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Original Article

Orthodontic force influences the activity of osteoclasts in alveolar bone remodeling by regulating EphB4/ephrinB2 expression

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Abstract: EphB4/ephrinB2 signaling plays an important role in bone remodeling, but the role of orthodontic force regulated EphB4/ephrinB2 expression in influencing osteoclastogenesis in alveolar bone remodeling remains to be studied. A rat alveolar bone remodeling model was induced by orthodontic force, and we observed microstructural changes in the alveolar bone using Micro CT and hematoxylin-eosin (HE) staining methods. EphrinB2 expression localization was detected by immunofluorescence. EphrinB2 and EphB4 protein expressions were tested by western blot. EphrinB2, EphB4, osteoprotegerin (OPG), and receptor activator of nuclear factor κB ligand (RANKL) mRNA levels were determined by real-time PCR. After placing the orthodontic device, the rats did not change their drinking and eating habits, and the orthodontic side gingival tissue and the surrounding mucosa were slightly congested. Micro-CT (HECT) and HE staining showed large and multinucleated osteoclasts near the orthotopically absorptive lacunae. Immunofluorescence exhibited strong positive ephrinB2 expression in the orthodontic pressure zone. Western blot and real-time PCR demonstrated EphrinB2 upregulation and EphB4 downregulation in the pressure zone. OPG expression was reduced, but the RANKL level was elevated on the orthodontic side. In the alveolar bone remodeling model, orthodontic force can regulate EphB4/ephrinB2 expression and affect the activity of osteoclasts. It provided a theoretical basis for the mechanism of stress zone remodeling during orthodontic tooth movement, and helped to discover substances that can regulate the activity of osteoblasts and osteoclasts, thereby regulating bone remodeling.

Keywords: Orthodontic force, alveolar bone remodeling, EphB4/ephrinB2 signaling

Introduction

With the improvement of the quality of life, more and more patients require orthodontic treatment [1, 2]. In order to further improve the orthodontic treatment level and reduce the periodontal tissue damage caused by orthodontic tooth movement, solving the problems surrounding changes in the physiological and pathological processes during orthodontic treatment caused by orthodontic force is urgently needed [3, 4].

Under the action of external force, correcting the various teeth deformities through the movement of the teeth in the bone tissue and control over jaw bone growth is the basic purpose of orthodontic treatment [5]. The teeth are connected to the alveolar bone through the periodontal ligament and fixed in the alveolar bone [6]. During orthodontic treatment, the mechanical forces acting on the teeth can cause alveolar bone remodeling, including the bidirectional action of bone deposition and bone resorption.

EphrinB2/EphB4 signaling plays an important role in bone remodeling [7]. Previous studies suggested that Ephrin ligands are mainly expressed in osteoclasts, while Eph receptors are mainly expressed in osteoblasts [8]. The interaction between cells expressing EphrinB2 and EphB4 is a prerequisite for the bidirectional signal transduction of EphrinB2/EphB4. The EphrinB2 to EphB4 signal can promote the dif-

ferentiation of osteoblasts, and they can inhibit the formation of osteoclasts [9]. Bidirectional activation of the signaling pathway can simultaneously inhibit osteoclast differentiation and promote osteoblast differentiation [10].

The number and function of osteoclasts are the main factors that determine the speed of teeth. RANKL is an essential factor for osteoclast differentiation and maturation. OPG and RANKL are important regulators of osteoclast differentiation. OPG can induce RANKL binding and restrain osteoclast differentiation [11]. Therefore, determining the ratio of OPG and RANKL is an important index for evaluating osteoblast and osteoblast activities.

Therefore, in this study, we examined the effect of orthodontic force on the expression of EphB4/ephrinB2 and its correlation with osteoclastic osteoclast activity by establishing an alveolar bone remodeling model induced by rat orthodontic force.

