treatment and the measurement at baseline. Given that maxillary growth also results in a change of incisor position relative to the third molar reference point, the amount of incisor retraction was calculated in terms of incisor position relative to that of untreated controls at day 21. The impressions did not include the incisors during the intra-study time points to avoid altering the springs involved in the promotion of tooth movement. Therefore, since the untreated controls showed a mean of 1.42 mm of forward incisor displacement, this value was added to all values of incisor movement derived from the models of the experimental animals.

Biopsy harvest and histological preparation

The animals were sacrificed by CO₂ euthanasia at 21 days following appliance placement. After taking final impressions, block biopsies of the right and left maxillae were harvested, immediately fixed and stored in 10% formalin for 48 h, and then transferred to 70% ethanol. Samples were divided randomly (right and left) so that half the samples were imaged using micro-computed tomography and the other half prepared for histology. Samples used for histology were decalcified with 10% vol./vol. EDTA and the embedded in paraffin. Sagittal

sections (4–5 μm thick) were obtained of the mesial root of the maxillary first molar. The mesial root was chosen because it is the largest of the first molar's five roots, is in approximately the same plane as the applied force and is most commonly evaluated in tooth movement studies [30,35–37]. The sections were stained with hematoxylin and eosin (H & E) for descriptive histology.

Immunohistochemical analysis

Tartrate-resistant acid phosphatase (TRAP) immunohistochemical staining was performed to identify and quantify osteoclasts. A minimum of six, randomly selected midsagittal sections per animal were deparaffinized and then incubated in DakoCytomation Target Retrieval Solution (Dako Corp., Carpinteria, CA) for 30 m. TRAP immunohistochemical staining was performed with a goat ABC Staining System kit (sc-2023; Santa Cruz Biotechnology) and anti-TRAP antibody (sc-30833; Santa Cruz Biotechnology).

Images of coded specimens were captured using a Nikon Eclipse 50i microscope (Nikon, Inc., Melville, NY) fitted with a Nikon Digital Sight DS U1 camera (Nikon, Inc., Melville, NY) for analysis using Image Pro Plus™ software (Media Cybernetics, Silver Spring, MD). A single masked, calibrated examiner examined the images and quantified tension side, compression side, and total osteoclasts (TRAP-positive cells) present on the alveolar bone surface adjacent to the entire mesial root.

Micro-computed tomography

Micro-CT analysis was done to quantify alveolar bone in the proximity of the first molar roots. Regions of fixed, non-demineralized rat maxillae were scanned in de-ionized water using a cone beam micro-CT system (GE Healthcare, London, ON, Canada). Each scan was reconstructed at a mesh size of $18\ \mu\text{m} \times 18\ \mu\text{m} \times 18\ \mu\text{m}$, allowing a three-dimensional digitized image to be generated for each specimen (Fig. 1B). Using GE Healthcare Microview Analysis + software, the images were rotated into a standard orientation and threshold to distinguish the degree of mineralization of tissues such as bone, tooth roots and tooth crowns. For each specimen, a gray voxel value histogram was generated to determine an optimal threshold value. An average of the individual threshold values was calculated and a representative value was used to threshold all images.

Measurements of the bone volume fraction (BVF) in the first molar furcation area were carried out as previously described [38]. The furcation area and root apex was chosen because they provide reproducible, morphological landmarks. Briefly, the most mesial and distal roots of the first molar were selected as endpoint landmark borders. Two-dimensional regions of interest (ROIs) were then drawn at regular intervals (mean = 144 μm) in order to quantify tooth-supporting alveolar bone.

Systemic measure of osteoclast activity was determined by assaying for TRAP-5b by ELISA (RatTRAP Assay, SBA Sciences, Finland), in accordance with manufacturer's instructions. TRAP-5b is specific for osteoclast activity thus providing a marker of bone resorption [40].

Statistical analysis

Descriptive statistics (mean, standard error) for each parameter were calculated for all groups. Each animal was used as the experimental unit. Comparisons of tooth movement were made using repeated measures, analysis of variance and Tukey's HSD post-hoc comparison. Osteoclast number, bone volume fraction, serum OPG, and serum TRAP-5b comparisons were made using analysis of variance (ANOVA) and Tukey's HSD post-hoc comparison. Kruskal–Wallis analysis of ranks was used to compare ratios of incisor retraction to molar movement. Differences with $p < 0.05$ were considered statistically significant.

