ABSTRACT
It has been shown that inhibiting the expression of certain cytokines decreases the rate of tooth movement. Here, we hypothesized that stimulating the expression of inflammatory cytokines, through small perforations of cortical bone, increases the rate of bone remodeling and tooth movement. Forty-eight rats were divided into 4 groups: 50-cN force applied to the maxillary first molar (O), force application plus soft tissue flap (OF), force application plus flap plus 3 small perforations of the cortical plate (OFP), and a control group (C). From the 92 cytokines studied, the expression of 37 cytokines increased significantly in all experimental groups, with 21 cytokines showing the highest levels in the OFP group. After 28 days, microcomputed tomography, light and fluorescent microscopy, and immunohistochemistry demonstrated higher numbers of osteoclasts and bone remodeling activity in the OFP group, accompanied by generalized osteoporosity and increased rate of tooth movement.

KEY WORDS: osteoperforation, cytokines, bone remodeling, tooth movement.

INTRODUCTION
Orthodontic forces induce an aseptic inflammatory response. During early stages of tooth movement, there is an increase in vascular permeability and cellular infiltration of leukocytes (Krishnan and Davidovitch, 2006; Meikle, 2006). Migrated immune cells along with native cells such as fibroblasts and osteoblasts produce inflammatory cytokines that include lymphocyte- and monocyte-derived factors, colony-stimulating factors, growth factors, and chemotactic factors (Ren and Vissink, 2008; Krishnan and Davidovitch, 2009). High concentrations of inflammatory cytokines such as interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-8, tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), and osteoclast differentiation factor have been found in the gingival crevicular fluid surrounding moving teeth (Alhashimi et al., 2000; Garlet et al., 2007; Ren et al., 2007).

The role of cytokines during tooth movement is not clear. It has been suggested that cytokines and other inflammatory markers, such as prostaglandin E2 (Saito et al., 1991), may activate bone remodeling characterized by bone resorption in the compression region and bone deposition in the tension region of the periodontal ligament (PDL) (Davidovitch et al., 1988; Garlet et al., 2007). This is in agreement with previous studies that demonstrated that bone injury which causes cytokine release leads to an accelerated bone turnover and a decrease in regional bone density (Frost, 1983, 1989a,b; Shih and Nordin, 1985; Yaffe et al., 1994). One possible mechanism through which inflammatory cytokines may affect bone remodeling is through recruitment of osteoclast precursors from the circulation, their maturation and activation. Many cytokines that promote osteoclast formation and activation, such as IL-1, IL-6, and TNFα (Glantschnig et al., 2003; Seidenberg and An, 2004; Yao et al., 2008), have also been found in crevicular fluid during orthodontic tooth movement (Uematsu et al., 1996; Basaran et al., 2006).

The effect of cytokine expression on bone remodeling is important, since the rate of tooth movement correlates with the efficiency of bone remodeling in the alveolar process. Studies of knockout mice deficient for TNFα receptors (Yoshimatsu et al., 2006) showed a slower rate of tooth movement in response to orthodontic forces. Previous reports have shown that anti-inflammatory medication can decrease the rate of tooth movement (Arias and Marquez-Orozco, 2006).

Based on these findings, we hypothesized that limited and shallow perforations of the buccal cortical plate of the maxilla would be sufficient to increase the expression of inflammatory cytokines, accelerating the bone remodeling process and therefore the rate of tooth movement.
MATERIALS & METHODS

Animal Study

Forty-eight adult male Sprague-Dawley rats (average body weight of 400 g, 120 days of age) were housed and treated according to a protocol approved by the New York University Institutional Animal Care and Use Committee. Animals were divided into 4 groups (12 rats per group): control, which received coil spring without activation (C), orthodontic force applied to the spring (O), orthodontic force and soft tissue flap (OF), and orthodontic force, soft tissue flap, and shallow perforations of the buccal cortical plate (OFP). The health status and body weight of the rats were evaluated daily, and no significant differences were observed among groups. From each group, 4 animals were used for gene expression studies, 4 for microCT and fluorescent studies, and 4 for demineralized histological studies. Procedures were performed on one side of the maxilla, which allowed the contralateral side to be used as an additional control.