Materials and methods

Main materials and reagents

Rabbit anti-rat EphB4 and EphrinB2 polyclonal antibodies were purchased from Abcam (California, USA). Takara Trizol Total RNA Extract was purchased from Sigma (Los Angeles, USA). Chloroform, isopropanol, and anhydrous alcohol were purchased from the Shanghai Reagent No. 1 Plant (Shanghai, China). Phosphate buffer was purchased from Boster (Wuhan, China). ECL Luminescent Kits were purchased from Beyotime (Jiangsu, China). BCA protein kits, RIPA total protein lysates, and protease inhibitor cocktails were purchased from KGI Biosciences, Inc. (Shanghai, China). PCR primers were purchased from Bio-Bio (Dalian, China).

Main instruments

A real-time PCR instrument was purchased from Applied Biosystems (London, UK). A vertical plate electrophoresis apparatus and DC stabilized electrophoresis apparatus were purchased from Bio-Rad (Shanghai, China). A brain stereotactic apparatus was purchased from Shenzhen Ruiwoode Life Science and Technology Co., Ltd. Company (Shenzhen, China). Ultra-low temperature refrigerators were purchased from NUAIRE (California, USA). A fluorescence microscope was purchased from Leica (Frankfurt, Germany). A dental portable low-speed phone was purchased from NSK Corporation (Tokyo, Japan). A thermostatic water bath was purchased from Beijing Chang'an Scientific Instrument Factory (Beijing, China).

Experimental animal grouping

6-8 week old female SD rats weighing 180-220 g were purchased from the Experimental Animal Center of Harbin Medical University. The rats were housed in a clean animal room with a temperature of about 24°C, a relative humidity of about 60%, and a 12 h day/night cycle. The rats had free access to food and water. The rats were divided into two groups, including a control group and an orthodontic group. The bilateral first molars of one rat were used for self-control.

The rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Shanghai Ninth People's Hospital.

Modeling

After the rats were anesthetized, a dental portable low-speed phone was used to make a fixed groove of about 0.2 mm on the bilateral labial margins of the maxillary teeth and the mesial axis of the first molar teeth. The stainless-steel ligation wire was ligatured to the coil spring between the maxillary teeth and the first molars so that the coil springs would each generate a 50 g tensile force. The maxillary first molars were pulled to move horizontally. The right side of each rat was selected as the experimental group, while the left side was used as a control. The orthodontic device of each group was closely monitored.

Micro CT

The microstructure of the alveolar bone was measured using microCT scanning technology. The instrument system parameters were: X-ray voltage 60 Kv, X-ray 30 W, exposure time 2.97 s, and aluminum filter 0.5 mm. CT scan data were analyzed by MedProjects software.

HE staining

The experimental rats were sacrificed on the 14th day. The maxillary first molars and the surrounding alveolar bone masses were taken,

Table 1. Primer sequences

Gene	*Primer sequence (5'-3')*
OPG	Forward: GAAGATCAGCCCAGACGAGATT
	Reverse: TGCTCGCTGGGTTTGCA
RANKL	Forward: AGCGCTTCTCAGGAGTTCCA
	Reverse: GCCGGGCCACATCGA
EphrinB2	Forward: CCCTCTCCTCAACTGTGCCAAACC
	Reverse: CAGCAAGAGGACCACCAGCGTGAT
EphB4	Forward: CGGCCAGGAACATCACAGCCAGAC
	Reverse: CACCTGCACCAATCACCTCTTCAATC
β-actin	Forward: TGGGTATGGAATCCTGTGGC
	Reverse: CAGACAGCACTGTGTTGGC

washed with physiological saline, fixed in 10% formaldehyde for 24 hours, and then decalcified using the EDTA method at 4°C for 4-6 weeks until a needle could penetrate without resistance. Then the sample was treated by conventional dehydration, paraffin embedding, and coronally sectioned in the distal part along the long axis. Paraffin sections were routinely dewaxed, hydrated, hematoxylin stained, 1% hydrochloric acid-alcoholic differentiated, eosindyed, gradient alcohol dehydrated, turpentine oil hyalinized, and mounted.

