Original Article

Expression of RANKL mRNA during Root Resorption Induced by Orthodontic Tooth Movement in Rats

Massoud Seifi, DDS, MSD*, Maryam Jessri, DDS

Orthodontic Department, Dental School, Shahid Beheshti University of Medical Sciences, Tehran, Iran

** Corresponding Address: Orthodontic Department, Shahid Beheshti University of
Medical Sciences, Tehran, Iran
Email: mseifi@dent.sbmu.ac.ir*

Abstract

Objective: Receptor activator for nuclear factor kappa B ligand (RANKL), which is also called osteoclast differentiation factor, is an important regulatory factor in osteoclast maturation. Knowledge of bone and cementum similarities and RANKL role in bone resorption suggests the possibility of a role for this protein in root resorption induced by orthodontic tooth movement. The aim of this study is to examine the expression of RANKL mRNA during root resorption induced by orthodontic tooth movement in rats.

Materials and Methods: In order to move maxillary right first molars mesially fixed Ni-Ti closed coil springs (Dentaurum®-Germany) were tightened to the teeth. Sample consisted of 20 male seven week old Wistar rats. For each animal, the contralateral tooth was used as, an internal control. At day 21 the rats were sacrificed. Tissues from 10 rats were embedded in paraffin for histologic examination. Scratched material from resorptive lacunae on mesial sides of the roots of the other ten rats was used for extracting mRNA by RT-PCR.

Results: The histologic sections, analyzed histomorphometrically, showed a significant increase in root resorption in the case group as compared to the control (p<0.001). Densitometric studies of RANKL mRNA expression band on gel electrophoresis showed significantly increased RANKL expression in the resorptive lacunae of the case group (p<0.001).

Conclusion: This observation indicates increased RANKL expression is associated with orthodontic tooth movement induced root resorption.

Keywords: RANKL, Root Resorption, Tooth Movement

Introduction

Despite extensive research, the mechanism of root resorption remains unclear. It is believed to be a multifactorial phenomenon. It has been shown that orthodontic tooth movement induces root resorption (1). Force magnitude (2-6), age (7), treatment duration (8), minerals (9), metabolites (10, 11) and genetic factors (12) have been shown to influence root resorption.

Application of orthodontic forces makes a significant change in the periodontal ligament (PDL) space on the pressure side (13). Bone remodeling and subsequent tooth movement are considered secondary to this change. Osteoclasts play a significant role in bone resorption (14) and their participation in bone resorption during orthodontic tooth movement is of clinical significance.

Physiologic and genetic studies of bone marrow cell kinetics have provided insight into the mechanism of osteoclast differentiation from hematopoietic precursors (15-17). Receptor activator for nuclear factor kappa B ligand (RANKL) binding with RANK receptor on preosteoclast is an essential step in its differentiation and subsequent bone resorption (18, 19). RANKL is a member of the TNF-receptor family and mediates signals leading to osteoclastogenesis (20).

PDL cells increase RANKL expression during intermittent mechanical forces (21). RANKL has been detected in crevicular fluid during orthodontic tooth movement. It has been suggested that RANKL is involved in bone resorption in response to compression force (22). In an *in situ* study in rats, RANKL and RANK have been shown to increase in pathologic conditions (23). Yamaguchi et al. hypothesized that in patients who experience severe external root resorption, because of orthodontic tooth movement, compressive forces dur-

ing orthodontic tooth movement may be responsible for the RANKL formation and up-regulation of osteoclastogenesis (24). RANKL over expression is higher during root resorption induced by heavy orthodontic forces on rat molars (25).

Although there have been a number of studies on the association of RANKL with bone and root resorption, little is known about orthodontic tooth movement induced root resorption and its relation to the expression of RANKL mRNA. The aim of the present study is to elucidate the involvement of RANKL in root resorption associated with orthodontic tooth movement.

Materials and Methods

Twenty, 7-week-old male Wistar rats with a body weight of 230 to 250 gr were obtained from the Pharmacology Department of Shahid Beheshti Medical Science University for this study (Tehran, Iran).

