Original Article

Molecular Markers of Early Orthodontic Tooth Movement

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ABSTRACT

Objective: To understand the molecular basis of early orthodontic tooth movement by looking at the expression of KI-67, runt-related transcription factor 2 (Runx2), and tumor necrosis factor ligand superfamily member 11 (RANKL) proteins.

Materials and Methods: We employed a rat model of early orthodontic tooth movement using a split-mouth design (where contralateral side serves as a control) and performed immunohistochemical staining to map the spatial expression patterns of three proteins at 3 and 24 hours after appliance insertion.

Results: We observed increased expression of KI-67, a proliferation marker, and RANKL, a molecule associated with osteoclastic differentiation, in the compression sites of the periodontal ligament subjected to 3 hours of force. In contrast, there was increased expression of KI-67 and Runx2, a marker of osteoblast precursors, in tension areas after 24 hours of force. Decreased KI-67 expression in the mesial and distal regions of the periodontal ligament was observed at the midpoint of the tooth root.

Conclusions: The early RANKL expression indicates that at this early stage cells are involved in osteoclast precursor signaling. Also, decreased KI-67 expression found near the midpoint of the tooth root is believed to represent the center of rotation, providing a molecular means of visualizing mechanical loading patterns. (*Angle Orthod.* 2009;79:1108–1113.)

KEY WORDS: Orthodontic tooth movement; Periodontal ligament; Precursor cells; Mice; Proliferation

INTRODUCTION

Orthodontic tooth movement (OTM) occurs upon the application of a controlled mechanical force and results in biologic reactions that model and remodel the surrounding dental and periodontal tissues.[1] During the first phase of OTM there is displacement of the tooth in the periodontal space followed by the lag phase, when movement ceases and resorption of the necrotic tissue formed during the initial phase is required.[2] While the later stages of tooth movement through bone are better understood, the cellular and molecular processes occurring in the early stages of OTM, the initial, and lag phases are relatively uncharacterized.

During OTM, movement of the tooth within the periodontal space causes stretch and compression of collagen fibers and changes in cellular activity.[1,3] Tooth movement only occurs when the areas of hyalinized (cell-free) tissue in the compression side, created due to a combination of inflammatory cytokines and a disruption in the blood flow[1,4] are removed.[1,2,4] Removal of this tissue and adjacent alveolar bone is achieved by circulating macrophages and bone marrow osteoclastic precursors that are recruited to the periodontal ligament (PDL).[1,4] Stresses generated on the tension side result in the differentiation of mesenchymal cells into osteoblasts.[1,5] Communication occurs between the osteoblast and osteoclast populations, eg, osteoblasts and their precursors aid in the differentiation of

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osteoclasts through expression of tumor necrosis factor ligand superfamily member 11, or receptor activator of nuclear factor kappa β ligand (RANKL), a transmembrane or soluble protein also found on fibroblasts, stromal cells, and T-cells.[6–8] Interaction between receptor activator of nuclear factor kappa β (RANK) on the preosteoclasts and RANKL promotes maturation and fusion of the preosteoclasts into activated osteoclasts.[6,9,10] RANKL and runt-related transcription factor 2 (Runx2), a transcription factor of osteoblast precursors, have been detected as early as 24 hours; however, as yet the spatial expression patterns of these proteins and cell proliferation have not been examined in detail or at earlier time points.[11–15] Our hypothesis is that mechanical loading on a tooth will activate proliferation of PDL cells and specific gene products within the PDL in the early phases of OTM.

MATERIALS AND METHODS

Rat Model of OTM

Ten grams, or 10 cN, of orthodontic force were applied in the mesial direction to the right maxillary first molar of male Sprague-Dawley rats in a split-mouth design, as described previously.[16] This force value was chosen because it was the smallest amount of force that could be accurately created in rats. In this way the model relates well to clinical applications where human teeth are much larger than those of rats. Twenty-two animals from the same litter were divided randomly into two test groups of 3 ($n = 10$) and 24 ($n = 12$) hours of force duration. The experimental design was approved by the Animal Ethics Committee of the University of Toronto.

