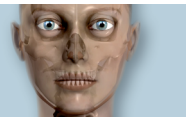
Orthodontics & Craniofacial Research



ORIGINAL ARTICLE

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Evaluation of BSP expression and apoptosis in the periodontal ligament during orthodontic relapse: a preliminary study

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Structured Abstract

Objective – To examine the expression of bone sialoprotein (BSP) and apoptosis in an *in vivo* orthodontic relapse model.

Materials and Methods – Male mice (10–12 weeks old), either transgenic [green fluorescent protein (GFP) driven by the BSP promoter] or wild type, were used in this study. To achieve orthodontic tooth movement (OTM), maxillary right first molars were moved mesially using closed-coil springs. Animals were divided into an OTM group (14 days continuous orthodontic force – 11 animals) or Relapse group (10 days of force application followed by 4 days of relapse – 8 animals). The control group was comprised of the contralateral maxillary molars. The periodontal ligament (PDL) was analyzed in areas of compression and tension for transgenic expression, osteoclast localization, and the presence of apoptotic cells.

Results – There was a significant decrease in GFP-labeled cells on the compression and tension sides of the PDL in the OTM group compared with control. In the relapse group, GFP-labeled cells were significantly decreased only on the old compression side. Osteoclasts were localized on the compression side of the OTM group, whereas in the Relapse group, they were present on both sides. PDL apoptosis significantly increased on the compression side in OTM and Relapse groups.

Conclusion – Both OTM and Relapse groups exhibited a decreased number of GFP-labeled cells in areas of compression and tension. There was significant PDL apoptosis in regions under compressive forces following OTM and to a lesser extent following relapse.

Key words: apoptosis; osteoblast; osteoclast; periodontal ligament; tooth movement

Introduction

Orthodontic tooth movement (OTM) occurs as a result of bony remodeling of the alveolar complex in response to an applied force. Osteoblasts and osteoclasts have been identified as key players in this remodeling process, and their involvement is well characterized (1). Bone is resorbed in areas of compression by osteoclasts, while in areas of tension, osteoblasts form osteoid tissue which is later calcified, forming new bone (2). This tissue turnover is characterized at the cellular level by rapid proliferation of progenitor cells and apoptosis of subpopulations of mature cells (3).

Relapse following OTM is an ongoing problem in orthodontics. According to a study by Little et al. (4), satisfactory alignment was maintained in less than 30% of orthodontic patients treated with fixed appliances, 10 years after the end of treatment. An increasing number of studies have recently focused on the prevention or minimization of orthodontic relapse (5–7).

The underlying biological mechanism for relapse remains unclear (8). Previous studies have implicated collagen and oxytalan fibril bundles of the transseptal group of fibers as key players in this process (9, 10). These fibers may be responsible for the tensile force that pulls an orthodontically moved tooth back to its original position. In an animal study, Yoshida et al. (11) detailed the changes in periodontal fibroblasts and the arrangement of osteoclasts during relapse but did not quantify these cells as compared to a control group. King et al. (12) found a significant increase in osteoclast surface percentage and osteoclast numbers on the new compression side at day 1 of relapse. No changes were detected in the number of osteoblasts.

To better understand the overall process of bone remodeling, the development of transgenic mice expressing green fluorescent protein (GFP) has played a pivotal role (13, 14). GFPs driven by different promoters that are expressed during defined phases of osteoblast lineage progression have been developed (15, 16). Bone sialoprotein (BSP) is one such non-collagenous protein expressed by a more differentiated subpopulation of osteoblasts beyond the pre-osteoblast stage that have started producing mineral (17, 18). The change in BSP expression during OTM has been demonstrated (19, 20), but the role of BSP during relapse has not been elucidated.

Apoptosis, or programmed cell death, during OTM has been investigated in rodent models, and apoptotic activity has been identified in osteocytes and periodontal cells under compression (21, 22). It has been hypothesized that cell-type-specific death may regulate periodontal remodeling at sites where excessive force is applied during OTM (23). Apoptosis in periodontal tissues has never been investigated during orthodontic relapse.

The goal of this study was to evaluate BSP expression in the periodontal ligament (PDL) during OTM and relapse using transgenic mice expressing GFP driven by the BSP promoter. We also examined the role of apoptosis in the PDL during OTM and relapse to correlate apoptosis with the underlying mechanism of bone remodeling involving BSP.

