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Locally limited inhibition of bone resorption and orthodontic relapse by recombinant osteoprotegerin protein

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Structured Abstract

Objectives – To determine minimal dose levels required for local inhibition of orthodontic relapse by recombinant OPG protein (OPG-Fc), while also determining effects of injected OPG-Fc on alveolar bone and long bone.

Setting and Sample Population – The Department of Orthodontics and Pediatric Dentistry at the University of Michigan. Eighteen male Sprague Dawley rats.

Materials & Methods – Maxillary molars were moved with nickel–titanium springs and then allowed to relapse in Sprague Dawley rats. Upon appliance removal, animals were injected with a single dose of 1.0 mg/kg OPG-Fc, 0.1 mg/kg OPG-Fc, or phosphate-buffered saline (vehicle) just distal to the molar teeth. Tooth movement measurements were made from stone casts, which were scanned and digitally measured. Alveolar tissues were examined by histology. Micro-computed tomography was used to quantify changes in alveolar and femur bone.

Results – Local injection of OPG-Fc inhibited molar but not incisor relapse, when compared to vehicle-injected animals. No significant differences in alveolar or femur bone were seen between the three treatment groups after 24 days of relapse.

Conclusions – Our results demonstrate that a single local injection of OPG-Fc effectively inhibits orthodontic relapse, with minimal systemic bone metabolic effects. Our results also show that a single injection of OPG-Fc will influence tooth movement only in teeth close to the injection site. These findings indicate that OPG-Fc has potential as a safe and effective pharmacological means to locally control osteoclasts, for uses such as maintaining anchorage during orthodontic tooth movement and preventing orthodontic relapse in humans.

Key words: alveolar resorption; bone density conservation agents; orthodontics; osteoclasts; X-ray microtomography

Introduction

Osteoclastogenesis is mediated by the ratio of nuclear factor kappa B ligand (RANKL) to osteoprotegerin (OPG), and inhibitors of RANKL can inhibit bone resorptive activity (1). In humans, injection with OPG-Fc or a monoclonal antibody to RANKL leads to diminished serum markers of bone resorption (2, 3), reduced fracture incidence, and increased bone mineral density in post-menopausal women (4, 5). *In vivo* evidence also supports a role for RANKL and OPG in control of mechanically induced bone resorption (6). Together, these studies demonstrate that inhibitors of RANKL can be used to systemically reduce bone resorption induced by biologic or mechanical perturbations of bone.

Local control of osteoclasts through limited delivery of RANKL inhibitors would be useful for situations in which inhibition of bone resorption is desirable only at specified locations. Orthodontic tooth movement serves as a well-utilized *in vivo* model of mechanically induced, local bone modeling activity that is responsive to biologic agents (7–9). That orthodontic tooth movement is mediated by RANKL and OPG is evidenced by previous findings showing that compressive orthodontic forces increase RANKL expression (10), and that manipulation of RANKL or OPG levels by gene delivery can alter the rate of orthodontic tooth movement (11, 12). Similarly, we previously showed that injection with OPG-Fc inhibits orthodontic tooth movement (13). As a proof of concept study, high and frequent doses were used and no attempt was made to limit affects to local tissues. Correspondingly, the results showed that the injected OPG-Fc entered the systemic circulation in amounts sufficient to influence osteoclasts distant from the site of injection.

Because orthodontic relapse involves tooth movement through bone, it is also a bone modeling and remodeling process that requires osteoclast activity. We previously investigated OPG-Fc as a potential mediator of orthodontic relapse. Using multiple high doses of locally delivered OPG-Fc, we found a robust inhibition of post-orthodontic relapse (14). However, at this dose and frequency, the OPG-Fc entered the systemic circulation and had effects at sites distant from the tooth being stabilized. From a clinical perspective, local inhibition of osteoclast activity could allow for enhanced control of individual teeth during orthodontic treatment and for spatially restricted effects in the prevention of relapse after orthodontic treatment. However, unless appropriate doses or technologies can be validated for localizing the control of bone resorption by RANKL inhibitors, the use of these agents for this purpose is not likely to be realized. To date, no studies have been performed to modulate the local regulation of bone turnover by RANKL inhibition. In this study, we utilized a rodent model of orthodontic relapse to determine whether a single injection of OPG-Fc will inhibit local alveolar bone resorption and orthodontic relapse, while having minimal effects on bones distant from the site of injection.