Histological and Immunohistochemical Procedures

Maxillae were dissected, formalin fixed, and decalcified in EDTA. Samples were dehydrated and paraffin embedded, and 6-μm thick horizontal sections were prepared. Tissue sections less than 180 μm from the furcation of the disto-palatal root of the right and left first maxillary molars were stained for RANKL and Runx2, using nine sections from each animal. Also, every third slide containing sections obtained from the furcation of the first maxillary molar disto-palatal roots down to the apex of the root were analyzed for KI-67 expression in order to look at expression along the entire tooth root. Sections were dewaxed, blocked for endogenous hydrogen peroxidase, subjected to antigen retrieval, incubated with serum (Sigma, Oakville, ON), and incubated with polyclonal goat anti-RANKL antibody (1:200, Vector Laboratories, Burlington, ON), polyclonal goat anti-Runx2 antibody (1:50, Vector Laboratories), or polyclonal rabbit anti-KI67 antibody (1:1000, Vector Laboratories). Biotinylated secondary antibodies against the primary antibodies (Vector Laboratories) were used, followed by avidin-biotin complex (Vectastain Universal Elite ABC kit, Vector Laboratories), diaminobenzidine (DAB, Vector Laboratories), and eosin. Negative controls were conducted in the absence of the primary antibody. To determine the specificity of the RANKL antibody, it was incubated with soluble recombinant mouse RANKL before application to the tissue sample for immunohistochemical detection. Several slides stained for RANKL were counterstained with DAPI to visualize the RANKL signal location. The presence of osteoclasts was determined with TRAP staining, as described previously[17] using one slide per animal containing sections close to the furcation of the disto-palatal root of the first maxillary molar.

Quantification and Analysis

Images were taken with an Infinity 2 color camera attached to an Olympus IX71 microscope at 60× magnification. Quantification was achieved using Image Pro software (Image Pro Plus 2.0). Areas of interest (AOIs) were defined as regions containing the mesial and distal portions of the PDL surrounding the disto-palatal root of the right and left first maxillary molars. All AOIs were of equal size, 208 μm by 52 μm, and positive cells were counted in the total area of these four AOIs. For AOIs that contained hyalinized tissues, the number of positive cells in the total AOI area was extrapolated from the density of positive cells in the nonhyalinized tissue area. The two AOIs on the experimental side were compared to their control AOIs with paired *t*-test (SPSS, version 15.0). The number of positive cells in the tension and compression AOIs were normalized to the number of positive cells in the contralateral controls by subtraction. To test if the protein expressions in the tension and compression AOIs were different from one another and if the duration of force (3 and 24 hours) affected the expression patterns, a Kolmogorov-Smirnov test was performed to show that the data were not normalized ($P > .05$) and was followed by a Mann-Whitney nonparametric test (SPSS, version 15.0). Sections along the length of the disto-palatal root slides spanning 70 μm at the middle, apex, and furcation were grouped together and compared to one another using one-way analysis of variance (ANOVA). Results were significant if $P < .05$.

RESULTS

RANKL, Runx2, TRAP, and KI-67 Expression Near the Furcation of the Disto-Palatal Root

Histological analysis of the tissues surrounding the orthodontically moved molar showed areas of taut col-

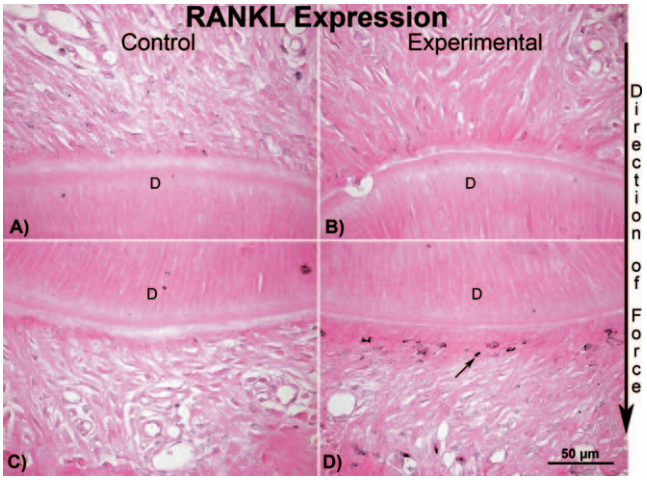

Figure 1. RANKL expression (black) in the distal (A and B) and mesial (C and D) areas of the periodontal ligament (PDL) after 3 hours of orthodontic force in the mesial direction. Arrow indicates RANKL-positive cell. * D indicates dentin.

lagen fibers and disorganized collagenous tissue in the PDL of the distal and mesial regions, respectively, as seen in Figures 1 through 3. Also, in the compression side of the PDL after 24 hours of orthodontic force, cell-free regions were present.