Materials and methods

Nineteen, 10- to 12-week-old male mice, either BSP-GFP transgenic (with GFP-labeled cells) or CD-1 wild type, were used for this study. The transgenic mice were generated (15) and kindly provided by Dr. Peter Maye's laboratory at the University of Connecticut Health Center, Farmington, CT, USA. All experiments were performed under an institutionally approved protocol for the use of animals in research.

Experimental animals were divided into two groups: (1) OTM – 14 days of OTM, chosen because, based on our observation, the majority of OTM in mice occurs within the first 2 weeks or (2) Relapse – 10 days of OTM followed by spring removal and 4 days of relapse movement (Fig. 1). Different durations of OTM were chosen to keep the end point of the experiment, similar.

Within the OTM group, seven transgenic mice with GFP-labeled cells were used to evaluate BSP expression and four wild-type mice were used to evaluate apoptosis. Within the Relapse

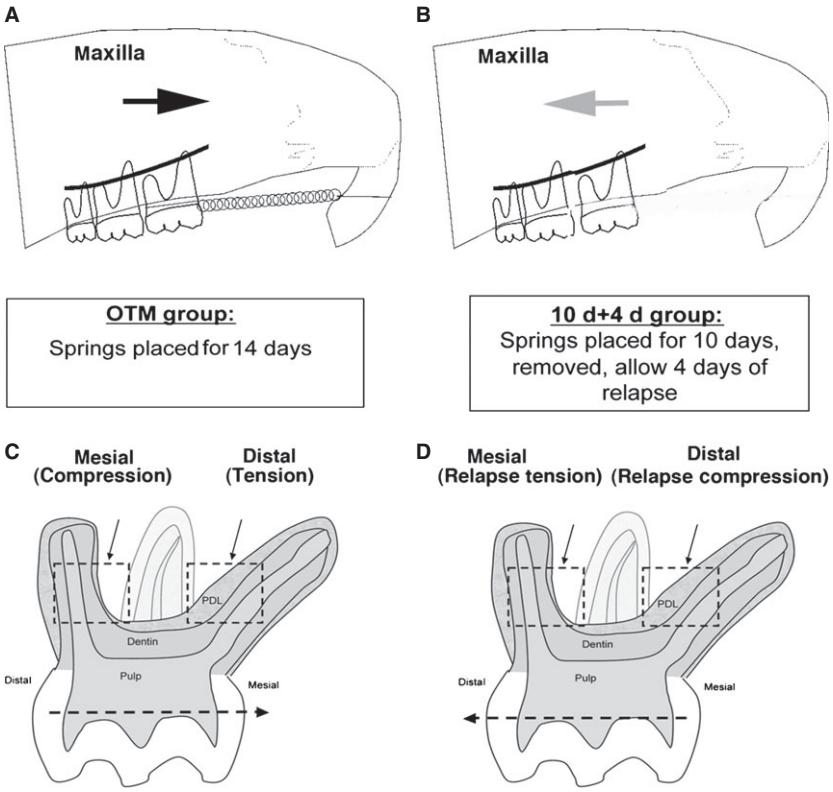

Fig. 1. Illustration of the tooth movement and relapse models used in this study. (A) The placement of NiTi coil spring in the orthodontic tooth movement (OTM) group. The spring was activated from the maxillary right first molar to the right incisor for 14 days. *Arrow* shows the direction of orthodontic force. (B) Relapse model. The spring was placed for 10 days and then removed to allow relapse tooth movement for 4 days. (C, D) Schematic representation of a maxillary molar sagittal section. Dotted squares indicate areas of compression and tension imaged at 20× magnification in OTM (C) and Relapse (D) groups.

group, four transgenic mice were used to analyze BSP expression and four wild-type mice were used to evaluate apoptosis. The number of transgenic animals was not equal in both groups because some animals were excluded due to problems with the retention of the appliance for the duration of the experiments. Data analysis included only the animals that completed the experimental period (n = 19). The contralateral sides of both groups were pooled and considered as the control group for statistical tests.

Orthodontic appliances were placed in animals under general anesthesia according to the protocol described by Olson et al. (24). The maxillary right first molar was subjected to orthodontic force, and the contralateral side served as the control. The appliances were checked daily in conscious animals, and additional bonding material was added after anesthetizing the animals when necessary.