Materials and methods
Animals and procedures

Orthodontic appliances were placed in eighteen male Sprague Dawley rats that were randomly assigned to three treatment groups of six animals each. One volumetrically equivalent bolus of 1.0 mg/kg OPG-Fc (rat recombinant osteoprotegerin; Amgen, Inc., Thousand Oaks, CA, USA), 0.1 mg/kg OPG-Fc, or phosphate-buffered saline (PBS) vehicle was injected immediately prior to appliance removal, into the palatal mucosa just distal to the maxillary first molars. Six animals served as pre-tooth movement controls. An additional six animals that received orthodontic appliances but no injections served as post-tooth movement controls. All procedures were performed in accordance with the University of Michigan's Committee on Use and Care of Animals.

Orthodontic appliance for tooth movement and relapse

Orthodontic forces were applied using a previously established rodent model of tooth movement and relapse (7, 14). During the tooth movement phase, maxillary first molars were moved mesially, with

nickel–titanium springs calibrated to provide 50 cN of force that were ligated from the molar to the ipsilateral incisor tooth. Appliances were removed after 28 days, and the teeth were allowed to relapse for 24 days. Tooth movement was measured at specific intervals during the tooth movement and relapse phases of the experiment.

Measurements of orthodontic tooth movement and relapse

Tooth movement was measured as previously described (14). Briefly, stone models made from polyvinylsiloxane impressions were scanned at 1200 dpi and magnified 300× and measured using commercial software (Adobe Photoshop CS6; Adobe Systems Inc. San Jose, CA, USA). The amounts of molar and incisor relapse were calculated as the difference between the post-relapse measurement and the post-tooth movement measurement, as well as a percent of initial tooth movement that accounted for differences in initial tooth movement between individual animals. The intra-examiner and interexaminer errors for tooth movement measurements were assessed by two repeat measurements of first molar mesial movement taken on 87 teeth, 2 weeks apart. A Pearson's correlation coefficient analysis demonstrated very high intra-examiner ($r = 0.99$; $p < 0.0001$) and interexaminer ($r = 0.98$; $p < 0.0001$) reliability.

Histological analysis

Block biopsies of hemi-maxillae were fixed, decalcified, and embedded in paraffin. Axial sections containing the first molar tooth roots were obtained. Sections were stained with hematoxylin and eosin for descriptive histology. Sections were stained for tartrate-resistant acid phosphatase (TRAP-5b) for osteoclast identification using commercially available reagents and following the manufacturer's protocol (Biocare Medical, Concord, CA, USA).

Micro-computed tomography

Hemi-maxillae and femurs were harvested, fixed in 10% formalin and transferred to ethanol. Samples were embedded in agarose and scanned using a micro-computed tomography system (μCT100 Scanco Medical, Bassersdorf, Switzerland) at voxel size 18 μm^3, 70 kVp, 114 μA. Scans were calibrated to the manufacturer's hydroxyapatite phantom. Analyses were conducted using commercial software (Scanco Medical, Brüttisellen, Switzerlandand) and are reported in accordance with the recommendations of Bouxsein et al. (15). Alveolar bone was analyzed in the maxillary first molar furcation region according to a previously established protocol (16), because this bone undergoes modeling and remodeling during and after orthodontic tooth movement. Long bone analysis was performed at the distal metaphysis of the femur.

Statistical analysis

Each animal was treated as a unit, and bilateral measures were averaged to give a single measure for each animal at each time point. Descriptive statistics were calculated for each parameter for all groups. A repeated measures analysis of variance (ANOVA) was performed at each time point to compare tooth movement and relapse data between groups and over time within a group, using statistical software (SPSS version 20; SPSS Inc., Chicago, IL, USA). Tukey's *post hoc* tests were performed to establish pairwise differences.

Results
Animal status

The animals tolerated the experimental procedures well, with no discernible effect on overall health. There were no significant differences in weight gain between the treatment groups at any time point during the study.