Surgical Procedure

On day 0, all groups were anesthetized with an intraperitoneal injection of ketamine-xylazine (0.09 mL/100 g), and anesthesia was verified by lack of response to toe-pinch. All groups were fitted with 50 cN Sentalloy closing coils (DENTSPLY GAC International, Bohemia, NY, USA) tied at both ends to holes drilled in the maxillary incisors and left maxillary first molar with 0.008-inch ligature wire; the coil was activated in groups O, OF, and OFP, but not in the C group. In the OF and OFP groups, a soft tissue flap was raised around the left first molar. Flaps were sealed with cyanoacrylate tissue adhesive (Vetbond, 3M, St. Paul, MN, USA). In the OFP group, the animals received 3 shallow perforations, approximately 0.25 mm in diameter (depth of 0.25 mm), 5 mm mesial to the left first molar with the use of a round bur and handpiece. Animals were checked under general anesthesia twice weekly, and any springs requiring retying (mostly due to continuous eruption of the maxillary incisors) were adjusted. Bone labeling by intra-root springs requiring retying (both Qiagen, Valencia, CA, USA) on a DNA Engine Optic 2 thermocycler (both Qiagen, Valencia, CA, USA) was performed with the TRIZOL reagent (Life Technologies, New York, NY, USA), and RNA clean-up was performed with an RNeasy Mini Kit (Qiagen Sciences, Valencia, CA, USA) according to the method of Erben (Erben, 1997). The samples were sectioned at 5- to 7-µm thickness on a Reichert-Jung Ultracut E microtome and viewed by fluorescent microscopy (Nikon Microscopy, NIS-Elements software, Tokyo, Japan).

Micro-CT Imaging

Hemimaxillae were scanned with a Scanco Micromet (µCT40, Scanco Medical, Bassersdorf, Switzerland). Results were analyzed utilizing µCT V6.0 software on the HP open platform (openVMS Alpha Version 1.3-1 session manager). The area extending from the coronal to the apical root third was analyzed for bony changes. Maxillae were analyzed in fixed coronal and sagittal zones. The ratio of bone volume to total volume (BV/TV) was calculated based on a threshold of 275.

Histology and Immunohistochemistry

Hemimaxillae were collected and fixed in 10% phosphate-buffered formalin and demineralized in a sodium formate (6.8%) and formic acid (50%) solution for 6-8 wks. Following demineralization, specimens were dehydrated in alcohol series and embedded in paraffin; 5-µm-thick sections were cut and stained with hematoxylin and eosin (H&E). Consecutive specimens were immunostained with antibodies for tartrate-resistant acid phosphatase (TRAP; Zymed antibodies, Invitrogen, Carlsbad, CA, USA), a marker of osteoclasts, and Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. As negative control, consecutive sections were exposed to pre-immune serum. Stained sections were scanned on a Scan Scope GL series optical microscope (Aperio, Bristol, UK) at 20x magnification. Osteoclasts were defined as TRAP-positive multinuclear cells on the bone surface. The area around the mesio-palatal root of the maxillary first molar was divided into mesial and distal halves, and osteoclasts in the mesial half were counted. Data were expressed as the number of TRAP-positive cells per 1000 µm² in the area of PDL and adjacent alveolar bone, excluding the marrow cavities and blood vessels.

For fluorescent microscopy, after formalin fixation, specimens were washed overnight in running water, dehydrated in alcohol, cleared in xylene, and embedded in methyl methacrylate according to the method of Erben (Erben, 1997). The samples were sectioned at 5- to 7-µm thickness on a Reichert-Jung Ultracut E microtome and viewed by fluorescent microscopy (Nikon Microscopy, NIS-Elements software, Tokyo, Japan).

RT-PCR Analysis

For RNA extraction, 4 animals from each group were killed by CO₂ narcosis at 24 hrs, and the hemimaxillae were dissected and frozen in liquid nitrogen. Isolation of total RNA was performed with the TRIZOL reagent (Life Technologies, New York, NY, USA), and RNA clean-up was performed with an RNeasy Mini Kit (Qiagen Sciences, Valencia, CA, USA) as described previously (Oliveira et al., 2009). All equipment and tools were cleaned with RNaseZap (Sigma, St. Louis, MO, USA). Ninety-two inflammatory cytokines and cytokine receptor genes were analyzed with primers specific for rat genes (see online Appendix for list of genes), with a QuantiTect SYBR Green RT-PCR kit (both Qiagen, Valencia, CA, USA) on a DNA Engine Optic 2 System (MJ Research, Waltham, MA, USA). Each mRNA specimen was tested 3 times. Relative levels of mRNA were calculated and normalized to the level of GAPDH and acidic ribosomal protein mRNA.

