

Inhibition of tooth movement by osteoprotegerin vs. pamidronate under conditions of constant orthodontic force

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The undesired movement of anchor teeth, and relapse of previously moved teeth, are major clinical problems in orthodontics. Dental implants are increasingly used to preserve anchorage, but these are costly and require invasive surgical procedures. An alternative strategy for maintaining anchorage may be the use of biological inhibitors of osteoclastic bone resorption. In the present study, we investigated the relative efficacy of pamidronate vs. osteoprotegerin (OPG) in inhibiting bone resorption and tooth movement, using a new orthodontic model in mice in which maxillary molars are moved for prolonged periods by near-constant, clinically relevant forces. Osteoclast influx to compression sites was initiated on day 3, was maximal on day 4, and persisted until at least day 12 after force application. Tooth movement paralleled osteoclast numbers. Minimal osteoclast apoptosis was observed, suggesting that recruitment, rather than programmed cell death, is a critical regulatory mechanism under conditions of constant force. Osteoclasts were reduced at compression sites by both OPG (95%) and pamidronate (70%); tooth movement was more dramatically inhibited by OPG (77% vs. 34%). Our findings indicate that constant orthodontic force regulates the recruitment, activation, and viability of osteoclasts, and that OPG could have clinical utility in preventing undesired tooth movement.

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Orthodontic tooth movement is induced by the controlled application of mechanical force, which results in bone resorption by osteoclasts on the compression side of teeth, and bone formation by osteoblasts on the opposing (tension) side. Despite the efficacy of clinical orthodontics, there are a number of circumstances under which treatment efficiency might be improved by modulating the activity of osteoclasts. In this regard, the undesired movement of anchor teeth, and the relapse of moved teeth, are major clinical problems that result from the limitations of available force delivery systems. Over the last decade, implants have been used to preserve anchorage in orthodontics (1, 2). However the placement of these temporary anchorage units requires an invasive and traumatic surgical procedure, adds cost, and requires a second surgery to remove the implants at the conclusion of treatment.

Recent research advances suggest that biological modulators which inhibit osteoclasts could be used to address these problems and provide new adjunctive approaches to orthodontic treatment. Several such inhibitors have been examined, including bisphosphonates (3–6) and osteoprotegerin (OPG), a soluble protein that inhibits receptor-activator of nuclear factor- κ B

ligand (RANKL) interactions with its cognate receptor, and prevents osteoclast differentiation and activation (7, 8). However, the relative efficiency of these inhibitors in preventing tooth movement is unknown.

In the present investigation, we evaluated the effectiveness of OPG vs. pamidronate in a novel mouse model of orthodontic tooth movement. In this system, near-constant forces are applied to maxillary molar teeth, resulting in predictable tooth movement over at least a 12-d period. Tooth movement is closely correlated with the influx of osteoclasts on the compression side of the periodontal ligament. Using this model we showed that the systemic administration of OPG is much more effective than a bisphosphonate in inhibiting molar tooth movement, suggesting potential utility for maintaining anchorage in clinical orthodontics.

Material and methods

Mice

Eight-week-old C57Bl/6 male mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and were

maintained on a 12-h light/dark cycle in the Forsyth Institute animal facility.

Orthodontic appliance design and placement

Mice were anesthetized by the intraperitoneal injection of 62.5 mg kg^{-1} ketamine HCl and 10 mg kg^{-1} xylazine in phosphate-buffered saline (PBS), and placed into a jaw retractor under a dissecting microscope. Orthodontic force was applied to the maxillary first molars using a 0.2-mm diameter coiled stainless steel wire (Supreme type, A.J. Wilcock Australian wire; G & H Wire Company, Greenwood, IN, USA) fashioned into a Y-shaped spring. The coil was placed around the maxillary incisors, with the ends of the spring bonded to the occlusal surfaces of the first molars (Fig. 1A). For spring activation ('constricted' groups), the wire ends were bent symmetrically towards each other until precisely 0.5 mm apart. The central cusp of the molar was flattened with a 0.25-mm diameter bur, and light-cured adhesive resin (Assure; Reliance Co., Orthodontic Products Inc., Itasca, IL, USA) was used to bond the re-opened wires to the enamel surface, and to lock the spring into position with resin applied to the incisor tips. Controls received an identically installed passive spring with the tips of the wires 3.5 mm apart (normal intermolar distance). The opposing lower first molars were extracted to prevent occlusal interference. Animals were fed powdered chow and milk (1% fat) during the experimental period, and their weights were monitored daily.