Results

Animal status

Appliance placement and injections did not appear to hinder the animals' ability to thrive. There were no significant differences in weight gain among the groups. The mean + SD starting weight of all animals at baseline was 272.6±13.4 g. At 21 days, the animals weighed 372.0±25.8 g, 377.4±15.8 g, and 353.5±25.5 g in the vehicle, 0.5 mg/kg OPG-Fc, and 5.0 mg/kg OPG-Fc groups, respectively. There was 100% appliance success rate as no tooth movement appliances broke or needed to be replaced over the 3-week experimental period.

Tooth movement

Local delivery of OPG-Fc resulted in a substantial decrease in mesial movement of the first molar tooth when compared to vehicle-injected control animals (Fig. 2A). At day 7, there was a significant decrease in molar movement in both the 0.5 mg/kg OPG-Fc group (0.15±0.02 mm, $p<0.05$) and 5.0 mg/kg OPG-Fc group (0.14±0.02 mm, $p<0.01$) when compared to controls (0.27±0.03 mm). However, at this time-point there was no significant difference in amount of molar movement between groups receiving the two doses of OPG-Fc. By day 14, not only was there a significant decrease in mesial molar movement in the low (0.37±0.05 mm, $p<0.05$) and high (0.16±0.03 mm, $p<0.001$) OPG groups compared to controls (0.54±0.05 mm) but there was also a significant difference between the low- and high-dose OPG groups ($p<0.05$). Only the high-dose OPG group (0.20±0.03 mm, $p<0.001$) showed a significant decrease in molar movement at day 21 compared to controls (0.93±0.07 mm). The high-dose OPG group also had significantly less ($p<0.001$) mesial molar movement than the low-dose OPG group (0.75±0.10 mm).

Using the data reported in Fig. 2A to determine percentages of tooth movement, it was noted that rats administered a dose of 5.0 mg/kg OPG-Fc demonstrated only 54.3%, 29.5%, and 21.3% of the total mesial molar movement compared to that observed in control rats at days 7, 14, and 21, respectively. These differences were all statistically significant. Rats receiving 0.5 mg/kg OPG-Fc had a statistically significant 56.2% and 68.2% of the molar movement relative to that in control rats at days 7 and 14, respectively. At day 21, animals treated with 0.5 mg of OPG-Fc did not demonstrate significant molar tooth movement compared to controls. At days 14 and 21, the 5.0 mg/kg OPG-Fc group showed significantly less ($p<0.05$) percent molar movement compared to the 0.5 mg/kg OPG-Fc group.

The amount of incisor retraction appeared also to be inhibited dose dependently (Fig. 2C). Animals without active springs demonstrated 1.42±0.06 mm of forward maxillary incisor movement over the 3-week experimental time period. The vehicle-injected animals with active springs showed 2.17±0.05 mm of incisor retraction relative to the untreated controls corrected for the abovementioned growth-associated anterior movement of the incisors. The 0.5 mg/kg OPG-Fc-injected group showed significantly less incisor retraction (1.53±0.03 mm, $p<0.01$) than the vehicle-injected group. Likewise, the 5.0 mg/kg OPG-Fc group showed significantly less incisor retraction (1.05±0.03 mm, $p<0.01$) than either the vehicle or 0.5 mg/kg OPG-Fc-injected animals. No tooth movement was noted for the three groups not receiving appliances (i.e., the no-treatment control, vehicle-control or high-dose OPG treatment control, data not shown).

In order to evaluate the relative effect of OPG on movement of the first molars and incisors, the ratio of incisor to molar tooth movement was calculated (Fig. 2D). In the vehicle-injected rats, the incisors were retracted 2.3 mm for every millimeter of anchorage loss at the first molar. Injecting 0.5 mg/kg OPG-Fc, allowed for only 2.0 mm of incisor retraction for every millimeter

of anchorage loss. Finally, the 5.0 mg/kg OPG-Fc group demonstrated 5.2 mm ($p<0.001$) of incisor retraction for every millimeter of molar anchorage loss.

Descriptive histology

The control and low-dose OPG groups showed many osteoclasts, inflammatory cells, and areas of bone resorption (Fig. 3). In contrast, very few osteoclasts and areas of bone resorption were found in the high-dose OPG group. The periodontal ligament appeared to be normal in all groups and there was no evidence of ankylosis. Isolated areas of root resorption were noted but did not appear to correlate with any particular treatment group (data not shown).