Immunofluorescence

Each slice was routinely deparaffinized and boiled in an 0.01 M citric acid buffer (pH 6.0) for 20 minutes for antigen repair. Then the samples were incubated in a 3% H_2O_2-methanol solution for 15 minutes to eliminate endogenous oxidase activity. Next, the slices were blocked in goat serum for 30 minutes at room temperature and incubated with EphrinB2 primary antibody working solution (1:50-1:200) at 4°C overnight. Finally, each slice was incubated in FITC-labeled secondary antibody working solution for half an hour at room temperature and photographed under a fluorescence microscope.

Real-time PCR

After grinding with liquid nitrogen, 100 mg alveolar bone tissue was mixed with 1 ml Trizol on ice for 5 minutes. The lysate was pipetted into a 1.5 mL Eppendorf (EP) tube and added to 200 μL of chloroform and then shaken vigorously for 15 sec and then left to stand at room temperature for 3 min. After being centrifuged at 12,000 g and 4°C for 15 min, the upper aqueous phase was carefully pipetted into a new EP tube and mixed with 500 μL isopropanol at room temperature for 10 minutes. After being centrifuged at 12,000 g and 4°C for 10 minutes, the sediment was washed with 1 mL of ethanol for three times. Finally, the sediment was added to 20 μL of DEPC water to obtain mRNA.

The PCR primer sequences were synthesized according to the reference (**Table 1**) [12, 13]. The PCR reaction was performed in a 50 μl system. The reaction procedure was at 50°C for 30 min, 95°C. for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 50 s, and finally 72°C extension for 5 min. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Total protein extraction

After grinding with liquid nitrogen, 100 mg alveolar bone tissue was mixed with 10 μL 100 mM PMSF and homogenized in 1000 μL lysate on ice for 5-10 min. After being centrifuged at 4°C and at 12000 rpm for 5 min, the supernatant was the whole protein solution.

Western blot

Western blot was performed according to the reference [14]. The extracted total protein solution was quantified by BCA and boiled for 5 minutes to denature. The sample was separated by electrophoresis on a 10% SDS-PAGE and transferred to PVDF membranes at 300 mA for 1 h. Next, the membrane was incubated in PTC, shh, VEGF, ANG-1, and ANG-2 polyclonal antibodies (1:1000) overnight at 4°C. After being washed three times with TTBS, the membrane was incubated with the corresponding secondary antibody (1:1000) at 37°C. for 2 h. The bands were visualized by chemiluminescence.

Statistical analysis

All data analyses were performed on SPSS 19.0 software. Measurement data were presented as the mean ± standard deviation and tested for normality and homogeneity of variance. The data were compared by *t*-test and one-way analysis of variance (one-way ANOVA).

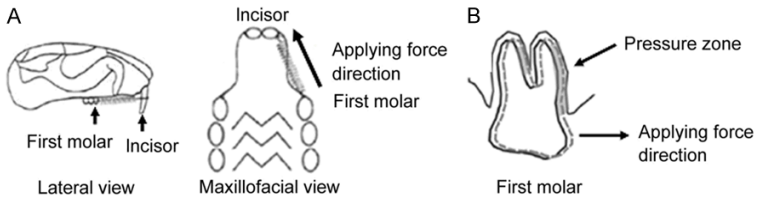

Figure 1. Schematic diagram of tooth movement under orthodontic force. A. Orthodontic device placement; B. Pressure during tooth movement.

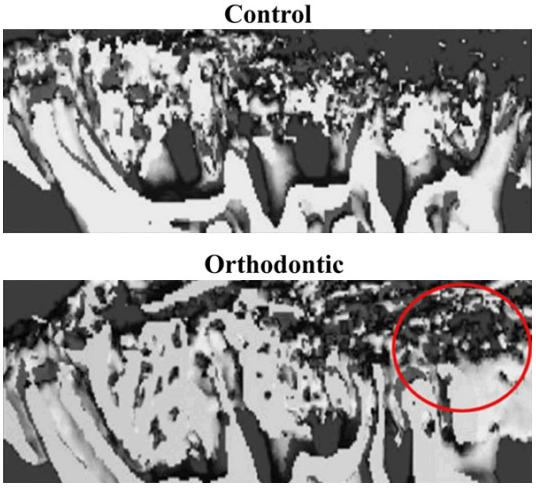

Figure 2. Micro-CT observation. Rats from the control and orthodontic groups were anesthetized followed by measuring the microstructure of the alveolar bone by microCT scanning technology. The CT scan data were analyzed using MedProjects software.