Immunohistochemical analysis of RANKL, an osteoclastic differentiation factor, showed RANKL was expressed by cells in the mesial side (compression) of the PDL surrounding the disto-palatal root of the maxillary first molar, after 3 hours of force (Figure 1). Specificity of RANKL antibody staining in these sections was confirmed by incubation of the RANKL antibody with soluble RANKL prior to the immunohistochemical procedures. RANKL, as expected of a soluble or transmembrane protein, was expressed in the cytoplasm and not in the nucleus when counterstained with DAPI (data not shown). The RANKL-positive cells were found within 30 µm of the tooth root, and were small

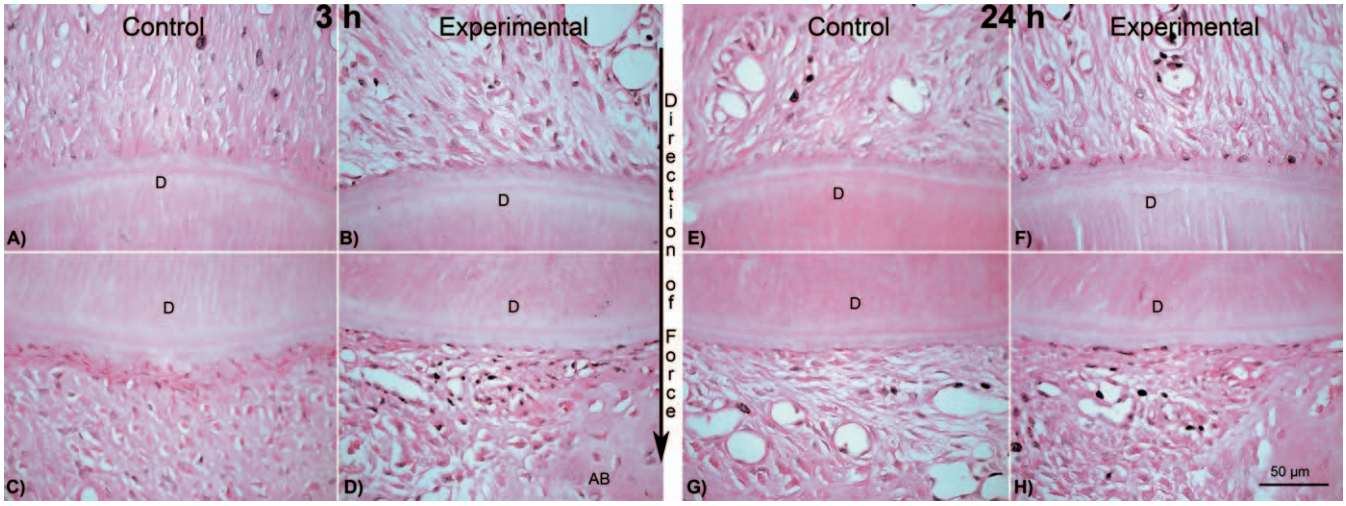

Figure 2. KI-67 expression (black) in the distal (A, B, E, and F) and mesial (C, D, G, and H) areas of periodontal ligament (PDL) of the disto-palatal root after 3 (B and D) and 24 hours (F and H) of orthodontic force in the mesial direction. * D indicates dentin; AB, alveolar bone.