Strain measurements

Appliances were tested at the Biomaterials Unit, Sydney Dental Hospital (University of Sydney, NSW, Australia), using a Shimadzu Autograph Calibrator AG-E (Shimadzu, Kyoto, Japan). The force produced by the constriction and deactivation of the spring was measured at $7.5 \mu\text{m}$ increments over the entire range of activation (0.5–3.5 mm). Readings were automatically recorded (Shimadzu Software, Version 3.0) until the point of complete loss of resistance. Tests were repeated five times for each spring in order to ensure reproducibility.

Delivery of osteoclast modulators

Pamidronate disodium was obtained from Sigma-Aldrich (St Louis, MO, USA). OPG was the kind gift of Amgen (Thousand Oaks, CA, USA). Both agents were dissolved in sterile saline and then injected subcutaneously at doses of 5 mg kg^{-1} and 10 mg kg^{-1} , respectively, daily, over a period of 8 d. Controls received sterile saline alone.

Tooth movement measurements

Radiographs were taken on days 0, 1, 4, 8, and 12 after appliance placement to quantify tooth movement. Low-speed dental X-ray film was exposed using an HP Faxitron (Hewlett Packard, McMinnville, TN, USA), with an exposure time of 30 s at 30 W. Developed films (Fig. 1C) were scanned as a tif file and digitized using COREL DRAW 11 software (Corel Corp. Ottawa, Canada). Images were magnified $\times 50$, and tooth movement was calculated as one-half of the intermolar distance between the bonded wire tips.

Quantification of osteoclasts

In an initial study, groups ($n = 6$) of constricted and control mice were killed by CO_2 inhalation on days 4, 8, and 12 after appliance placement. In a second study to refine the kinetics of the osteoclast response, groups ($n = 5$) of mice were examined on days 0, 1, 2, 3, and 4 after appliance placement. The maxilla was isolated, fixed in 4% paraformaldehyde overnight, washed in 50% ethanol, demineralized in 10% EDTA for 30 d, dehydrated, and embedded in paraffin. Transverse sections ($6 \mu\text{m}$) were cut anterior-posteriorly and stained with hematoxylin and eosin. Sections of the central aspects of the distal root, demonstrating a patent root canal and apical foramen of the first maxillary molar, were stained with tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts. Osteoclasts were counted under high-power microscopy ($\times 200$ magnification) on the compression (palatal) and tension (buccal) sides of the distal root (a minimum of five sections per animal). In experiments comparing the effects of pamidronate and OPG, groups of mice ($n = 8$)