Immunohistochemical analysis

Osteoclasts were enumerated along the compression and tension sides of the mesial tooth roots of the maxillary first molars (Fig. 4). The 5.0 mg/kg OPG-Fc group showed significantly fewer osteoclasts (3.2 ± 1.7) than either the vehicle- (23.3 ± 2.9 , $p<0.001$) or 0.5 mg/kg OPG-Fc-injected (16.0 ± 4.2 , $p<0.05$) groups in terms of osteoclasts along the compression side of the mesial root (Fig. 4A). Along the tension side, the high-dose OPG group also showed fewer osteoclasts (3.1 ± 1.2) compared to the low-dose OPG group (13.4 ± 2.5 , $p<0.05$), but was not significantly different than controls (8.9 ± 2.0) (Fig. 4B). Finally, the high-dose OPG group had significantly less total osteoclasts (6.3 ± 2.8) than either the control (32.2 ± 3.3 , $p<0.001$) or the low-dose OPG (29.4 ± 4.7 , $p<0.001$) groups (Fig. 4C). One animal in each of the low- and high-dose OPG groups could not be evaluated because the tissue sections demonstrated histological artifacts abrogating analysis.

Micro-computed tomography

BVF was measured in the first molar furcation area. Compared to the baseline group that received no treatment (0.524 ± 0.020), there were no significant differences in BVF (Fig. 5) in the vehicle (0.515 ± 0.029) and 0.5 mg/kg OPG (0.374 ± 0.133) groups. The 5.0 mg/kg OPG group (0.666 ± 0.049), however, demonstrated a significant ($p<0.001$) increase in BVF compared to all other groups. In addition, the low-dose OPG group showed significantly ($p<0.01$) less BVF than the control group. One animal in the low-dose OPG group was excluded because the specimen was damaged during biopsy retrieval.

ELISA of serum TRAP-5b

At baseline, animals showed 1.9 ± 0.4 , 1.7 ± 0.3 , and 1.5 ± 0.2 U/L of serum TRAP-5b in the control, low-dose OPG, and high-dose OPG groups, respectively (Fig. 6). By day 7, the high-dose group (0.0 ± 0.0 U/L) demonstrated a significant decrease ($p<0.05$) compared to controls (1.2 ± 0.2 U/L). The low-dose group showed a non-significant decrease (0.5 ± 0.4 U/L). At day 14, the high-dose group remained significantly lower (0.3 ± 0.2 U/L, $p<0.05$) than the control group (2.1 ± 0.5 U/L). Serum TRAP-5b levels in the low-dose group rebounded to 2.5 ± 0.6 U/L. At day 21, the animals demonstrated 1.5 ± 0.3 , 2.2 ± 0.5 , and 0.9 ± 0.4 U/L of serum TRAP-5b in the control, low-dose OPG, and high-dose OPG groups, respectively. The differences were not statistically significant.

Discussion

The binding of RANKL to RANK is critical for osteoclastogenesis and bone resorption. By preventing this binding, the soluble decoy receptor OPG inhibits osteoclast formation, activation, and survival [41]. Considering OPG is a competitive inhibitor, the ratio of RANKL to OPG ultimately is responsible for the activation of RANK and inducing osteoclast progenitor cells to form mature osteoclasts. At the cellular and molecular levels, tooth movement is the result of the interaction between bone-forming osteoblasts and bone-resorbing osteoclasts.

Given the critical role osteoclasts play in tooth movement, it follows that the RANK/RANKL/OPG pathway is likely to be critical in this process. Indeed, it has been shown that application of orthodontic forces results in the expression of RANKL protein in osteoblasts, osteocytes, fibroblasts, and osteoclasts [42]. In our studies, we utilized the knowledge of the basic biologic mechanisms of osteoclastogenesis and the important role played by the OPG–RANKL axis in osteoclast function to test the hypothesis that OPG participates in mechano-modulation of osteolysis to diminish orthodontic tooth movement and could therefore serve as a biologic mediator for enhancing orthodontic anchorage.

Bearing in mind the shortcomings of traditional orthodontic anchorage approaches, a number of different groups have attempted to alter rates of tooth movement with a variety of bioactive molecules. Bisphosphonates [15,19], nitric oxide synthase inhibitor [17], echistatin [16], and matrix metallo-proteinase inhibitors [18] have all been shown to decrease tooth movement. Bisphosphonates are not indicated for orthodontic use currently, and osteonecrosis of the jaw has recently become a concern with bisphosphonate therapy [43,44]. Bisphosphonates incorporate directly into bone where they reside for many years, and their effects are not readily reversible. In contrast, RANKL inhibitors including OPG are not incorporated into bone and have reversible effects on bone resorption [23]. As such, RANKL inhibitors may be desirable when transient osteoclast suppression is desirable in clinical situations such as with tooth anchorage.