Statistical Analysis

Significant differences between test groups and controls were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed with Tukey’s post hoc test.
Two-tailed p-values were calculated; p < 0.05 was set as the level of statistical significance.

RESULTS

Osteoperforations Increase the Rate of Tooth Movement

Coil springs were used for mesial movement of the first maxillary molar crown (Fig. 1A). Three shallow perforations were made in the cortical bone, 5 mm mesial to the molar (Fig. 1B). At 28 days, the average crown movement (measured in 12 rats per group) was 0.29 mm in the O and OF groups (Fig. 1C), significantly different from controls (p < 0.05). The OFP group showed the greatest mean tooth movement, 0.62 mm, which was significantly higher (p < 0.05) than that of the C, O, and OF groups (Fig. 1C).

Osteoperforations Increase Expression of Inflammatory Cytokines

Expression of 92 different cytokines/cytokine receptors was studied by RT-PCR, 24 hrs after force application. The expression of 37 cytokines/cytokine receptors increased more than two-fold in the left maxilla of rats in the O, OF, and OFP groups when compared with that in the C group (data not shown). Differences between O and OF groups were not statistically significant. From these 37 cytokines, expression of 21 cytokines/cytokine receptors was statistically higher in the OFP group than in the O or OF group (p < 0.05) (Fig. 2), with 8 cytokines showing a 1.6- to 2.7-fold increase (Fig. 2A), 5 chemokines showing a 1.6- to 2.8-fold increase (Fig. 2B), and 8 receptors showing a 1.7- to two-fold increase in expression (Fig. 2C). All cytokines/cytokine receptors expressed in the OFP group were also expressed in the O or OF group. Expression of cytokines in the contralateral side of all groups showed no statistically significant differences from group C (data not shown).

Osteoperforations Increase Osteoclast Activity

In both the O and OF groups, application of the orthodontic force stimulated an increase in alveolar bone resorption in the direction of tooth movement and, consequently, an increase in PDL thickness (Fig. 3A, top row). The OFP group showed increased alveolar bone resorption in the direction of tooth movement (Fig. 3A, top row). Immunohistochemical staining for TRAP-positive osteoclasts (Fig. 3B) revealed an increase in osteoclast number in the OFP group, compared with the OF and O groups (Fig. 3A, bottom row). Quantitative analysis of osteoclasts in the pressure side (mesial) of alveolar bone adjacent to the mesio-palatal root of the maxillary first molar demonstrated a three-fold increase in numbers of osteoclasts in comparison with the O and OF groups (p < 0.05) (Fig. 3C). The difference between numbers of osteoclasts in the O and OF groups was not statistically significant.

Osteoperforations Increase the Rate of Bone Remodeling and Generalized Osteoporosity

Sagittal sections of specimens viewed under fluorescent microscopy showed more prominent fluorescence in the OFP group.
Figure 2. Osteoperforations increased expression of inflammatory markers. Mean “-fold” increase in expression of cytokines (A), chemokines (B), and inflammatory receptors (C) in the orthodontic group (O, white bars) and the orthodontic force plus flap plus perforations group (OFP, black bars) compared with controls. Data expressed as mean ± SE of 3 experiments. All values in the orthodontic (O) group showed a statistically significant increase from controls. *Significantly different from orthodontic (O) group, p < 0.05.

(Fig. 4A), indicative of heightened bone remodeling activity. We used microCT quantification to evaluate the effects of osteoperforations on induction of osteoporosis during tooth movement. Comparison of the OFP group with the other groups revealed significant findings on all planes of analysis (Fig. 4B). Bone volume fraction (BV/TV) levels in the OFP group were significantly lower (p < 0.05, ANOVA) than in the C, O, or OF group (see Appendix Table). The BV/TV fraction in the C group was, on average, as high as 82% around the first maxillary molar, while in the OFP group these values decreased to 33%. Both the O and OF groups also exhibited statistically significant changes in BV/TV levels (p < 0.05, ANOVA) when compared with the C group. Interestingly, in comparison with other groups, the BV/TV fraction in the OFP group decreased significantly (p < 0.05) around all left maxillary molars (from 33% to 35%). This effect was limited to the left hemimaxillae, and no change in BV/TV fractions was observed in contralateral hemimaxillae (p > 0.05).