Upon completion of the experiments, mice were euthanized by CO_2, followed by cervical dislocation. Maxillae were dissected, hemisected, and placed in 10% neutral buffered formalin for 5 days at 4°C. During that period, maxillae were scanned (μCT, 40 SCANO MEDICAL, Bassersdorf, Switzerland). The distance between the first and second molars was measured in the midsagittal plane by reformatting the scans to sagittal cuts transecting all three molars. Measurement slices were chosen from the image that revealed the most root structure (mid-root sections), and the measurement points were selected at the greatest heights of contour of the molars at the cemento-enamel junctions (CEJ). A region of interest (ROI) for alveolar bone analysis was defined between the distal surface of the mesiobuccal root and the mesial surface of the distobuccal root of the maxillary first molar (480 μm) including the compression and tension sides.

After fixation, maxillae from the transgenic mice were washed in PBS and placed in 30% sucrose overnight. They were embedded in Shandon Cryomatrix embedding resin (Thermo Fisher Scientific, Kalamazoo MI, USA), flash frozen in 2-methylbutane, and stored at −20°C. A Leica CM1900 Cryostat (D-69226; Leica, Inc., Nussloch, Germany) was used to obtain 5-μm sagittal sections, which included the mesiobuccal and distobuccal roots of the maxillary first molars.

The frozen sections were analyzed for GFP in the PDL using appropriate filter cubes on the Zeiss Axiovert 200 microscope (Carl Zeiss, Thronwood, NY, USA). Three to four sections per animal were imaged from the tension and compression sides, and mean ratios were calculated. The specimens were counterstained with DAPI nuclear stain to visualize all cells. The ratio of GFP-labeled cells for each section was calculated as the positive cells/total cells. Mean ratios were calculated for the compression and tension sides of each animal.

The maxillae from the wild-type mice were similarly dissected and decalcified in 14% EDTA for 5 weeks. These were dehydrated in a series of ethanol concentrations, cleared in xylene, and embedded in paraffin. A Leica RM 2125R7 microtome (Leica, Inc.) was used to obtain 5-μm sagittal sections.

Immunostaining for BSP in paraffin sections was carried out as follows: Rehydrated sections were blocked with 10% normal goat serum in 1% bovine serum albumin (BSA) and incubated overnight with 1:2500 dilution (in 1% BSA) of primary rabbit polyclonal anti-BSP II antibody (AB 1854; Chemicon, Temecula, CA, USA) at 4°C. Sections were then incubated with a secondary antibody (biotinylated goat anti-rabbit) for 1 hour at room temperature, and the signal was developed with a biotin/avidin system. The secondary antibody and developing reagents were from the Vector Elite ABC kit (PK6101; Vector Laboratories, Burlington, CA, USA).

To qualitatively evaluate osteoclasts in the furcation of the maxillary first molars, staining for tartarate-resistant acid phosphatase (TRAP) activity was performed with an acid phosphate leukocyte kit (Sigma Chemical, St Louis, MO, USA) according to the manufacturer's instructions. Sections were imaged at 20× magnification.

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation resulting from apoptotic signaling cascades. Paraffin sections were stained with the DeadEnd Fluorometric TUNEL System (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions and imaged. DAPI staining was performed to quantify total cells. Similar to GFP quantification, the ratio of apoptotic cells was calculated as TUNEL-positive cells/total cells. Three to four sections per animal were analyzed from each side, and mean ratios were calculated for the compression and tension sides.

Statistical analyses were carried out with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was determined by the Kruskal–Wallis nonparametric test followed by the Dunn's multiple comparison post hoc test. Significance was set at $p < 0.05$.

Results

Micro-CT measurements showed significantly greater tooth movement in the OTM group compared with the control (Fig. 2). The mean distance between the first and second molars after 14 days of OTM was 397 ± 126 μm (mean ± SD). In the Relapse group, this space was significantly smaller than the OTM group with a mean distance of 209 ± 25 μm and was similar to the control group (206 ± 36 μm).

The OTM group demonstrated a significant decrease in GFP-labeled cells on both, mesial side of the distobuccal root (compression) and distal side of the mesiobuccal root (tension) compared with the controls (Fig. 3). In the Relapse group, GFP-labeled cells were significantly decreased on the mesial side of the distobuccal root. Immunostaining for endogenous BSP showed a similar trend. In the control, BSP was detected in some osteocytes, cementoblasts, and osteoblasts lining the alveolar bone surface. During OTM, there was a decrease in BSP-stained osteoblasts on the alveolar bone surface on both the compression and tension sides. During relapse, endogenous BSP was increased on both sides, but was diffuse within the PDL (Fig. 4).