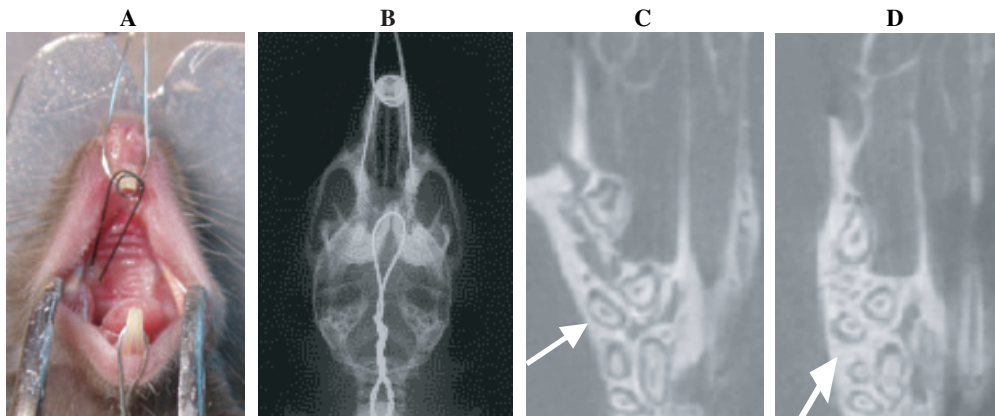


Fig. 1. Novel appliance for controlled movement of molar teeth in mice. (A) Y-shaped spring appliance bonded to the right maxillary first molar; note constricted spring with 0.5 mm distance between the ends. (B) Radiograph of the installed appliance bonded to both molars for quantification of the intermolar distance. (C) Micro-computed tomography of the control (passive) appliance, 4 d. The periodontal ligament (PDL) space is uniform around all roots of the maxillary first molar. (D) Active (constricted) appliance, 4 d. The PDL space is reduced on the palatal aspect of all three roots of the first molar.

were killed on day 8, and specimens were processed in an identical manner.

Apoptosis assay

Osteoclast apoptosis was assessed in day-8 sections, from mice treated with activated appliances, by the TdT-mediated biotin-dUTP nick-end labelling (TUNEL) assay (Apoptag kit; Chemicon, Temecula, CA, USA), in accordance with the manufacturer's instructions. Sections were counterstained for TRAP, and the numbers of osteoclasts with labeled nuclei and total osteoclasts were counted under light microscopy.

Statistical analysis

Time-related differences in tooth movement and osteoclast numbers were analyzed by the *t*-test.

Results

Novel mouse model of orthodontic tooth movement

We developed and characterized a new mouse model of orthodontic tooth movement that employs a Y-shaped spring appliance which is capable of moving mouse maxillary molar teeth in either a buccal ('expansion') or a palatal ('constriction') direction (Fig. 1A,B). In preliminary experiments, 8 d of buccally directed force resulted in molar tooth movement, but also caused separation of the mid-palatal suture, thus confounding quantification of actual tooth movement (data not shown). In contrast, palatally directed force produced movement of $\approx 100 \mu\text{m}$ per tooth over 8 d, with no histological changes in either the integrity of the midline suture or in the numbers of osteoclasts in sutures (data not shown).

The effect of palatal force application was confirmed using microcomputed tomography. As shown in Fig. 1D, all three roots of the maxillary first molar contacted bone on the palatal aspect of the periodontal ligament (PDL), consistent with a compressive force, whereas a widened PDL was evident on the tension side. No PDL asymmetry was seen in control teeth with a non-activated control appliance (Fig. 1C).

Force characteristics of the appliance

Prior to extensive *in vivo* studies, the force characteristics of the appliance were evaluated. The initial force exerted by the spring when it was maximally opened (from 0.5 to 3.5 mm) was 22.4 g. This value decreased only marginally, to 20.1 g (10.2%), when the spring was allowed to constrict to 3.2 mm, representing the working range of the appliance over a 12-d period. Thus, the force developed using this device is nearly constant and is similar in magnitude to the forces used clinically to move human teeth.

Kinetics of tooth movement

The kinetics of tooth movement was initially evaluated on days 1, 4, 8, and 12 after force application. As shown

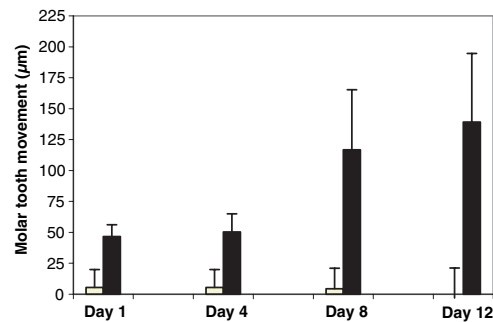


Fig. 2. Kinetics of molar tooth movement. Groups of mice ($n = 6$) were treated with passive (white bars) or active (black bars) appliances, and tooth movement was quantified by radiography. 'Tooth movement' on day 1 represents compression of the periodontal ligament (PDL) without bone resorption. Results are expressed as mean \pm standard deviation. * $P < 0.01$ vs. day 1 active appliance.

in Fig. 2, during the first 24 h the intermolar distance decreased by $\approx 46 \mu\text{m}$ per tooth, which represented compression of the soft connective tissues of the PDL without changes in the bone. The intermolar distance did not change on days 2 or 3 following this initial compression event (A. Keles, P. Stashenko, unpublished observations). However, by day 4 after force application, a small amount of tooth movement through bone was seen, which increased linearly on days 8 and 12 (both $P < 0.001$). The rate of tooth movement through bone between days 4 and 12 was $\approx 10 \mu\text{m}$ per day. Control animals fitted with an identical, but non-activated, appliance exhibited no change from baseline over the 12-d period. These data indicate that, in this model, tooth movement occurs after a lag phase of ≈ 3 –4 d in a continuous manner.