Orthodontic tooth movement and relapse movement

The amount of tooth movement achieved at the time of appliance removal was similar in the three groups of injected animals (0.61 ± 0.25 mm for vehicle control, 0.73 ± 0.18 mm for 0.1 mg/kg OPG-Fc and 0.74 ± 0.33 mm for 1 mg/kg OPG-

Fc). Representative micro-CT isosurface images of pre-tooth movement, post-tooth movement and post-relapse PBS, and OPG-Fc-injected animals are depicted in Fig. 1A. During the relapse phase, the 0.1 mg/kg OPG-Fc-injected animals showed significantly less linear (Fig. 1B) and percent molar (Fig. 1C) relapse than the control animals at days 12, 18, and 24 days after appliance removal. The 1.0 mg/kg OPG-Fc-injected animals had significantly less linear and percent molar relapse than the vehicle-injected animals at all time points following appliance removal.

When comparing intragroup changes in molar relapse over time, all of the treatment groups showed significant distal molar relapse move-ment from day zero to day three after appliance removal. This result is expected because upon appliance removal the tooth undergoes a rebound shift within the tooth socket that does not require osteoclast activity (17). Importantly, whereas the vehicle-injected animals continued to exhibit significant molar relapse movement beyond this initial period, the OPG-Fc-injected animals did not. Therefore, these results demonstrate that OPG-Fc effectively inhibited molar relapse beyond the original constraints of the tooth socket.

To establish whether or not the effects OPG-Fc were localized to the molars and did not affect teeth distant from the site of injection, we also
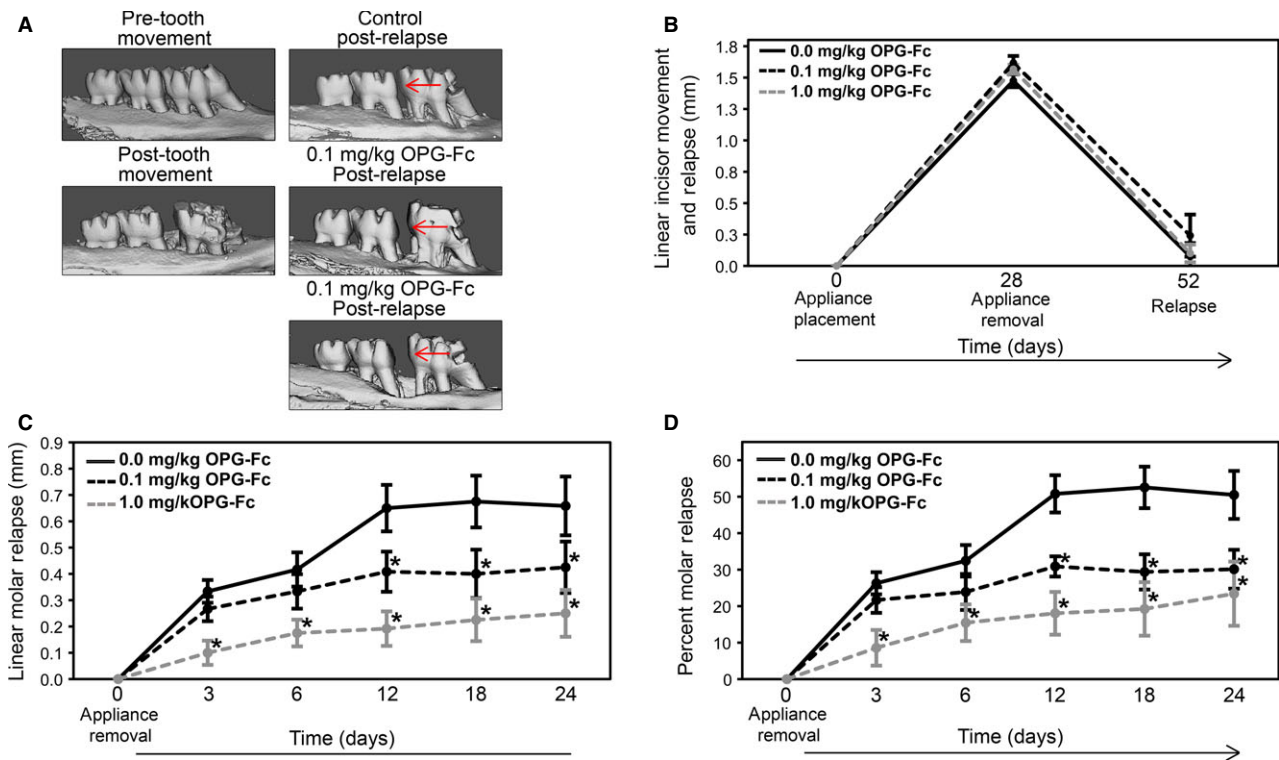