Manipulation and treatment of the animals was performed according to the approved protocol of the Institutional Animal Care and Usage Committee and the approval of the Ethical Committee of the Shahid Beheshti University of Medical Sciences Dental School. To minimize the risk of trauma or discomfort to the rats and displacement of the appliances, the animals were fed with ground laboratory chow and drank water ad libitum. The animals were weighed before and after treatment to assess the whole body effects of the intervention.

All 20 rats received orthodontic therapy and were randomly divided into two groups (each containing 10 animals). The specimens of the first group were used to extract RANKL via RT-PCR and the second group specimens were apportioned for histological studies. In order to elucidate the influence of intervention, we considered the maxillary left teeth as an internal control group for the right teeth.

Orthodontic treatment

On the first day of the experiment, the animals were anesthetized with 50 mg/kg ketamine hydrochloride (Ketamine Hydrochloride, Gedeon Richter Ltd., Budapest) and local anesthesia with 6 mg/kg xylazine HCL injection (xylazine hydrochloride, Rompun, Bayer Leverkusen).

Modified King and Fischlschweiger method (26) was used to apply mesial traction force to the maxillary right first molars. Phosphoric acid gel 37.4% (Dentaurum® Group, Ispringen, Germany) was applied for 30 seconds to etch the mesial, palatal and buccal surfaces of the maxil-

lary right first molars. The teeth were then wiped with a disposable brush (Dentaurum® Group, Ispringen, Germany) soaked in distilled water and dried with an air syringe. A closed Ni-Ti coil spring was used to drift the first molar mesially. A force measuring gauge (Dentaurum® Group, Ispringen, Germany), was used to assure each spring delivered 60 gf. The springs were tightened to the cervical third of the first maxillary right molars and then fixed anteriorly by using ligature-wire loops (Dentaurum® Steel ligature wire 0.010 inch, Dentaurum® Group, Ispringen, Germany) around the cervical third of the maxillary incisors. Anatomic position and structural development of both upper incisors suggested these teeth were appropriate anchorage units for protraction of the first molars. In order to prevent mucosal trauma, the ligature wire ends were covered and secured with composite resin (Self cure Degufill, Degussa AG, Frankfurt, Germany).

On the 21st day of the experiment, the rats were sacrificed with carbon dioxide asphyxiation and the maxilla was immediately removed. Orthodontic tooth movement was measured using a filler gauge (Mituto Co., Japan) to reveal the distance between the first and second molars. This distance was initially zero. The measurements were done by a single operator and checked by an assistant. In the case of report inconsistency, the first operator was asked to repeat the measurement and report the average of the two measurements.

RT-PCR examinations

To isolate high quality ribonucleic acid (RNA), we used a modification of the method described by Low et al. (25). The first molars of 10 rats were separated from the surrounding bone with a dissecting microscope, high speed fine diamond discs and burs. In order to locate the resorptive lacunae on mesial surfaces of the case group, the teeth were studied by the stereomicroscope. The resorptive lacunae on the mesial surfaces of the case group roots and the whole mesial root surfaces of the control teeth were scratched with a fine diamond bur. The obtained material was then snap-frozen in liquid nitrogen and stored at -70ºC for later extraction of mRNA.

Under liquid nitrogen, and by using a mortar and pestle, the tissues were homogenized. After evaporation of the suspension in disposable petri dishes, 1mlit/gr tissue of RNXPLUS buffer was added to the suspension and incubated for 5 minutes at room temperature. Tissue was further homogenized using an ultrasonic homogenizer (Biologics Inc.,Virginia, USA) for 15 minutes.

Table 1: Primers used in RT-PCR for RANKL and GAPDH

Gene sequence mRNA	Nucleotide sequence 5' to 3'	Accession no.	Product size
Rat GAPDH	ACCACAGTCCATGCCATCACT CCACCACCACCCTTGCTGTACCA	AB017801	454
Rat RANKL	ACGCACATTGCAGGACTCGAC TTCGTGCTCCCTCCTTTCATC	AF019048	493

200 ml chloroform was added to the mixture. It centrifuged for 15 minutes in 4°C for 12000 rpm. The upper phase was transferred to an Eppendorf microtube containing 1.5 mg/L tissue isopropyl alcohol was added. After incubation for 10 minutes and washing with 70% ethanol, the deposit was centrifuged for 2 minutes at 12000 g.