Tartarate-resistant acid phosphatase staining revealed the appearance of osteoclasts on the compression side during OTM, whereas the tension side showed no TRAP-positive cells. The relapse-tension side still showed osteoclasts after

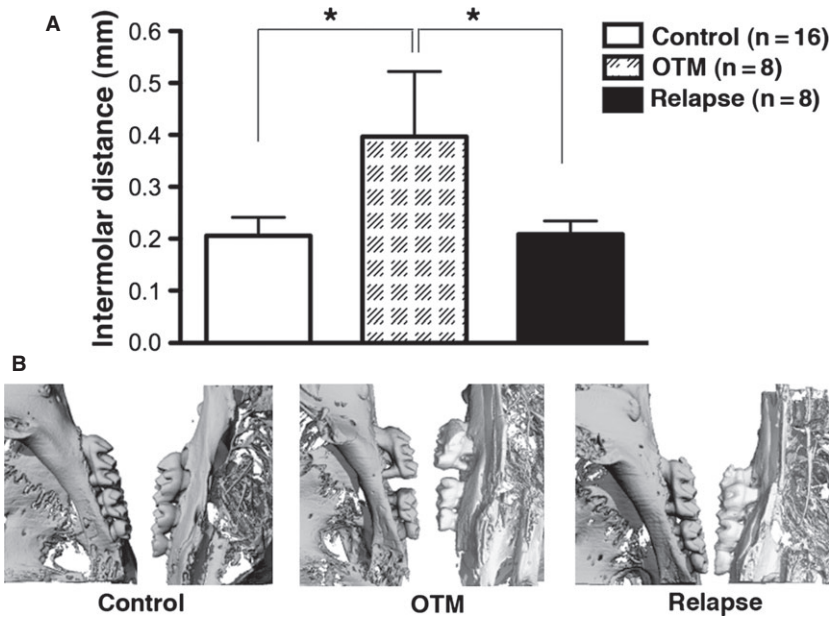

Fig. 2. Tooth movement observed by micro-CT. (A) Graph showing the intermolar distances (CEJ-CEJ) in control, orthodontic tooth movement (OTM), and Relapse groups. Each value represents the mean ± SD. Significantly greater tooth movement was observed in the OTM group compared with the control and Relapse groups ($p < 0.001$). (B) 3-D images of the maxillary region in all three groups.