DISCUSSION

Analysis of our data demonstrated that shallow perforations of the cortical plate increased the levels of cytokine expression in response to orthodontic forces without changing their pattern of expression. Indeed, markers of lymphocytes (CCL20, CCR1) (Han et al., 2000; Kao et al., 2005), T-cells (LTα, IL-3, CCL5, CCR5, CX3CR1, IL-18rb, IL-1r1) (Ito et al., 1999; O’Hara et al., 2006), monocytes (IL-1, IL-6, IL-11, IL-18, IL-6ra) (Adachi et al., 1994; Lean et al., 2002; Arend et al., 2008; Dale et al., 2008; Dienz and Rincon, 2009), and macrophages (IL-1, TNF, IL-6, IL-11, IL-18, IL-6ra) (Adachi et al., 1994; Yamamoto et al., 2006; Arend et al., 2008; Dale et al., 2008) were found elevated in the OFP group in comparison with the O group, 24 hrs after initiation of the experiment.

Higher levels of expression of cytokines and their receptors are important, since it has been shown that inflammatory cytokines play an important role in recruitment of osteoclasts and activation of the bone remodeling machinery (Alhashimi et al., 2000; Ren and Vissink, 2008; Krishnan and Davidovitch, 2009). The fact that the number of osteoclasts and the bone remodeling rate were higher in the OFP group in comparison with the O and OF
groups supports the possible role of inflammatory cytokines in recruiting osteoclasts into the area.

Similar to previous studies (Verna et al., 1999), we demonstrated that the increase in bone remodeling rate was not limited to the area of the loaded tooth, but extended to the tissues surrounding adjacent teeth. This generalized increase in bone turnover was accompanied by osteoporosis, as reflected by a decrease in bone density around all upper left molars. While a limited number of osteoperforations had a generalized effect, the effect was not robust enough to cross to the contralateral side.

Since bone remodeling controls the rate of tooth movement, the increase in rate of bone remodeling and osteoporosity in response to bone perforations may explain the increase in the rate and magnitude of tooth movement observed in this study. One may argue that the effects of the shallow perforations on tooth movement were not a response to an increased cytokine expression, but rather were due to weakening of the bone structure. While we cannot ignore the effects that perforations can have on the physical properties of the bone, this study was designed to minimize this possibility. First, the perforations were small and limited (only 3); therefore, the majority of the cortical bone remained intact. In addition, the perforations were placed far away from the tooth, and could still be observed at the end of the study with remaining bone (about 4 mm) between perforations and the moved tooth. These results further suggest that the perforations do not need to be close to the tooth to be moved to accelerate the rate of movement.

It is important to mention that inflammation is “a two-sided sword”; and while it can work to our benefit by accelerating bone remodeling and tooth movement, if uncontrolled, it may also have a destructive effect on the periodontium and tooth structure. We are currently investigating root resorption in response to osteoperforation. While extensive injury to the cortical plate bone, also referred to as corticotomies, is currently being used to accelerate orthodontic tooth movement in private practice, analysis of our data suggests that this approach could be simplified to minimize deleterious side-effects. Therefore, we propose the use of flapless minimal cortical perforations as a means of fine-tuning inflammation levels for enhanced tooth movement, enabling orthodontists to provide more efficient treatment for their patients.

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Figure 4. Osteoperforations increased bone remodeling rates and generalized osteoporosity in the entire length of the hemimaxillae. (A) Sagittal sections of maxillae from the 4 groups viewed under fluorescent microscopy showed the rate of bone remodeling in the entire hemimaxillae. The increased intensity of the label in most of the trabecular surface of the OFP group in comparison with other groups indicates that extensive bone remodeling has taken place at 28 days post-treatment. White arrows demonstrate the direction of force application. B = control; O = orthodontic force alone; OF = orthodontic force plus flap; OFP = orthodontic force plus flap plus perforations.