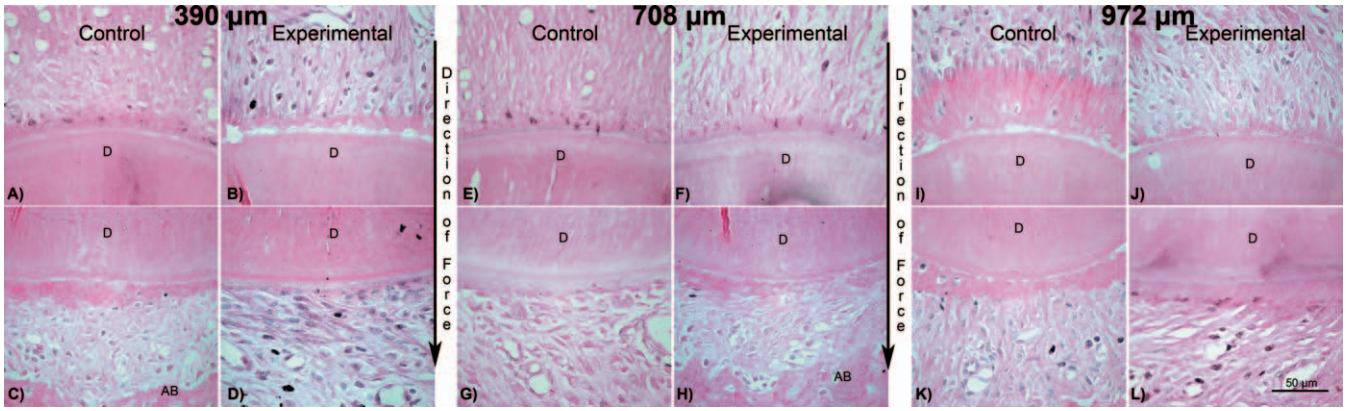

Figure 3. KI-67 expression (black) in the distal (A, B, E, F, I, and J) and mesial (C, D, G, H, K, and L) areas of periodontal ligament (PDL) of the disto-palatal root 390 (A–D), 708 (E–H), and 972 (I–L) µm apical from the tooth root furcation after 3 hours of orthodontic force in the mesial direction. * D indicates dentin; AB, alveolar bone.

Table 1. Number of KI-67–Positive Cells (Given in Cells/AOI_{area}) in Each Region of the PDL[a] Down the Length of the Tooth Root at 3 and 24 Hours is Presented as Mean ± Standard Deviation, $P < .05$ with Mann-Whitney Nonparametric Test of Compression Compared to Tension Region, n = 3

Apical Distance, μm	3 Hours of Force			24 Hours of Force		
	Distal PDL	Mesial PDL	*P*	Distal PDL	Mesial PDL	*P*
360	14.0 ± 3.0	21.0 ± 0.0	.019	19.0 ± 1.0	13.0 ± 3.5	.045
680*	4.3 ± 2.5	5.0 ± 1.0	.690	3.7 ± 1.2	5.0 ± 2.0	.374
1000	18.7 ± 3.1	13.0 ± 1.0	.038	13.7 ± 2.2	23.7 ± 2.1	.016

[a] PDL indicates periodontal ligament.
* $P < .05$.

and circular in shape. The mean ± standard deviation of positive cells on the mesial side was found to be 7.8 ± 2.2 cells/AOI_{area} and 1.0 ± 0.6 cells/AOI_{area} on the distal side. After 24 hours of force, RANKL was no longer detected. Runx2, a marker of osteoblast precursors, was present in three of eight animals examined in only the distal PDL after 24 hours, but not after 3 hours (data not shown).

The presence of osteoclasts was determined through TRAP staining, where TRAP-positive cells were found only on the distal side of the experimental and control PDL at both time points (data not shown).

KI-67, a proliferation marker, was found in sections taken within 180 μm apical to the furcation of the disto-palatal root of the moved first maxillary molar (Figure 2). Those cells that were KI-67–positive were located throughout the PDL and comprised many different cell types based upon cell shape. Quantification of the KI-67–positive cells on the mesial and distal sides of the PDL showed that there were approximately twice the number of KI-67–positive cells in the compression area after 3 hours of orthodontic force (26.7 ± 0.6 cells/AOI_{area}) when compared with the tension area (13.3 ± 0.6 cells/AOI_{area}). After 24 hours of force, about twice as many cells were KI-67–positive in the tension region (23.0 ± 2.7 cells/AOI_{area}) compared with the compression region (12.3 ± 3.2 cells/AOI_{area}). Two-way ANOVA with interaction revealed the tension and compression AOIs were statistically different, $P < .05$.

KI-67 Expression Along the Length of the Disto-Palatal Root

The expression of KI-67 by cells in the PDL was also determined along the coronal-apical length of the disto-palatal root of the orthodontically moved molar, a distance of roughly 1400 μm. It was found that fewer cells were KI-67–positive at approximately 700 μm from the root furcation compared to those tissue sections above and below this point (Figure 3). The KI-67 expression patterns that were seen less than 180 μm from the furcation of the root were also seen along the rest of the root. Towards the apex of the tooth root after the point where a negligible number of KI-67–positive cells were present, the expression patterns were reversed when comparing the mesial and distal regions of the PDL, where the pattern expected in the mesial region was observed in the distal region. It was found that there were approximately three times fewer KI-67–positive cells above and below this point both in the distal and mesial PDL at both time points (Table 1).