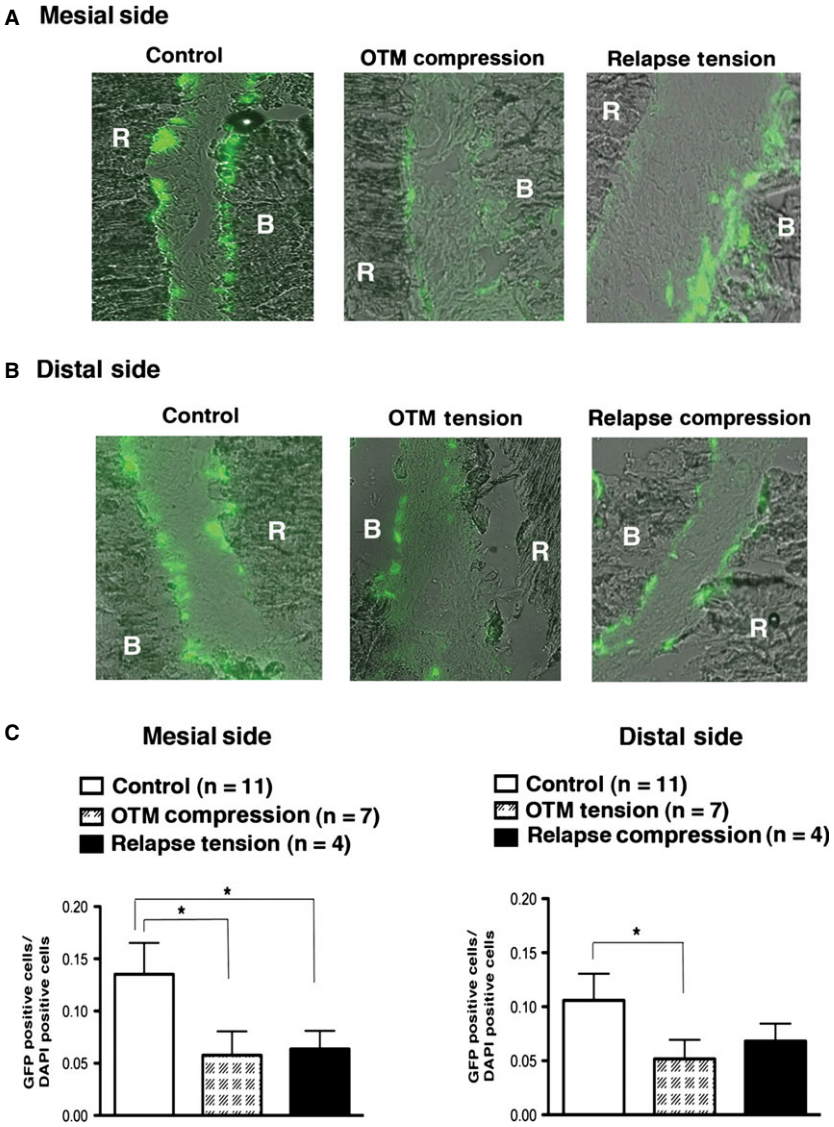

Fig. 3. Green fluorescent protein (GFP) expression during orthodontic tooth movement (OTM) and relapse. (A) Mesial side of the distobuccal root in control, OTM, and Relapse groups. (B) Distal side of the mesiobuccal root. Note the decreased number of GFP-positive cells in the OTM group on both compression and tension sides. B = bone, T = tooth. (C) Graph of GFP quantification showing mesial and distal sides. Significant decrease of GFP-labeled cells in the OTM group compared with the control on both sides. GFP-labeled cells increased slightly in the Relapse group, but their number was still lower than the control group.

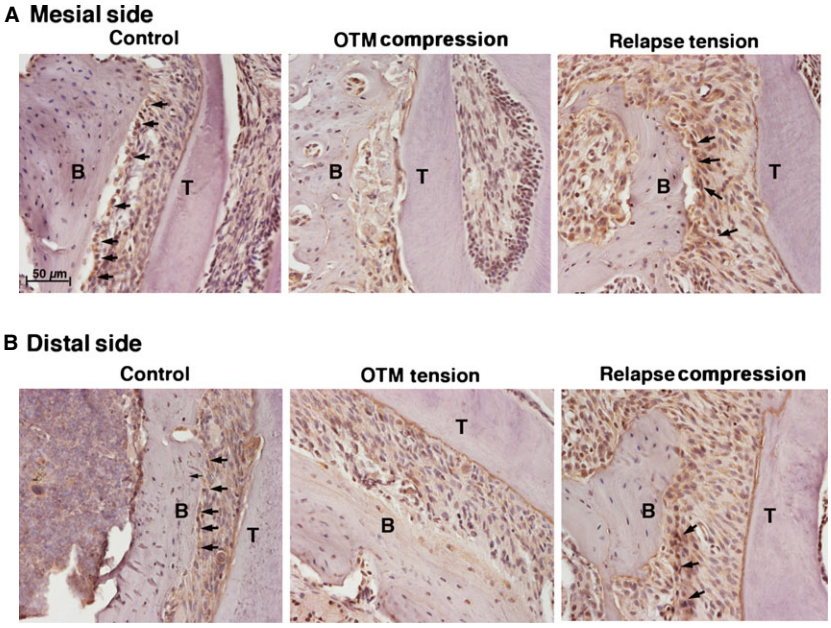

Fig. 4. Bone sialoprotein (BSP) immunostaining on sagittal histological sections of control, orthodontic tooth movement (OTM), and Relapse groups. Upper panel represents 20× images of the mesial side of the distobuccal root (A), and lower panel represents 20× images of the distal side of the mesiobuccal root (B). In the control group, BSP immunostaining was localized in some osteocytes, cementoblasts, and osteoblasts lining the alveolar bone surface (arrows). Note the decreased number of osteoblasts expressing endogenous BSP on the alveolar bone surface in the OTM group on both compression and tension sides. On the relapse-compression and relapse-tension sides, immunostained cells were more apparent than in the OTM group. B = bone, T = tooth.