$P < 0.05$ was considered a significant difference.

Results

Establishment of a rat alveolar bone reconstruction model

A rat model of alveolar bone remodeling was established. As shown in **Figure 1A**, a coil spring was added between the incisor and the first molar. The direction of applying force and the loading of the first molar are shown in **Figure 1B**.

General observation after placing orthodontic device

After the orthodontic device was placed, there was no significant change in the rats' drinking and behavioral activities. The color of the gums and the surrounding mucous membranes around the moved teeth were slightly red, especially on the orthodontic side.

Micro-CT observation

From the results of the micro-CT, it was found that there is a gap between the first and second molars, indicating that the maxillary first molar underwent a mesial shift. In the pressure zone, the alveolar bone was rough and lacunar resorption was observed. Large and multinucleated osteoclasts were found near the absorptive lacunae and no osteoblasts were seen in the pressure zone (**Figure 2**).

HE staining

The orthodontic force caused tension zones and pressure zones to occur in the periodontal ligament (**Figure 3**). In the tension zone, the periodontal ligament was widened, collagen fibers were elongated, a large number of fibroblasts proliferated, and osteoblasts appeared on the alveolar bone surface. In the pressure zone, the width of the periodontal ligament was narrowed and the microcirculation was disturbed, leading to a small amount of ischemia, bleeding, and degeneration. Polynuclear osteoclasts and the absorption lacunae could be seen near the alveolar bone wall. It was shown that the remodeling of the alveolar bone under the effect of orthodontic force was accompanied by osteogenic osteoclast activity.

Immunofluorescence

Since no osteoblasts were seen in the pressure zone, only the EphrinB2 was immunofluorescently stained. An inflammatory expression of EphrinB2 was observed in both alveolar bone fibroblasts both in the control and orthodontic groups. In the control group, EphrinB2 expression was evenly distributed in periodontal fibroblasts. In the pressure zone of the orthodontic group, EphrinB2 expression was strongly expressed and mainly distributed in the mature osteoclasts (**Figure 4**).

EphB4 and EphrinB2 expression changes

The expression levels of EphB4 and EphrinB2 in the pressure area were examined. The EphrinB2 level increased, but EphB4 signifi-

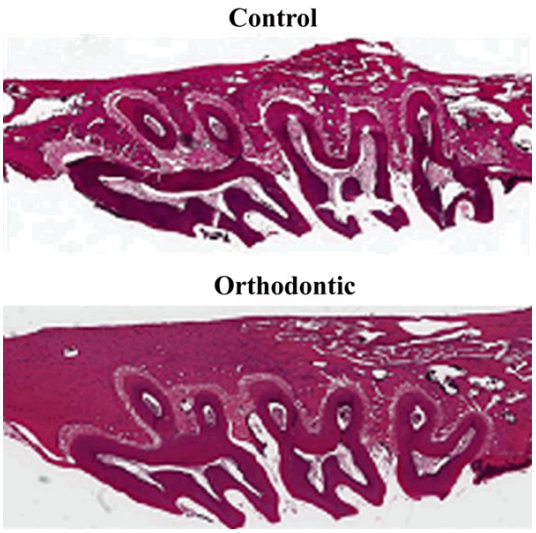

Figure 3. HE staining. The experimental rats were sacrificed on the 14th day followed by isolation of the maxillary first molars and the surrounding alveolar bone masses, which were then fixed and decalcified, followed by being embedded in paraffin, dewaxed, hydrated, hematoxylin stained, 1% hydrochloric acid-alcoholic differentiated, eosin-dyed, gradient alcohol dehydrated, turpentine oil hyalinized, and mounted.