Kinetics of osteoclast recruitment and maintenance

TRAP-positive osteoclasts were found in resorption lacunae on the compression side of the distal root after 4, 8, and 12 d (Fig. 3A,B). The distal root was chosen for evaluation because it is the most perpendicular, of the three maxillary first molar roots, to the direction of force. None of the sections displayed bleeding, extensive root resorption, or other obvious tissue damage. Significantly increased numbers of osteoclasts were present by day 4 on the compression side (Fig. 4A). Osteoclast numbers were maintained at nearly identical levels on days 8 and 12. In a second experiment, the kinetics of osteoclast influx was examined between days 0 and 4 in order to define more precisely the critical period of cell recruitment. As seen in Fig. 4B, significant numbers of osteoclasts were first visible on day 3 after force application, indicating that the critical signals which regulate migration are generated before this time point. Most osteoclasts at all time points were multinucleated (approximately fourfold more numerous than mononucleated TRAP-positive cells; data not shown). On the tension side, the PDL was widened and collagen fibers were parallel to the vector of force, but the osteoclast numbers remained low (Fig. 4A,B).

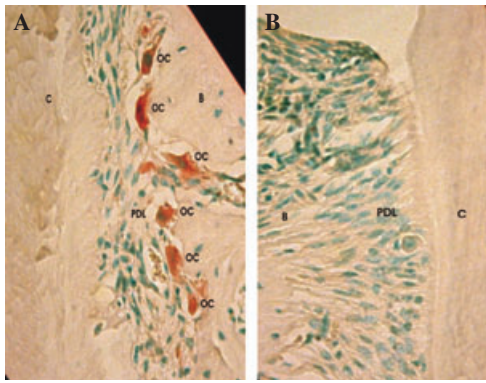


Fig. 3. Osteoclast recruitment during tooth movement. (A) Representative section of the compression (palatal) side of the first molar distal root. B, alveolar bone; C, cementum; OC, osteoclasts; PDL, periodontal ligament. OC are visible as tartrate-resistant acid phosphatase (TRAP)-positive cells (stained red). Counterstain: hematoxylin & eosin. (B) The tension (buccal) side of the same tooth. Magnification, $\times 200$.

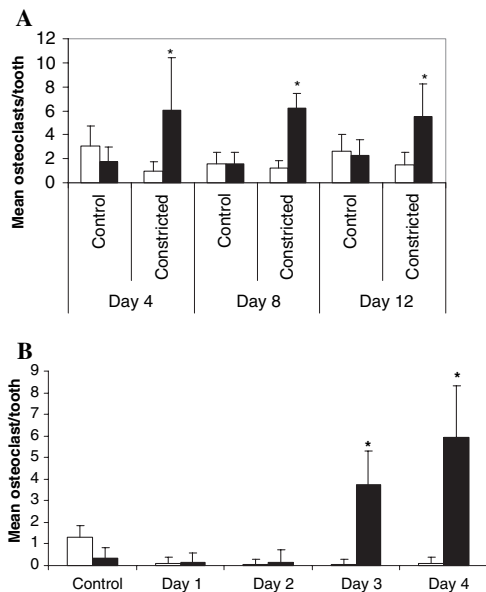


Fig. 4. Kinetics of osteoclast recruitment. (A) Osteoclasts were evaluated on days 4, 8, and 12 after force application. (B) Detailed kinetics of osteoclast recruitment on days 0–4. Results are expressed as mean \pm standard deviation. Control: passive appliance; Constricted: active appliance. Black bars, compression (palatal) side; white bars, tension side. * $P < 0.05$ vs. control.