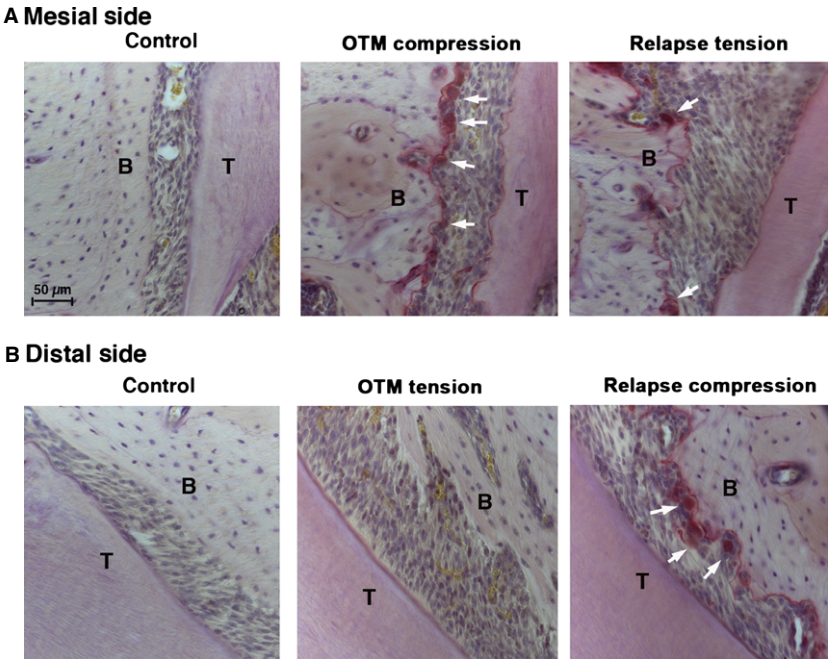

Fig. 5. Tartarate-resistant acid phosphatase (TRAP) staining on sagittal histological sections of control, orthodontic tooth movement (OTM), and Relapse groups. (A) Mesial side of the distobuccal root-20× magnification. (B) Distal side of the mesiobuccal root. Multiple TRAP-positive, multinucleated cells (arrows) were observed in the alveolar bone on the compression side in the OTM group. No osteoclasts were seen on the tension side in the OTM group. In the Relapse group, residual osteoclasts were still present on the relapse-tension side. At this time, osteoclasts were also observed on the relapse-compression side. B = bone, T = tooth.

4 days of relapse. At this time, osteoclasts could also be detected on the relapse-compression side (Fig. 5).

Micro-CT analysis of bone volume fraction (BVF: bone volume/total volume) and apparent density of alveolar bone showed a significant decrease in the OTM group compared with the control. These parameters increased slightly in the Relapse group. Tissue density showed a similar trend (Fig. 6).

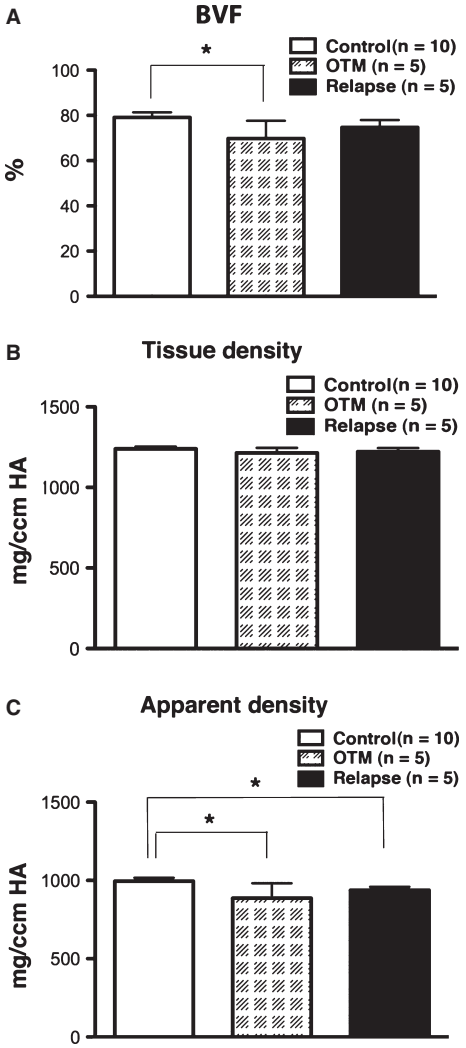

Fig. 6. Micro-CT analysis of the alveolar bone of the maxillary first molars. A region of interest (ROI) for alveolar bone analysis extended from the mesial side of the distobuccal root to the distal side of the mesiobuccal root including both compression and tension areas. (A) BVF = bone volume fraction (bone volume divided by total volume) ($p = 0.0105$). (B) Tissue density ($p = 0.0858$). (C) Apparent density ($p = 0.0027$). Note the significant decrease in BVF and apparent density in the orthodontic tooth movement group and a slight increase in the Relapse group.