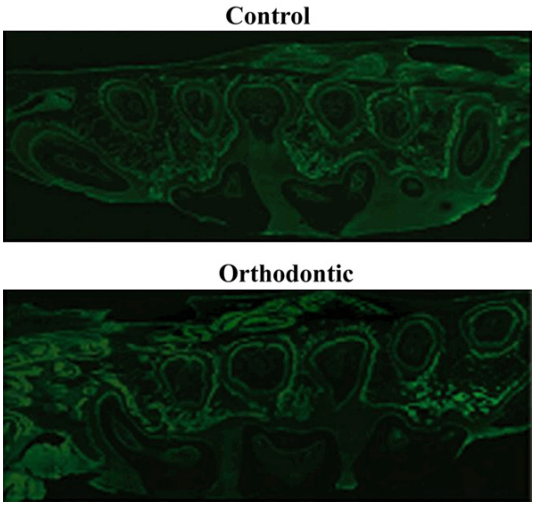

Figure 4. Immunofluorescence. Each slice was routinely deparaffinized and boiled followed by incubation with a H_2O_2-methanol solution and subsequent blocking in goat serum. After that, the slice was incubated with an EphrinB2 primary antibody followed by incubation with an FITC-labeled secondary antibody. Images were taken under a fluorescence microscope.

cantly declined in the orthodontic group compared with the control. It was consistent with the increase in osteoclasts observed in the micro-CT and HE staining results (**Figure 5**).

EphB4 and EphrinB2 mRNA expression changes and osteoblast activity (OPG/RANKL) changes

EphB4, EphrinB2, OPG, and RANKL mRNA levels were measured by real-time PCR (**Figure 6**). Compared with the control group, the expression levels of EphB4 and EphrinB2 in the orthodontic group were obviously downregulated and upregulated, respectively. The reduction of OPG expression and the elevation of RANKL levels were also found in the orthodontic group compared with the control, confirming that the osteogenic activity was reduced and the osteoclastic activity was enhanced in the pressure zone where orthodontic forces acted, which was consistent with the results observed by micro-CT and HE staining.

Discussion

In this study, the expression of EphB4/ephrinB2 signaling and its effect on osteoblast osteoclast activity under orthodontic force were studied. A gap between the first and second molars on the left maxilla was found by histological examination, indicating that the forced maxillary first molars underwent a mesial shift. Microscopy showed that obvious tension and pressure zones were formed around the moving teeth, which showed that the periodontal ligament was narrowed, fibrils were closely and disordered arranged, and bone resorption lacunae appeared on the surface of the alveolar bone in the pressure zone. The periodontal ligament was widened, collagen fibers were stretched, and the osteogenic phenomenon was active in the tension zone. It revealed that the orthodontic tooth movement model established in this experiment was successful and reliable.

The EphB4 receptor conducts a positive signal and also acts as a receptor that conducts reverse signals in its own cells. The forward signal transduction of EphB4 is dependent on Eph kinase activity and kinase-dependent signaling, whereas the reverse signal of ephrinB2 is dependent on the Src family kinases and other effector molecules [13, 15]. It was confirmed that Eph/ephrin-mediated bidirectional signal transduction plays an important role in neural development and regeneration, immune function, stem cell differentiation, cell migration, tumor development, and bone remodeling [16].

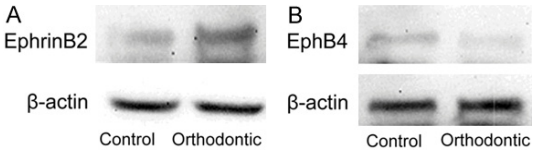

Figure 5. EphB4 and EphrinB2 expression changes. Total proteins were extracted from the alveolar bone tissue of rats from the control or orthodontic group followed by separation on 10% SDS-PAGE and subsequent measurement of EphrinB2 and EphB4 after the addition of corresponding antibody by western blot.