Reversal lines were evident in bone adjacent to the PDL, indicative of new bone formation (data not shown). The non-activated control group did not demonstrate any differences in osteoclast numbers between the two sides of the root (Fig. 4A,B), and reversal lines were absent from the tension side of controls (data not shown).

Assessment of osteoclast apoptosis

In transient force models, osteoclasts are removed by apoptosis, coincident with the decay in force (9, 10). To

determine apoptosis as an important regulator of osteoclast numbers in our constant force model, we assessed osteoclast apoptosis at compression sites, after 8 d of tooth movement, by the TUNEL assay. We found that no apoptotic osteoclasts were present at the compression sites after the application of constant force (Fig. 5). This finding suggests that recruited osteoclasts represent a relatively stable cell population, that once recruited, maintain their viability and activation for extended periods under conditions of constant force.

Effect of OPG and pamidronate on osteoclast numbers and tooth movement

Both bisphosphonates and OPG inhibit osteoclastic bone resorption and have been reported to reduce tooth movement, although their relative efficacy in this system is unknown. We administered, subcutaneously, the bisphosphonate, pamidronate (5 mg kg^{-1} per day), and OPG (10 mg kg^{-1}) at doses previously shown to have maximal inhibitory effects on bone resorption *in vivo* (11, 12). As shown in Table 1, pamidronate treatment resulted in a 70% reduction in osteoclast numbers compared with controls ($P < 0.01$) and reduced tooth movement by 34% after 8 d. The inhibition of tooth movement by pamidronate, although indicating a trend, was not statistically significant ($P = 0.10$). In contrast, OPG (10 mg kg^{-1} per day) had a much more profound effect, inhibiting osteoclast numbers by 95% and tooth movement by 77% vs. controls (Table 1, $P < 0.01$). These data demonstrate that osteoclast accumulation and tooth movement in this constant-force model can be largely prevented by the systemic administration of OPG, which is a much more potent blocker of tooth movement than pamidronate.

Discussion

The movement of anchor teeth is an undesired complication in orthodontics, which may result in prolonged treatment time and/or a less than optimal clinical outcome. In the present study we evaluated the relative efficacy of two anti-osteoclastic agents – the bisphosphonate,

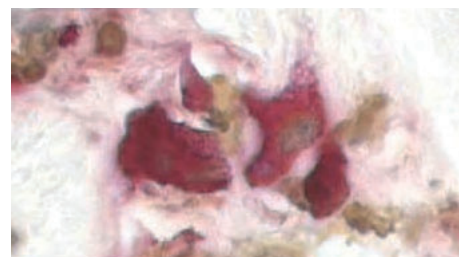


Fig. 5. TdT-mediated biotin-dUTP nick-end labelling (TUNEL) staining of osteoclasts at the compression site. A representative section of the compression (palatal) side of the first molar distal root is shown. Note the lack of brown/black-stained nuclei in osteoclasts [stained for tartrate-resistant acid phosphatase (TRAP); red].

Table 1
Inhibition of tooth movement and osteoclasts by pamidronate vs. osteoprotegerin (OPG)

Experiment	Treatment	Molar tooth movement (μ)	No. of osteoclasts per tooth	
			Compression sites	Tension sites
1	Control	60.5 \pm 22.3	4.2 \pm 2.9	0.5 \pm 1.0
	Pamidronate	39.7 \pm 13.1 (34)	1.2 \pm 1.9 (70)*	0.4 \pm 1.1 (20)
2	Control	72.1 \pm 24.0	11.0 \pm 7.0	1.9 \pm 3.5
	OPG	16.4 \pm 6.0 (77)*	0.6 \pm 0.8 (95)*	0.2 \pm 0.4 (89)*

The results are expressed as mean \pm standard deviation; values in parenthesis represent percentage inhibition vs. control.

* $P < 0.01$ vs. control.

pamidronate, and the RANKL inhibitor, OPG – in reducing molar tooth movement in a novel, quantitative mouse model. Our results demonstrate that OPG is a more powerful inhibitor of osteoclast recruitment and activity than pamidronate, with a reduction in osteoclast numbers and tooth movement of 95% and 77%, respectively. These data suggest that a biologic approach to preserving tooth anchorage may be a feasible alternative to implants and extra-oral devices (such as headgear) in clinical orthodontics.