Fig. 1. A single local injection of OPG-Fc locally inhibits post-orthodontic molar relapse. (A) Sagittal micro-CT isosurface images of representative pre-tooth movement, post-tooth movement, and vehicle, low-dose, and high-dose OPG-Fc-injected animals after 24 days of relapse post-appliance removal are shown. Note the difference in interdental distance visible between the first and second molar teeth in pre-tooth vs. post-tooth movement and in vehicle vs. OPG-Fc-injected animals. Red arrows indicate the direction of molar distal relapse following appliance removal. (B) Incisor distal movement (0–28 days) and incisor mesial relapse movement (29–52 days) of vehicle, 0.1 mg/kg OPG-Fc and 1.0 mg/kg OPG-Fc-injected animals are shown. Results are expressed as the mean $\pm$ SEM. All three groups achieved similar magnitudes of incisor movement after 28 days of orthodontic force, and significant incisor movement after 28 days was found for all three groups ($p < 0.001$, pre-tooth movement vs. post-tooth movement). Significant incisor relapse movement after 24 days was also found for all three groups ($p < 0.001$, post-relapse vs. post-tooth movement). No significant differences between any of the three groups were found at any time point after appliance removal, indicating that the injected OPG-Fc did not inhibit incisor relapse. (C) Linear maxillary first molar distal relapse movements after appliance removal are shown for the vehicle, 0.1 mg/kg and 1.0 mg/kg injected animals. Results are expressed as the mean $\pm$ SEM. *$p < 0.05$ between the indicated group and vehicle-injected animals. (D) Molar distal relapse is presented as a percentage of initial mesial molar movement to account for differences in initial tooth movement between individual animals. Results are expressed as the mean $\pm$ SEM. *$p < 0.05$ between the indicated group and vehicle-injected animals.

measured incisor orthodontic tooth movement and relapse (Fig. 1D). The vehicle-injected, 0.1 mg/kg OPG-Fc-injected and 1.0 mg/kg OPG-Fc-injected animals exhibited very similar incisor relapse movement following appliance removal. This finding, combined with the fact that incisor relapse reached close to 95% for all of the treatment groups, indicates that the injected OPG-Fc did not inhibit relapse of teeth that were more distant from the site of injection.

Descriptive histology

Alveolar tissue sections from immediate post-tooth movement animals showed a widened PDL space with a loss of supporting alveolar bone and isolated areas of root resorption in representative samples, as compared to the pre-tooth movement samples (Fig. 2A–C). These findings are consistent with the known process of orthodontic tooth movement (7, 18). Alveolar tissue sections from post-tooth movement animals also exhibited a higher presence of Trap5b-stained osteoclasts as compared to sections from pre-tooth movement animals (Fig. 2D). Following 24 days of relapse after appliance removal, tissue sections from animals of all three injection groups demonstrated a return to near baseline presentation, with fewer osteoclasts and an increase in alveolar bony support. Trap5b staining was diminished in all relapse groups as compared to that seen in post-tooth movement animals. OPG-Fc-injected animals did not show reduced levels of Trap5b staining as compared to vehicle-injected animals after 24 days of relapse.

Alveolar bone micro-computed tomography

To determine whether a single dose of OPG-Fc alters bone near the injection site, we analyzed alveolar bone investing the first molar tooth roots (Table 1). Multiple measurements showed trends toward decreased alveolar bone quantity and quality in the post-tooth movement animals as compared to the pre-tooth movement animals. Multiple measurements showed significantly increased bone quantity and quality after

the relapse period in vehicle-injected animals, when compared to the values for the post-tooth movement animals. Of greatest relevance to this study, no significant differences were seen between the three treatment groups for any of the measurements at the end of the relapse period.

Femur micro-computed tomography

Measures of femur bone in the three treatment groups at the end of the relapse period were similar, and no significant differences between any of the three groups for any of the measured bone parameters were evident (Table 2).

Discussion

Our group has focused upon the use of OPG-Fc for regulating orthodontic tooth movement and for mitigating relapse after tooth movement (14), because OPG-Fc was previously shown to be both safe and efficacious for inhibiting bone resorption in humans (2). Our previous studies showed that while serial injection of OPG-Fc can significantly inhibit orthodontic tooth movement and relapse after tooth movement, it also introduces OPG-Fc into the general circulation and therefore has the capacity to influence bone systemically (13, 14). In this study, we sought to determine whether a single local injection of OPG-Fc could inhibit orthodontic relapse without leading to systemic effects upon bone.