DISCUSSION

The Rat OTM Model Accurately Replicates the Cellular and Molecular Changes Observed During Early Phases of OTM

Histological analysis of the tissues surrounding the first molar displayed features of tissues that have been subjected to orthodontic force, confirming that OTM has been established, as described previously by other groups.[18,19]

The expression of RANKL, a ligand associated with osteoclastic differentiation, by PDL cells indicates that the orthodontic force is being transduced to the PDL. The expression of this ligand is only observed in the mesial areas of the PDL surrounding the tooth root, areas under compressive forces. Based on cell shape, we believe that the RANKL-positive cells (Figure 2D) most likely represent preosteoblastic cells which have been shown to express RANKL and have a round morphology in other studies.[20,21] The RANKL-positive cells in our samples displayed a round morphology, suggesting that they are not fibroblasts, which appear spindle-like, nor are they osteoblasts, which assume a cuboidal shape.[22,23] Other groups have noted the in vivo expression of RANKL by PDL cells through immunohistochemistry and RT-PCR in the compression side after one day of orthodontic force, but did not look at earlier time points.[5,14] In our model, we see RANKL expression after only 3 hours of force. After 24 hours, however, the ligand is no longer expressed, likely due to interaction with RANK or release of the ligand into

the extracellular matrix. The difference in temporal expression of RANKL in our study vs those of others could be due to the light orthodontic force we applied (10 grams as opposed to 20–60 grams).[24] It is possible that a lighter orthodontic force is conducive, allowing blood to flow within the PDL enabling cell survival and RANKL expression.

Osteocytes, mechanosensory cells in bone, are considered to be highly involved in mechanical loading and unloading, producing nitric oxide as a response.[25,26] Previously, we have looked at the differential expression of nitric oxide synthases in rat maxilla during early OTM and have found that it is not osteocytes that are involved with early nitric oxide signaling, but cells of the PDL.[16] These results agree with our findings that the cells involved in early signaling are not osteocytes, but cells within the PDL.

The presence of Runx2, a marker expressed by osteoblast precursors, in some animals (three of eight examined) after 24 hours of force in the tension region of the PDL indicates that mesenchymal cells are undergoing differentiation. Previously, other studies have noted the expression of Runx2 at or before 24 hours of force application, confirming that we have a model depicting early OTM cellular processes.[13,15] The inconsistency of Runx2 expression is most likely due to the inconsistent magnitudes of force applied by the orthodontic springs. Although we do not see expression of Runx2 by those cells that were RANKL-positive, others have shown that as preosteoblastic cells mature, expression of RANKL decreases and Runx2 expression increases.[20,27]

Prediction of Axis of Rotation of Orthodontically Moved Tooth

Cell proliferation is a hallmark of a changing extracellular environment, which is produced during OTM.[28] The fewer KI-67–positive cells in the compression region observed after 24 hours of force application corroborated the work of others that showed fewer cells present during the lag phase in the compression region.[1,4] However, as yet to be described by other groups, cell proliferation was greater after 3 hours of orthodontic force in the compression region.

The lack of cell proliferation observed at approximately 700 μm apically from the root furcation of the orthodontically moved tooth suggests that little or no force is being placed on the tissues in this area. We speculate that this area represents the axis of rotation (AOR) of the tooth, defined as the line in a tooth about which it rotates in a nontranslational displacement.[29] The absence of cell proliferation could also indicate areas of excessive force; however, we do not think that this is the case because areas of compression were not observed. The AOR was about 700 μm apical from the furcation of the root, near the midpoint of the root. It is noted that, apical to the AOR, we saw a reversal in the expected pattern of KI-67 expression of cells in the mesial and distal regions of the PDL when compared to the PDL coronal to the AOR. This pattern of KI-67–positive cells confirms that the tooth has not moved in a translational direction, but has tipped within the periodontal space. Therefore, analysis of KI-67 expression in the PDL of teeth subjected to an orthodontic force may provide potential in studies to correlate force with gene/protein expression along the axis of a tooth that has undergone OTM.

CONCLUSIONS

- After only 3 hours of orthodontic force, cells of the PDL are responding to the mechanical forces and are involved in osteoclast precursor signaling.
- Mechanical force distribution in the PDL due to orthodontic treatment can be determined through KI-67 protein expression in periodontal cells.

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