By using reverse transcriptase (Super-Script II; Bibco, Wickliffe, Ohio) and 2 μlit of hexamer primers, cDNA was synthesized at 42°C for 1 hour. The resulting cDNA was amplified in a thermocycler (Bioer Technology Co. Ltd., Hangzhou, China) by taq DNA polymerase (Amplitaq® DNA polymerase; Applied Biosystems, California, USA), PCR buffer, specific primers for RANKL and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma Aldrich, India) (Table 1), Mg-Cl$_2$ (Perkin Elmer/Cetus) and for 31 cycles for GAPDH and 40 cycles in the case of RANKL; all at 94°C for denaturation, 55°C for annealing and 72°C for extension.

PCR products were then resolved by electrophoresis on a 2% wt/vol agarose gel, and stained with ethidium bromide. The images were captured using a UV transilluminator. For loading control, the amplified products were represented as a ratio of the respective PCR product relative to GAPDH PCR product.

Histological examinations

Maxillas of the rats from the second group were fixed in 10% neutral buffered formalin. The first molar and surrounding bone (in both the case and control groups) was block dissected from the maxilla and decalcified in 10% EDTA for 7 days. Prior to being dehydrated with increasing gradients of alcohol and methylsalicylate, the specimens were rinsed in 0.01 M phosphate buffered saline (pH=7.4). The dehydrated tissues were then embedded in paraffin (melting point = 58-60°C). Sagittal serial sections, with 5μm thickness, were prepared and stained with hematoxylin and eosine (H&E). Sections were analyzed histomorphometrically to evaluate and compare the amount of root resorption.

Results

The animals had a 15% weight gain during the experiment; which shows that the orthodontic appliances did not interfere with their nourishment. Statistical analysis of tooth movement shows a significant change in the case group (Table 2).

Table 2: Orthodontic tooth movement in study groups (mm)

Study Group	Mean Orthodontic Tooth Movement
Control	0.242 ± 0.010 mm
Case	0.831 ± 0.028 mm

Resorptive lacunae and multinucleated giant cells were observed in histologic examinations on the mesial surfaces of the case group teeth. Whereas in the control teeth sections, which were not subjected to orthodontic forces, multinucleated giant cells and resorptive lacunae were absent. Data confirms that the model was able to induce orthodontic tooth movement and root resorption.

We used the method described by Parfitt et al. (27) to analyze specimens histomorphometrically. In this analysis the reports are based on the distance between two reference points. The external points of the resorptive lacunae were chosen as our reference points. The line joining the reference points and the internal wall of the resorptive lacunae inscribe a space we considered it to be resorptive lacunae. According to histomorphometric analysis of the tissue sections, the mean root resorption area (square millimeter) was measured at 4.9870×10^{-11} in the case group (Fig 1) and 1.6547×10^{-11} in the control group. The standard deviation was 5.3451×10^{-11} for the former and 1.5326×10^{-11} for the latter. The t-test showed a statistically significant difference ($p < 0.001$).
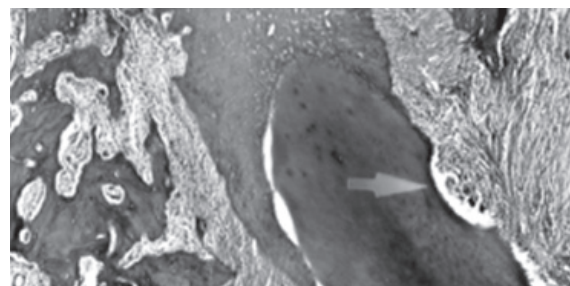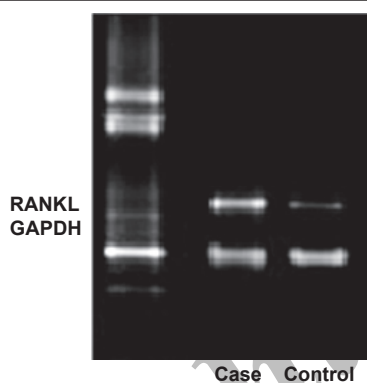

Fig 1: Resorptive lacuna in case group following orthodontic tooth movement.