Histologic staining of the alveolar bone tissue showed that Trap5b staining for osteoclasts was not reduced in OPG-Fc injected, as compared to vehicle-injected animals 24 days after the injection. This staining pattern suggests that the locally administered OPG-Fc was no longer active after 24 days of relapse. This finding is not surprising given the relatively short half-life of OPG-Fc of several days (19). In this study, we also found no significant differences in micro-CT-based parameters of local alveolar bone quality between any of the three injection groups at the end of the 24 day relapse period, indicating that a single local injection of OPG-Fc
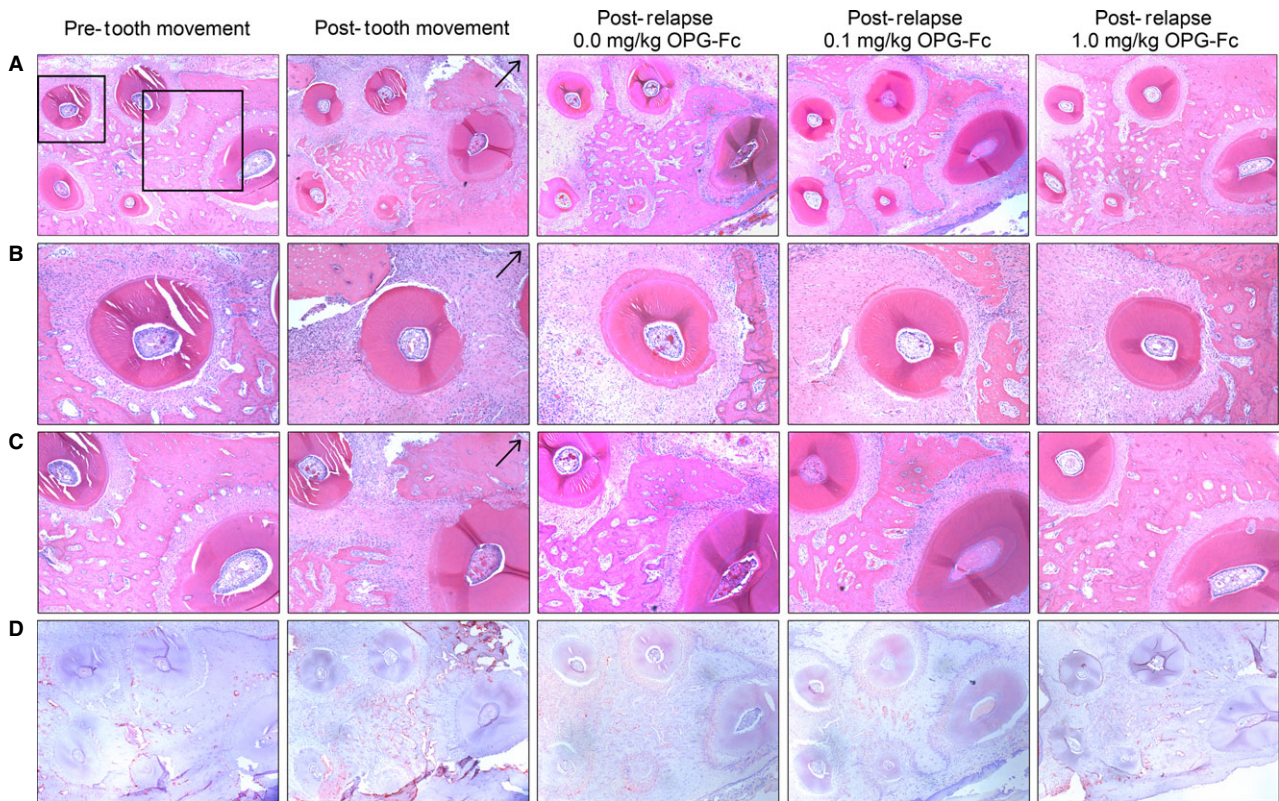
Fig. 2. Alveolar bone changes due to orthodontic tooth movement and relapse after movement. Representative photomicrographs of hematoxylin-and-eosin-stained sections of the alveolar bone investing the first molar roots before tooth movement, after tooth movement, and after relapse are shown. Arrows indicate the mesial direction of orthodontic tooth movement. Box outlines in A indicate areas shown in B and C. (A) 4× magnification of alveolar bone investing the first molar roots showing all five roots of the maxillary molar. (B) 10× magnification showing alveolar bone surrounding the disto-palatal molar root outlined in A. (C) 10× magnification showing alveolar bone between the mesial molar root and the palatal molar root, as outlined in A. (D) Representative photomicrographs of alveolar bone sections investing the first molar roots stained with Trap5b for osteoclasts and osteoclast precursors, before tooth movement, after tooth movement, and after relapse are shown. Note that Trap5b staining is increased in the local alveolar bone after 28 days of tooth movement and is then decreased 24 days after appliance removal. Also note Trap5b staining is not reduced in 0.1 mg/kg or 1.0 mg/kg injected animals to a greater extent than vehicle-injected animals. Box outlines in A indicate areas shown in B. 4× magnification of alveolar bone investing the first molar roots showing all five roots of the maxillary molar.