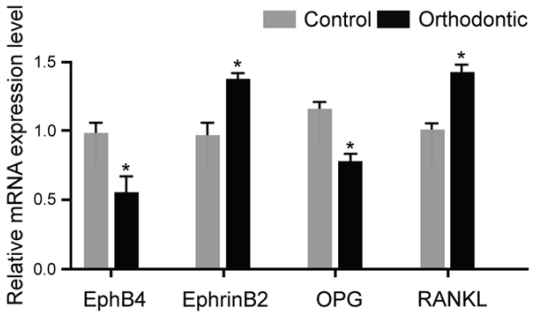

Figure 6. EphB4 and EphrinB2 mRNA expression changes and osteoblast activity (OPG/RANKL) changes. *P < 0.05, compared with control. Total RNA was isolated from the alveolar bone tissue of rats from the control and orthodontic groups followed by an analysis of the mRNA expression of EphB4, EphrinB2, OPG and RANKL by RT-PCR. The relative mRNA expression was quantified as a ratio relative to internal control.

It was suggested EphrinB2-EphB4 mediated bidirectional signal transduction between osteoblasts and osteoclasts in bone tissue [17]. Specifically, bone remodeling and metabolism are performed in the alveolar bone under dynamic interaction between osteoclasts and osteoblasts. The Eph-ephrin signaling pathway plays a key regulatory role. The researchers found that EphrinB2 is present on the surface of osteoclasts and EphB4 is present on the surface of osteoblasts. EphrinB2 and EphB4 on these two types of cells can activate each other to perform both positive and negative signal transduction. The positive signal is produced by the EphrinB2 ligand stimulating EphB4. Activated EphB4 can reduce RhoA activity and promote differentiation to accelerate osteoblasts maturation [18]. The reverse signal is EphB4 on osteoblasts to activate EphrinB2 on osteoclasts, and activated EphrinB2 can reduce the expression of c-Fos and NFATcl [19].

Bone remodeling occurs under orthodontic force, which presents enhanced osteoclast differentiation in the pressure zone. Osteoclasts are tissue-specific multinucleated macrophages that are differentiated from precursor cells of the multinuclear or macrophage system on the surface of bone or near the bone surface. In this study, a high expression of EphrinB2 was found in the pressure zone where orthodontic forces acted, and meanwhile the absorption lacunae and nearby large and multinucleated osteoclasts were observed in the micro-CT, which was consistent with the findings of previous studies [20]. It was showed that various mature osteoclasts and significant bone resorption occurred in the pressure zone of the periodontal tissue during orthodontic tooth movement. EphrinB2 is highly expressed in mature osteoclasts, suggesting that EphrinB2 may be involved in alveolar bone remodeling in the pressure zone during orthodontic tooth movement.

The ratio of OPG/RANKL plays a crucial role in the process of bone remodeling. The ratio of OPG/RANKL is the key to mediating the coupling of osteogenesis and osteoclasts and can be used to evaluate the condition of bone remodeling [21]. It was reported that the ratio of OPG/RANKL decreased in the rat osteoporosis model, suggesting that it enhanced the activity of osteoclasts. Conversely, after the administration of drugs, it can promote osteogenic differentiation by increasing the OPG/RANKL ratio, thus treating osteoporosis [22]. In the present study, it also shows that the osteoclastic activity increases and the ratio of OPG/RANKL is reduced in the pressure zone where orthodontic force acted.

In normal rat periodontium, EphrinB2 expression is weak. EphrinB2 is highly expressed in mature osteoclasts, and EphB4 expression is decreased in the periodontal tissue of the pressure zone of the rat orthodontic tooth, indicating that the EphB4/EphrinB2 signaling pathway is involved in the reconstruction of the alveolar bone in the pressure zone during orthodontic tooth movement. It may provide a theoretical basis for the study of the mechanism of stress zone remodeling during orthodontic tooth movement and will also help to discover substances that can regulate the activity of osteoblasts and osteoclasts, thereby regulating bone

remodeling. However, the specific role of EphB4/ephrinB2 needs to be further studied in cell experiments.

Conclusion

In the alveolar bone remodeling model, orthodontic force can regulate EphB4/ephrinB2 expression and affect the activity of osteoclasts. It has provided a theoretical basis for the mechanism of stress zone remodeling during orthodontic tooth movement and has helped to discover substances that can regulate the activity of osteoblasts and osteoclasts, thereby regulating bone remodeling.

Disclosure of conflict of interest

None.

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