OPG is a logical candidate for this application, given the key role of its ligand RANKL in osteoclast formation and activation (2,7). RANKL is expressed in PDL osteoblasts, osteocytes, and fibroblasts following force application (13). PDL cells subjected to compressive forces *in vitro* induce osteoclastogenesis through the up-regulation of RANKL expression (14). OPG-deficient mice exhibit extensive destruction of alveolar bone as soon as 5 d after force application (15), and local OPG gene transfer inhibits orthodontic tooth movement (14). RANKL is thus a central mediator in the mechanotransduction cascade, and its inhibition by OPG clearly reduces osteoclast function.

Bisphosphonates have been reported to reduce tooth movement significantly and to inhibit the relapse of moved teeth in rats when administered either systemically or by local injection (3, 4, 6, 16). The amount of inhibition in those studies was in the range of 50–60% (3,4), compared with the 34% inhibition observed in the present study. This inhibition, although indicative of a trend ($P = 0.10$), would probably have been statistically significant if the group size had been larger than $n = 6$. Nevertheless, in none of these studies did bisphosphonates reduce osteoclast accumulation and tooth movement to the same degree as observed for OPG. Although the *in vivo* potency of nitrogen-containing bisphosphonates differs substantially based upon the structure of the R(2) side-chain (17), the dose of pamidronate (5 mg kg⁻¹) used in the present study is considerably higher than that used clinically in humans and, furthermore, was previously found to be optimal in inhibiting hypercalcemia caused by parathyroidhormone (PTH) administration or by adenocarcinoma in mice (11). In that study, neither pamidronate nor zoledronic acid (also used at 5 mg kg⁻¹) was as effective as OPG in reducing hypercalcemia, mirroring the results of the present study. The lower potency of bisphosphonates vs. OPG may be a

result of the requirement that bisphosphonates should first be incorporated into bone in order to inhibit osteoclast activity efficiently (18).

A quantitative comparison of the properties of these inhibitors was made possible in our newly developed mouse model of constant force-induced tooth movement. This model uses a spring appliance that moves teeth in a palatal direction, with clinically relevant force properties. Tooth movement was initiated by days 3–4 after force application, coincident with the maximal influx of osteoclasts on the compression side of molar roots, which is similar to previous studies (19–21). Osteoclast numbers were maintained at similar levels, and tooth movement continued until at least day 12. The latter finding is quite unique in orthodontic models, which have largely employed appliances that undergo rapid decay of force, resulting in the disappearance of osteoclasts, in part via apoptosis (9,10) and in cessation of tooth movement by days 7–10 (19). New bone formation was also detected on the tension side of teeth by gross histological observation in samples from days 8 and 12, consistent with previous studies (20).

Only a few previous investigations have used mice for tooth movement studies (15, 21–23). A major advantage of mice is that there are a vast array of antibodies, recombinant proteins, and transgenic and knockout strains available, which will be essential in elucidating mechanotransduction mechanisms. The major drawback of this model is that placement of the appliance is technically challenging. However, with experience, placement becomes quite efficient, taking ≈ 30 min per animal.

Characterization of the force characteristics of the Y-shaped appliance showed that the forces developed are similar to those used in human clinical orthodontics. Moreover, the decay of force was minimal over the working range of the appliance. This contrasts with other experimental systems, including elastic bands forced between teeth (15,24), which generate high initial forces that decay very rapidly to a fraction of the original levels. Open coiled springs are also commonly used, which are attached to the incisors and apply mesially directed forces to the maxillary first molars (19–21, 25). Force levels also decay fairly rapidly by days 5–7 when using these devices. Osteoclast numbers at compression sites are thus dependent upon the force delivered, suggesting that force, and the downstream mechanotransduction

mechanisms that it activates, are required for the maintenance of osteoclast numbers and activity.

Taken together, our findings provide proof-of-principle data that osteoclast modulators are effective in preventing molar tooth movement, and hence have potential applicability for maintaining anchorage during orthodontic treatment. Further studies are needed to develop appropriate modes of localized delivery of OPG to sites where anchorage preservation is desired, as well as for prevention of relapse.

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