does not have long-term effects on alveolar bone. It is possible that the injected OPG-Fc had significant short-term effects on alveolar bone that were lost in the long term, due to loss of drug activity over time. In future studies, it will be important to measure changes in alveolar bone quality at earlier time points after appliance removal to establish more accurately the timing of the biologic processes of bone remodeling and maturation after cessation of tooth movement, and to determine the best therapeutic window for pharmaceutical prevention of orthodontic relapse.

The effects of injected OPG-Fc remained local, as is supported by the findings that both OPG-Fc and vehicle-injected animals exhibited up to 95% relapse of incisor tooth movement and that no differences in incisor relapse were noted between the three treatment groups. This finding is in stark contrast to our previous results, which showed that multiple injections of OPG-Fc had significant inhibitory effects upon incisor relapse (14). Results presented here demonstrate that a single, locally injected dose of OPG-Fc can be expected to have effects that are limited to the area surrounding the injection site. From a clinical viewpoint, these findings suggest that a single injection of OPG-Fc could potentially be utilized to enhance orthodontic anchorage locally, without inhibiting overall tooth movement. The findings may also suggest that the retentive effect of a low-dose OPG-Fc injection is limited to teeth immediately surrounding the

Table 1. **Local alveolar bone volume, density, and structural parameters**

	Pre-tooth movement	Post-tooth movement	Control post-relapse	0.1 mg/kg OPG-Fc post-relapse	1.0 mg/kg OPG-Fc post-relapse
Bone volume (mm^3)	2.8 ± 0.3	2.6 ± 0.9	3.7 ± 0.6[a,b]	3.4 ± 0.3[a,b]	3.8 ± 0.8[a,b]
Bone volume fraction	0.81 ± 0.03	0.77 ± 0.07	0.91 ± 0.04[a,b]	0.91 ± 0.02[a,b]	0.91 ± 0.03[a,b]
Bone mineral density (mg/cc)	831 ± 23	785 ± 65	906 ± 29[a,b]	892 ± 35[a,b]	924 ± 33[a,b]
Tissue mineral density (mg/cc)	1022 ± 23	986 ± 42	1004 ± 22	988 ± 34	1027 ± 17
Trabecular number	4.4 ± 0.3	4.6 ± 0.6	4.0 ± 0.6	4.4 ± 0.4	4.0 ± 0.5
Trabecular thickness	0.29 ± 0.02	0.27 ± 0.04	0.38 ± 0.07[a,b]	0.33 ± 0.04[a,b]	0.37 ± 0.05[a,b]
Trabecular spacing	0.18 ± 0.03	0.17 ± 0.04	0.09 ± 0.01[a,b]	0.08 ± 0.02[a,b]	0.09 ± 0.03[a,b]
Connectivity density	15.1 ± 4.3	33.5 ± 15.8[a]	10.2 ± 6.6[a,b]	11.4 ± 7.5[a,b]	13.0 ± 9.4[a,b]

[a]$p < 0.05$ vs. pre-tooth movement.
[b]$p < 0.05$ vs. post-tooth movement.