RANKL mRNA band was detected in rats of the case and control groups according to Table 3. Wilcoxon signed-rank test showed a significant difference (Fig 2) (p<0.001).

Table 3: RANKL / GADPH expression index in control and case groups

#	RANKL - Case	RANKL- Control	GADPH- Case	GADPH- Control
1	2	0	2	2
2	3	0	2	3
3	3	0	1	3
4	3	0	2	2
5	3	2	3	3
6	3	0	2	2
7	3	0	2	2
8	3	3	2	2
9	3	0	2	3
10	3	0	3	2

RANKL
GAPDH

Case   Control

Fig 2: The product was electrophoresed on agarose gel and visualized using ethidium bromide under UV Transilluminator.

Discussion

Root resorption has been extensively studied but the precise mechanism of root resorption remains unknown. Factors such as mineral (9) and bone metabolites (10, 11), the inflammatory nature (14, 28) of the root resorption; and genetic control of the participating cells (12) are considered to be some of the mechanisms involved in this process.

This is the first study that has shown a relationship between RANKL and root resorption due to orthodontic tooth movement.

To eliminate occlusal trauma, decrease manipulation and surgical intervention of animals in the present study; the methods used by Ashizawa and Sahara (29) and Kameyama et al. (30) were combined and modified to make a new approach to induce orthodontic tooth movement.

Multinucleated giant cells were observed in the histologic sections. These cells were present in the PDL space and in association with resorptive lacunae. This finding was consistent with the reports of Hasegawa et al. (31), who detected these cells in connective tissue of resorptive lacunae, and Kohno et al. (32) who reported multinucleated cells in the PDL space.

Histomorphometric analysis confirmed this model as a suitable method to induce root resorption due to orthodontic tooth movement. This is consistent with the observations of Seifi et al. (33) and Low et al. (25) and in contrast with Ashizawa et al. (29) findings.

Though Low et al. (25) has arranged and model to induce orthodontically, tooth movement and root resorption. The amount of induced orthodontic tooth movement and root resorption have not been revealed in the manuscript. As previously shown by King and Fischlschweiger (26), delivering 100gf by the springs to induce orthodontic tooth movement is more efficient in inducing resorption than tooth movement. Low has based the reports on the application of heavy orthodontic force, while in the present study, we have applied light orthodontic force.

Ashizawa and Sahara (29) have not reported the amount of orthodontic tooth movement; instead the volumes of newly formed bone on the alveolar wall surrounding the distolingual root surface of the maxillary right first molars were reported. In his study there was no significant difference between the experimental groups. The use of a square metal plate, cemented on the occlusal surface of the maxillary first molar in Ashizawa (29) method, could be a source of periodontal trauma and also interfere with the animals' nourishment. Also, the duration of our experiment is longer than Ashizawa's study (29); these could explain the inconsistency between results in the two experiments.

Fukushima et al. investigated expression of RANKL/RANK in physiological root resorption. They found RANKL was expressed significantly higher in PDL cells derived from resorbing deciduous teeth (34).

Nilforoushan et al. (35) has conducted a study to investigate the role of opioid systems on orthodontic tooth movement in cholestatic rats. The amount of the tooth movement reported in Nilforoushan's (35) study was almost half when compared to ours. The difference could be explained by the difference in method used for measurement of the tooth movement and a different experiment duration; 21 days in our study and 15 days in Nilforoushan's (35).

A common phenomenon or side effect during

orthodontic treatment is a varying degree of root resorption and this process; if it coincides with a gene that expresses itself during bone resorption is another similarity between bone and root structure and an emphasis on their isomorphism.