While multiple studies have demonstrated the ability to alter rates of tooth movement, the agents used affect osteoclasts indirectly rather than directly targeting the RANK/RANKL/OPG pathway. Using an *in vivo* gene transfer approach, molar movement was decreased by 47.8% ($p<0.001$) after 3 weeks of twice weekly OPG plasmid DNA injections [14]. In contrast to RANKL plasmid DNA injections that resulted in 31.6% ($p<0.01$) more molar movement [13]. These two gene therapy studies showed the potential of directly targeting this pathway in altering tooth movement.

Using the recombinant fusion protein, OPG-Fc, we successfully inhibited molar movement by 78.7% ($p<0.001$) with a 5.0 mg/kg twice weekly local injection protocol. Even with a 10-fold reduction in dose (0.5 mg/kg), there was still a significant reduction ($p<0.05$) in molar movement until day 21. It is likely that the 0.5 mg/kg OPG-Fc-treated animals' inability to maintain anchorage throughout the study was due to an inadequate dose compared to the level of RANKL expression that is induced during tooth movement. As we were using a human protein in a rat, we used higher and more frequent dosing than would likely be required for a human in order to overcome the animal's immune response to foreign human OPG-Fc. In a human study, using the same protein, a single subcutaneous dose (3.0 mg/kg) of OPG-Fc demonstrated a half-life of 6–7 days and remained effective for at least 30 days [22]. In addition, AMG 162 [21], a specific fully human monoclonal antibody to RANKL based on OPG, has demonstrated an 81% suppression in bone turnover six months after a single injection of 3.0 mg/kg. However, this human antibody cannot be studied in the rat model to directly compare its efficacy to humans.

It is also of interest to note that the majority (57%) of the total molar movement in both OPG groups occurred within the first 3 days, corresponding with the displacement phase of tooth movement. The displacement phase is the initial movement caused by physical compression of the viscoelastic periodontal ligament. As it is not the result of tissue remodeling, the amount of movement in this phase is dependent on its biophysical limitations. During the remaining phases (days 3–21), the high OPG group demonstrated almost no additional molar movement.

Considering the substantial decrease in molar tooth movement, it is likely that local OPG-Fc injections were effectively inhibiting osteoclastogenesis. This was indeed the case as the

animals in the high OPG group demonstrated 86% ($p < 0.001$) fewer osteoclasts along the compression side of the mesial root when compared to controls. The low-dose OPG group also showed a non-significant trend towards decreased osteoclasts.

One of the advantages of the mesial molar movement model that was used in the present study is that incisor retraction also can be measured. This allowed for evaluation of the effects of OPG on neighboring teeth. While there was a decreased rate of incisor retraction with increased levels of OPG, the overall treatment efficacy as measured by the ratio of incisor retraction to molar anchorage loss was greatly improved in the 5.0 mg/kg OPG-Fc group (incisor:molar movement=5.2:1) compared to the low-dose OPG group (2.0:1) or controls (2.3:1). As differentially regulating rates and magnitudes of tooth movement is the ultimate goal in maintaining orthodontic anchorage, this was an encouraging observation. This finding also demonstrates relative localization of the effects of the OPG-Fc-injected in proximity to the molar teeth. While these findings are promising, future research is recommended in order to determine the optimal dose regimen (quantity/frequency) that permits the greatest amount of incisor retraction with the least molar movement, and optimal time-release methods for administering OPG-Fc to obtain highly localized and long-term pharmacologic effects.

Using three-dimensional imaging, we were also able to evaluate the quantity of tooth-supporting alveolar bone. Not only did the animals in the high OPG group show greater BVF than the control and low OPG groups but they were also actually higher than the baseline sacrificed animals that never received any tooth movement appliances or tooth movement (Fig. 5). The modest but significant reduction in BVF associated with the low dose of OPG-Fc is consistent with the possibility that anti-human OPG-Fc antibodies that were induced by repeated injections resulted in some cross-reactive neutralization of endogenous rat OPG as well. Rat and human OPG are highly homologous and cross-reactive antibodies have been observed in previous rat studies (P. Kostenuik, personal communication). The potential for this phenomenon in the current study is supported by data obtained at the end of the study, wherein serum TRAP-5b values were slightly elevated in the low-dose OPG-Fc group, drug levels were not different from vehicle controls, and BVF was reduced. It is likely that this low dose produced pharmacologic effects for the first half of the study, followed by rapid immune-related clearance of the drug, and possible neutralization of endogenous OPG at the end of the study.