Table 2. **Femur bone volume, density, and structural parameters**

	Control post-relapse	0.1 mg/kg OPG-Fc post-relapse	1.0 mg/kg OPG-Fc post-relapse
Bone volume (mm^3)	9.9 ± 2.0	8.8 ± 2.5	10.7 ± 2.4
Bone volume fraction	0.60 ± 0.09	0.52 ± 0.12	0.61 ± 0.08
Bone mineral density (mg/cc)	392 ± 81	380 ± 78	429 ± 70
Tissue mineral density (mg/cc)	708 ± 17	717 ± 37	701 ± 71
Trabecular number	5.3 ± 0.6	4.7 ± 0.5	5.6 ± 0.3
Trabecular thickness	0.14 ± 0.01	0.14 ± 0.02	0.15 ± 0.04
Trabecular spacing	0.15 ± 0.03	0.19 ± 0.05	0.14 ± 0.02
Connectivity density	74.4 ± 6.2	75.2 ± 16.3	80.3 ± 9.8

No significant differences between the three relapse groups were found.

injection site. On a more global scale, these results demonstrate that exogenous inhibitors of RANKL have potential for local control of osteoclast activity *in vivo*. While treatment with the inhibitory monoclonal antibody to RANKL (Denosumab; Amgen, Inc.) may be more beneficial for systemic bone effects in osteoporotic patients due to its longer half-life of approximately 28 days, treatment with OPG-Fc may be more appropriate for achieving more limited and local effects, in part due to its shorter half-life of 5–7 days (3, 19).

Results of this study demonstrate that a single injection of OPG-Fc upon appliance removal significantly inhibits relapse after orthodontic tooth movement and that a single injection of 1.0 mg/kg OPG-Fc inhibits relapse to a greater extent than an injection of 0.1 mg/kg OPG-Fc. Notably,

relapse in the 1.0 mg/kg OPG-Fc-injected animals remained at approximately 20% of the initial tooth movement at 24 days after appliance removal. This striking inhibition of relapse is similar to that which was reported for multiple injections/week of OPG-Fc (14) and indicates that a single injection of OPG-Fc is as efficacious for preventing relapse as multiple injections of OPG-Fc for at least 24 days after appliance removal. Future studies are required to determine the extent of longevity of this effect. Future studies are also required to determine whether inhibition of orthodontic relapse requires sustained inhibition of osteoclasts, or if transient inhibition of osteoclasts during a critical time-limited period after orthodontic appliance removal is sufficient.

Limitations of this study include the fact that the period post-appliance removal was limited to

28 days of relapse. Future studies are required to establish how long a single local injection of OPG-Fc can inhibit relapsed tooth movement. This study also did not include analysis of tissues at multiple time points of relapse. Additional studies investigating bone, PDL, and tooth changes over time during relapse could further illuminate biologic processes underlying tooth movement post-orthodontic treatment. Such knowledge would guide future studies inhibiting specific aspects of these processes. This investigation was also performed in rodents, as opposed to larger mammals, primates, or humans. It remains unknown if a local injection of OPG-Fc will inhibit orthodontic relapse in humans.

A primary goal of this study was to determine the minimum effective dose of OPG-Fc for achieving maximal local clinical efficacy while minimizing potential systemic side effects. Toward this goal, we utilized micro-CT to assess changes in long bone quality. Measures of femur bone were similar after relapse, for all of the three injection groups. While further studies are required to definitively establish both immediate and long-term effects of locally delivered OPG-Fc on long bones, our results indicate that a single injection of low-dose OPG-Fc to prevent relapse will likely have minimal long-term effects on long bone volume, density, and structure.

Conclusions

A single local injection of OPG-Fc can inhibit orthodontic relapse in rodents, with minimal systemic bone metabolic effects. A single injection of OPG-Fc will influence tooth movement only in teeth close to the injection site. These findings indicate that OPG-Fc has potential as a safe and effective pharmacological means to control osteoclasts locally, for uses such as maintaining anchorage during orthodontic tooth movement and preventing orthodontic relapse. Future studies are required to establish efficacy in humans.

Clinical relevance

Post-treatment relapse is a significant challenge in clinical orthodontics. When delivered at doses sufficient to inhibit osteoclasts systemically, recombinant OPG protein (OPG-Fc) was previously shown to inhibit orthodontic tooth movement and relapse. While systemic inhibition of osteoclast activity is beneficial for systemic disorders of bone such as osteoporosis, local control of osteoclasts would be useful for situations in which inhibition of bone resorption is desirable only at specified locations. The purpose of this study was to determine the minimum effective dose of OPG-Fc for locally preventing orthodontic relapse, while minimizing potential systemic side effects.

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