Conclusion
The study results show that RANKL mRNA has increased significantly in resorptive lacunae during root resorption induced by orthodontic tooth movement. This finding suggests that this glycoprotein may play a pivotal role in root resorption. In this study PCR studies suggest a role for RANKL in root resorption induced by orthodontic tooth movement in rats. This study has been conducted in an animal model and in a controlled laboratory environment. It could not fully reflect the situation in human beings. Considering the important role of RANKL in bone and root resorption and the importance of root resorption as an unintended consequence of orthodontic treatment, further studies in humans are needed to explore a similar correlation.

Acknowledgments

References
1. Abuabara A. Biomechanical aspects of external root resorption in orthodontic therapy. Med Oral Patol Oral Cir Bucal. 2007; 12(8): 610-613.
2. Gonzales C, Hotokezaka H, Yoshimatsu M, Yozgatian JH, Darendeliler MA, Yoshida N. Force magnitude and duration effects on amount of tooth movement and root resorption in the rat molar. Angle Orthod. 2008; 78(3):502-509.
3. Pizzo G, Licata ME, Guiglia R, Giuliana G. Root resorption and orthodontic treatment. Minerva Stomatol. 2007; 56(1-2): 31-44.
4. Weiland F. External root resorptions and orthodontic forces: correlations and clinical consequences. Prog Orthod. 2006; 7(2):156-163.
5. Harris DA, Jones AS, Darendeliler MA. Physical properties of root cementum: part 8. Volumetric analysis of root resorption craters after application of controlled intrusive light and heavy orthodontic forces: a microcomputed tomography scan study. Am J Orthod Dentofacial Orthop. 2006; 130(5): 639-647.
6. Al-Qawasmi RA, Hartsfield JK Jr, Everett ET, Weaver MR, Foroud TM, Faust DM, et al. Root resorption associated with orthodontic force in inbred mice: genetic contributions. Eur J Orthod. 2006; 28(1): 13-19.
7. Owman-Moll P. Orthodontic tooth movement and root resorption with special reference to force magnitude and duration. A clinical and histological investigation in adolescents. Swed Dent J Suppl. 1995; 105: 1-45.
8. Taithongchai R, Sookkorn K, Killiany DM. Facial and dentoalveolar structure and the prediction of apical root shortening. Am J Orthod Dentofacial Orthop. 1996; 110(3): 296-302.
9. Seifi M, Eslami B, Saffar AS. The effect of prostaglandin E2 and calcium gluconate on orthodontic tooth movement and root resorption in rats. Eur J Orthod. 2003; 25(2).199-204.
10. Oguchi H. In vitro studies of bone resorption by the root-resorbing tissue from the bovine deciduous tooth. Bull Tokyo Med Dent Univ. 1975; 22(2).175-183.
11. Vázquez-Landaverde LA, Rojas-Huidobro R, Alonso Gallegos-Corona M, Aceves C. Periodontal 5'-deiodination on forced-induced root resorption the protective effect of thyroid hormone administration. Eur J Orthod. 2002; 24(4): 363-369.
12. Abass SK, Hartsfield JK Jr, Al-Qawasmi RA, Everett ET, Foroud TM, Roberts WE. Inheritance of susceptibility to root resorption associated with orthodontic force in mice. Am J Orthod Dentofacial Orthop. 2008; 134(6): 742-750.
13. Kohno T, Matsumoto Y, Kanno Z, Warita H, Soma K. Experimental tooth movement under light orthodontic forces: rates of tooth movement and changes of the periodontium. J Orthod. 2002; 29(2): 129-135.
14. Kominsky SL, Abdelmagid SM, Doucet M, Brady K, Weber KL. Macrophage inflammatory protein-1 delta: a novel osteoclast stimulating factor secreted by renal cell carcinoma bone metastasis. Cancer Res. 2008; 68(5): 1261-1266.
15. Thomas GP, Baker SU, Eisman JA, Gardiner EM. Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. J Endocrinol. 2001; 170(2): 451-460.
16. Teitelbaum SL. Osteoclasts and integrins. Ann N Y Acad Sci. 2006; 1068: 95-99.
17. Liao TS, Yurgelun MB, Chang SS, Zhang HZ, Murakami K, Blaine TA, et al. Recruitment of osteoclast precursors by stromal cell derived factor-1 (SDF-1) in giant cell tumor of bone. J Orthop Res. 2005; 23(1): 203-209.
18. Bilezikian JP, Raisz LG, Rodan GA. Principles of bone biology.2nd ed. UK: Academic Press; 2002; 7: 109-126.
19. Chikazu D, Katagiri M, Ogasawara T, Ogata N, Shimoaka T, Takato T, Nakamura K, Kawaguchi H. Regulation of osteoclast differentiation by fibroblast growth factor 2: stimulation of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor expression in osteoblasts and inhibition of macrophage colony-stimulating factor function in osteoclast precursors. J Bone Miner Res. 2001; 16(11): 2074-2081.
20. Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, et al. RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. Biochem Biophys Res Commun. 1998; 18; 253(2): 395-400.
21. Nakao K, Goto T, Gunjigake KK, Konoo T, Kobayashi S, Yamaguchi K. Intermittent force induces high RANKL expression in human periodontal ligament cells. J Dent Res. 2007; 86(7): 623-628.
22. Nishijima Y, Yamaguchi M, Kojima T, Aihara N, Nakajima R, Kasai K. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement

and effect of compression force on releases from peri-odontal ligament cells in vitro. Orthod Craniofac Res. 2006; 9(2): 63-70.

23. Ogasawara T, Yoshimine Y, Kiyoshima T, Kobayashi I, Matsuo K, Akamine A, et al. In situ expression of RAN-KL, RANK, osteoprotegerin and cytokines in osteoclasts of rat periodontal tissue. J Periodontal Res. 2004; 39(1): 42-49.

24. Yamaguchi M, Aihara N, Kojima T, Kasai K. RANKL increase in compressed periodontal ligament cells from root resorption. J Dent Res. 2006; 85(8): 751-756.

25. Low E, Zoellner H, Kharbanda OP, Darendeliler MA. Expression of mRNA for osteoprotegerin and receptor activator of nuclear factor kappa beta ligand (RANKL) during root resorption induced by the application of heavy orthodontic forces on rat molars. Am J Orthod Dentofacial Orthop. 2005; 128(4): 497-503.

26. King GJ, Fischlschweiger W. The effect of force magnitude on extractable bone resorptive activity and cemental cratering in orthodontic tooth movement. J Dent Res. 1982; 61(6): 775-779.

27. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res. 1987; 2(6): 595-610.

28. Wigen TI, Agnalt R, Jacobsen I. Intrusive luxation of permanent incisors in Norwegians aged 6-17 years: a retrospective study of treatment and outcome. Dent Traumatol. 2008; 24(6): 612-618.

29. Ashizawa Y, Sahara N.Quantitative evaluation of newly formed bone in the alveolar wall surrounding the root during the initial stage of experimental tooth movement in the rat. Arch Oral Biol. 1998; 43(6): 473-484.

30. Kameyama T, Matsumoto Y, Warita H, Soma K. Inactivated periods of constant orthodontic forces related to desirable tooth movement in rats. J Orthod. 2003; 30(1): 31-7; discussion 21-2.

31. Hasegawa N, Kawaguchi H, Ogawa T, Uchida T, Kurihara H. Immunohistochemical characteristics of epithelial cell rests of Malassez during cementum repair. J Periodontal Res. 2003; 38(1): 51-56.

32. Kohno T, Matsumoto Y, Kanno Z, Warita H, Soma K. Experimental tooth movement under light orthodontic forces. rates of tooth movement and changes of periodontium. J Orthod. 2002; 29(2): 129-135.

33. Seifi M, Jessri M, Vahid-Dastjerdi E. Identification of a novel root resorptive function of osteopontin gene. JDT. 2008; 5(4): 142-149.

34. Fukushima H, Kajiya H, Takada K, Okamoto F, Okabe K. Expression and role of RANKL in periodontal ligament cells during physiological root-resorption in human deciduous teeth. Eur J Oral Sci. 2003; 111(4): 346-352.

35. Nilforoushan D, Shirazi M, Dehpour AR. The role of opioid systems on orthodontic tooth movement in cholestatic rats. Angle Orthod. 2002; 72(5): 476-480.