Terminal deoxynucleotidyl transferase dUTP nick end labeling-positive apoptotic cells in the PDL were significantly increased on the compression side in the OTM group compared with the control. There was no difference on the tension side. The Relapse group showed a significant increase in the number of apoptotic cells on the relapse-compression side compared with the control; however, this increase was not as prominent as that on the compression side of OTM group (Fig. 7).

Discussion

Although few studies of relapse have been published, none have investigated osteoblast markers or apoptosis. In this study, we developed an *in vivo* mouse model of orthodontic relapse that allows us to easily visualize and quantify osteoblasts. Significant tooth movement was seen in the OTM group, whereas in the Relapse group, no intermolar space was seen, suggesting that a relapse of tooth movement had occurred. Considering our experimental model, it may be possible that OTM started after day 10 and no relapse occurred in the Relapse group. However, this is unlikely as we have previously demonstrated PDL changes after 12 h (24), and a significant increase in osteoclast activity after 72 h of OTM (25), suggesting that tooth movement starts very early. We also observed that majority of murine relapse occurs within 3–4 days after OTM.

In our study, GFP-labeled cells were significantly decreased on the compression side after 14 days of OTM. A similar trend of reduced BSP expression on the compression side at earlier time points of OTM was previously reported by Olson et al. (24). Surprisingly, a significant decrease in GFP-labeled cells was also seen on the tension side during OTM. According to the current literature, osteoblasts should increase on the tension side (1). BSP has been shown to label mature osteoblasts (16, 17) and is predominantly expressed during later stages of osteoblast differentiation. Based on this, decreased BSP on the tension side could be due to an abundance of immature osteoblasts in newly deposited osteoid that have not yet started producing BSP. These cells might instead be producing earlier markers of osteoblast lineage progression such as smooth muscle actin-α [SMA] (26) and the 3.6-kb fragment of the type I collagen promoter [Col3.6] (27). Transgenic reporter mice expressing these markers have been developed and characterized and will help to elucidate the role of various osteoblast subpopulations during OTM and relapse.

The micro-CT analysis of alveolar bone parameters in this study in the region including both