While the low-dose group had no significant effect on serum TRAP-5b, there was significant suppression of this resorption marker in the high-dose group (Fig. 6). It is likely that the reduced TRAP-5b suppression with low-dose OPG is related to an immune response to the foreign protein. In other words, it is likely that there was significant serum TRAP-5b suppression during the first part of the study, but at some point (probably between days 10 and 14), the immune response was sufficient to clear or neutralize the drug. With the high-dose group, an immune response also probably occurred but enough protein was delivered to overwhelm the immune response and maintain therapeutic circulating levels of OPG-Fc to effectively suppress bone resorption. By 21 days the TRAP-5b suppression was reduced, indicating that the immune response was likely eventually sufficient to neutralize even the high dose administration.

The effects of the local gingival delivery method employed in this study were not entirely contained as demonstrated by TRAP-5b assays (Fig. 6). Given its transient effect, the minimal safety concerns with systemic RANKL inhibition, and potential improvement in bone mineral density, this result does not appear to be of significant concern [23,24]. However, the targeted, primarily localized effects of OPG particularly with the high-dose group were demonstrated for therapeutic tooth anchorage. This phenomenon suggests that local OPG concentrations at the site of anchorage may have been higher than at other sites, perhaps due to simple diffusion of the protein within the gingivae.

Inhibition of tooth movement using pharmacological anchorage could have many applications for orthodontics and dentistry beyond simply maintaining molar anchorage during incisor/canine retraction and may have benefits in molar distalization, molar protraction, vertical anchorage of posterior teeth and other applications where dental anchorage is critical for a favorable functional and esthetic result. Overall, this study demonstrates for the first time that local delivery of the recombinant RANKL inhibitor, OPG, effectively inhibits mechanically induced osteoclastogenesis resulting in improved bone quantity, orthodontic anchorage, and would likely lead to enhanced treatment efficacy.

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Fig. 1.

(A) Intraoral photographs of orthodontic appliances in place at day 21 prior to sacrifice. Animals received twice weekly injections of vehicle, 0.5 mg/kg OPG-Fc, or 5.0 mg/kg OPG-Fc into the mucosa just mesial to the maxillary first molars. Note the amount of space visible between the first and second molar teeth. (B) Three-dimensional coronal and sagittal micro-computed tomography views of the same animals shown in panel A. Note the interdental distance visible between the first and second molar teeth as well as the differences in bone quantity around the first molars. Arrows indicate 2-D force direction.

Fig. 2.

Inhibition of tooth movement by local delivery of OPG-Fc. (A) Molar tooth movement over the course of time in vehicle, 0.5 mg/kg OPG-Fc, and 5.0 mg/kg OPG-Fc injected groups. No tooth movement was noted in sham control animals (inactive spring) (data not shown). (B) Incisor retraction measured at day 21. (C) Ratio of incisor retraction to molar tooth movement at day 21. All results are expressed as the mean $\pm$ SEM, $n = 10$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Comparisons made versus time-matched vehicle groups.

Fig. 3.

TRAP stained histological sections (4–5 μ m) counterstained with hematoxylin taken from the mesial tooth root of the maxillary first molar. (A) TRAP-positive multinucleated osteoclasts (40 $\times$ magnification); (B) control group (4 $\times$ magnification); (C) low-dose OPG group (4 $\times$ magnification); (D) high-dose OPG group (4 $\times$ magnification), which lacks osteoclasts. MR, mesial root; Co, compression side alveolar bone; T, tension side alveolar bone; OC, osteoclast. Arrows indicate osteoclasts.

Fig. 4.

Osteoclasts per root surface on the tooth compression surface (A), tension side (B), and total osteoclasts (C) along the alveolar bone adjacent to the mesial root of the maxillary first molar. All results are expressed as the mean $\pm$ SEM, $n = 10$ (vehicle), 9 (low-dose OPG, high-dose OPG): * $p < 0.05$; *** $p < 0.001$. Comparisons made versus time-matched groups.

Fig. 5.

Comparison of bone volume fraction measured from the furcation area to tooth root apex of the maxillary first molar with micro-computed tomography. Specimens were placed on a rotating stage allowing polychromatic X-rays to penetrate the sample, pass through an image intensifier, and be captured by a CCD camera, producing 2-D slices of 18 μm thickness. These cross-sectional images were then reconstructed into a 3-D structure by GE Healthcare reconstruction utility. Results are expressed as the mean $\pm$ SD, $n=3$ (baseline), $n=10$ (vehicle, high-dose OPG), $n=9$ (low-dose OPG): * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Comparisons made versus time-matched groups.

Fig. 6.

Concentrations of TRAP-5b in serum as measured by ELISA. Blood was drawn from the lateral tail vein at days 0, 7, 14, and 21 prior to the next administration of OPG-Fc or vehicle. Results are expressed as the mean $\pm$ SEM, $n = 10$; * $p < 0.05$. Comparisons made versus time-matched groups.