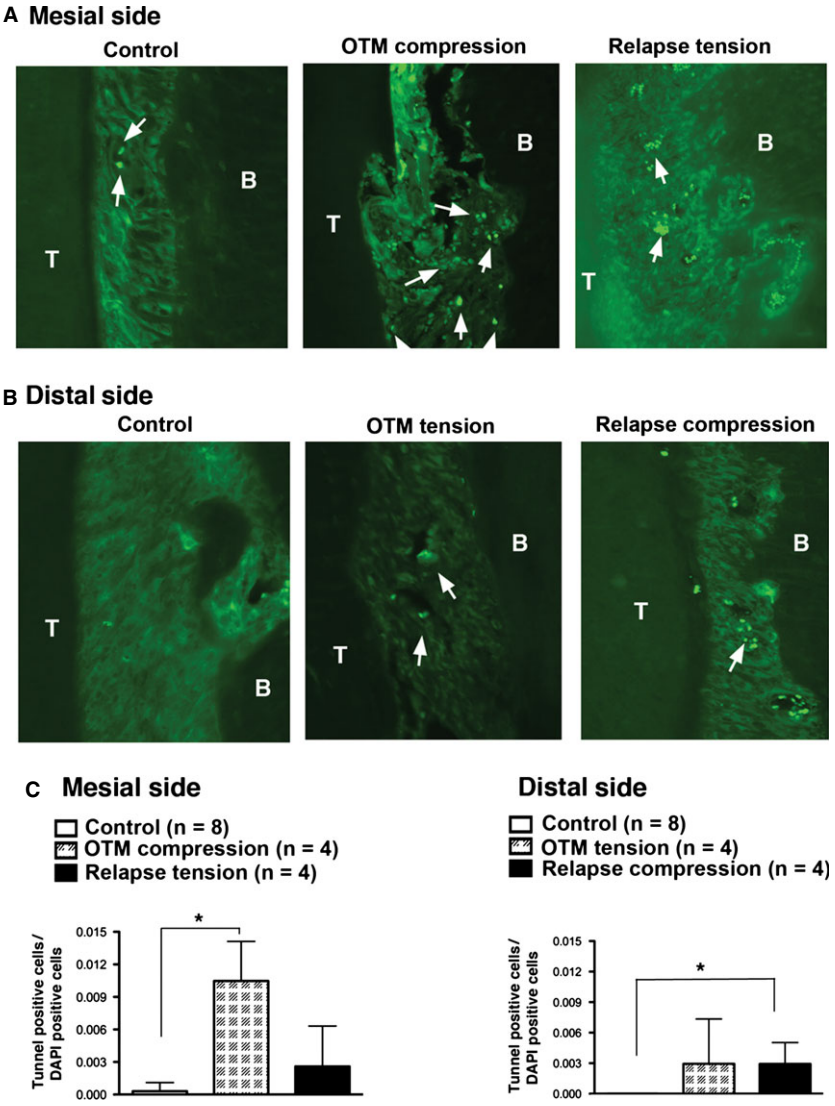

Fig. 7. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay in the periodontal ligament (PDL). (A) Histological sections of the PDL on the mesial side of the distobuccal root. Apoptotic cells are labeled green (arrows). Note the increased number of apoptotic cells in the orthodontic tooth movement (OTM) group compared with the Relapse group. (B) Distal side of the mesiobuccal root. B = bone, T = tooth. (C) Quantification of apoptotic cells in the PDL. Apoptotic cells were significantly increased on the compression side in the OTM group ($p = 0.0068$).

tension and compression sides (Fig. 6) demonstrated a significant decrease. Another recent study in rats in our laboratory also showed significant decrease in micro-CT alveolar bone parameters (BVF, tissue and apparent densities) on the tension side after 14 days of OTM (28). Considering the role of BSP in enhancing osteoblast differentiation and its expression by osteoblasts during initial bone mineralization (29, 30), it is possible that OTM causes an overall osteopenia of the alveolar bone. This could explain the decrease in osteoblast differentiation and mineralization on the tension side that subsequently decreases BSP expression.

Following removal of the spring for 4 days, both the distal side of the mesiobuccal root (relapse-compression side) and the mesial side of the distobuccal root (relapse-tension side) showed a slow recovery in the numbers of GFP-labeled cells. Orthodontic relapse was recently shown to be associated with increased bone density (BVF) in rats after administration of osteoprotegerin (OPG-Fc) (6). Increase in BVF was detected even in vehicle-treated rats as well, but to a lesser extent. Based on this, we expected to see an increase in osteoblasts during relapse as evidenced by the increase in BSP expression on both sides (Fig. 3C) and by the slow recovery of the micro-CT bone parameters (Fig. 6).

The qualitative results of TRAP staining demonstrated osteoclasts in areas under compression during OTM and relapse. There were TRAP-positive cells remaining on the relapse-tension side as it had just been under compression during the

past 10 days of OTM. The presence of osteoclasts on the relapse-compression side where there previously were none during OTM indicates a clear reversal of bone remodeling during relapse.

This study aimed to evaluate apoptotic activity during orthodontic relapse. Significant apoptosis was noted on the compression side after 14 days of OTM. This is consistent with previous studies (22, 31, 32) that demonstrated apoptosis on the compression side as a result of OTM. The lack of TUNEL staining on the tension side during OTM was also in agreement with previous studies. While there were some apoptotic cells on the relapse-compression side after 4 days of relapse, the number was not as high as seen on the compression side in OTM group. This suggests that there is less apoptosis during relapse than during OTM. Compressive forces within the PDL may be less during relapse than during OTM due to previous alveolar bone resorption or simply because the collagenous ligaments responsible for relapse may exert lower forces than orthodontic appliances. Future studies with various durations of relapse may show higher levels of apoptosis at different time points.

Taken together, the data from this study demonstrate an *in vivo* mouse model of orthodontic relapse. Better understanding of the mechanisms regulating relapse could lead to the development of new strategies and tools for its prevention in the clinic.

Conclusions

The use of an *in vivo* mouse model of orthodontic relapse allows us the investigation of the agents of relapse tooth movement. BSP-GFP-labeled osteoblasts decrease during OTM on both compression and tension sides and increase slightly during relapse. Apoptosis mostly occurs as a result of compression during OTM and to a much lesser extent during relapse-compression.

Clinical relevance

Relapse following orthodontic treatment is an undesirable outcome that, in extreme cases, necessitates retreatment. The underlying biology of the relapse of tooth movement is not clearly understood, and recent studies are attempting to bridge this knowledge gap. Improving our understanding of the role of osteoblasts and osteoclasts in this process is essential prior to the development of cell-targeted therapies for preventing relapse. The aim of this study was to develop a mouse model of orthodontic relapse tooth movement and study the early changes on both the compression and tension sides of the tooth when relapse occurs.

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