Wnt5a Stimulates Bone Resorption during Orthodontic Tooth Movement

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Abstract

Background Wnt signaling is valuable protein network involved in bone metabolism; however, its relationship in bone resorption and formation during orthodontic treatment is currently unknown.

Purpose This study examined Wnt5a expression on the compression side in an *in vivo* experimental rat tooth movement model. The study then investigated the relation of compression force (CF) to Wnt5a expression from human periodontal ligament (PDL) cells.

Material and Methods *In vivo*, to movement the maxillary first molar, we added 10 g of orthodontic force to twenty male rats for seven days. We assessed the production of tartrate-resistant acid phosphatase (TRAP) and Wnt5a protein in rats alveolar bone through an immunohistochemical analysis. *In vitro*, we examined the effect of compression force (CF) on Wnt5a expression using the human PDL (hPDL) cells.

Results We observed resorption lacunae with the multinuclear cells in rat alveolar bone during tooth movement in the 10g group. We observed Wnt5a immunoreactivity on day seven with orthodontic force in the PDL tissue. Wnt5a expression increased for time-dependent manner after addition of CF in hPDL cells.

Conclusion Given these findings, the Wnt signaling response to optimum orthodontic force appears to stimulate resorption of alveolar bone in the orthodontic tooth movement.

Introduction

Bone metabolism is based on modeling and remodeling, maintaining bones through bone formation and resorption. Bone modeling is an unbonded mechanical process that changes the bone shape, size and position by activation-reabsorption (catabolism) and activation-formation (assimilation) at the bone surface (1). In contrast, bone remodeling is a firmly bonded local process, start to bone resorption and subsequently reversal stage and formation stage continue, replacing old bone with new (2, 3).

Orthodontic tooth movements (OTM) is caused by multi-stage biological processes. This is characterized by sequential reactions of periodontal tissues such as periodontal ligament (PDL) and alveolar bone with biological forces (4). In alveolar bone remodeling, recruitment and balanced

activation of osteoclast precursor cells and osteoblast progenitor cells around the PDL are essential (5, 6). When the light orthodontic force applied, alveolar bone are direct resorption in pressure side. Conversely heavy orthodontic force causes in excessive compression force (CF) in the PDL, inducing tissue hyalinization and local ischemia, and then cell death (7).

Wnt signaling is a highly conserved protein network that regulates proliferation and differentiation. The Wnt protein is a soluble glycoprotein and one of many growth factors, and it plays an important role in multicellular function and mediating developmental activity and homeostasis (8, 9). In recent years, Wnt signaling has attracted attention as a cell signaling pathway involved in bone metabolism. Wnt signaling has two signaling pathways, canonical pathway and noncanonical pathway. The canonical pathway is β -catenin-dependent pathway, whereas the noncanonical pathway is β -catenin-independent pathway. In the canonical

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pathway, Wnt proteins bind to receptor complexes consisting of LRP 5/6 and Frizzled protein and finally causes nuclear translocation of the β -catenin. In the noncanonical pathway, Wnt proteins bind to various receptors and mediate a wide range of activities several of which oppose the canonical pathway. Maeda et al.(12) reported that the canonical pathway activates osteogenesis by binding Wnt3a to LRP 5/6, whereas the noncanonical pathway promotes receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastogenesis by Wnt5a binding to ROR2. Lim et al.(13, 14) demonstrated that PDL is a Wnt-dependent tissue and regulates PDL homeostasis. Isogai et al.(15) suggested that orthodontic force stimulates Wnt5a expression in the tension side of the PDL. These studies suggest that Wnt signaling is valuable in bone formation during OTM.

However, at present, the detailed relationships of Wnt signaling and OTM in the compression side is unclear. We therefore explored the relationship between Wnt signaling and OTM. In our animal experiment with rats, we studied Wnt5a production during experimental tooth movement. *In vitro*, to reproduce the pressure condition during OTM, we examined the effect of CF on Wnt5a production by use of human PDL (hPDL) cells.

Materials and Methods

The studies by using rats

Animals

The study was approved for animal experiments at the Nihon University School of Dentistry at Matsudo, Japan (approval no. AP16MD 013-1). Twenty male 6-week-old Wistar rats (body weight, 180 ± 10 g; Sankyo Labo Service, Tokyo, Japan) were randomly assigned to two experimental groups: a control group ($n = 10$), where rats received no appliances, and a CF group ($n = 10$), where rats were subjected to the optimum CF.

Application of the orthodontic devices

In both groups, the animals were anesthetized with an intraperitoneal injection of three mixed anesthetic agents: medetomidine hydrochloride (Domitor), midazolam, and butorphanol tartrate (Betorphanol) (0.15 mg/kg body weight) prior to the application of the orthodontic devices. We employed the methods of Asano et al.(16) as reference for the experimental tooth movement, using a closed-coil spring (thickness : 0.005 inch, diameter : $1/12$ inch ; Accurate, Inc.,

Tokyo, Japan) ligated to the maxillary incisor and first molar using 0.008 inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan). The upper right first molar was moved to the mesial side using a closed-coil spring with a force of 50 g. The experimental tooth movement was performed for seven days (Fig. 1).

Tissue preparation

The rats were anesthetized with the three mixed anesthetic agents. The maxilla was immediately dissected, and the specimens were decalcified, then dehydrated through ethanol washes, and finally embedded in paraffin using the standard preparation methods. Each sample was sliced continuously into $4\text{-}\mu\text{m}$ -thick sections in the horizontal direction and prepared for hematoxylin and eosin and immunohistochemical staining. We observed sagittal orientation sections using the methods of Asano et al.(16).

Immunohistochemistry

The tissue sections were deparaffinized, and the endogenous peroxidase activity was quenched via incubation in 3% H_2O_2 in methanol for 30 min at room temperature. The sections were washed in Tris-buffered saline and then incubated with rabbit polyclonal tartrate-resistant acid phosphatase (TRAP) antibody and rabbit polyclonal Wnt5a antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 18 h at 4°C . The TRAP and Wnt5a were stained using the Histofine[®] Simple Stain MAX-PO kit (Nichirei Co., Tokyo, Japan). We observed negative reactivity in the control samples.

In vitro studies

hPDL cell culture

The hPDL cells were prepared according to the method by Somerman et al.(17). Informed consent was obtained from the patients or guardians prior to the sampling. The hPDL tissues were then taken from the roots of premolars extracted from healthy young volunteers (3 males, 3 females; $14\text{--}16$ years of age) during the course of orthodontic treatment. The study protocol was reviewed and approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 18-15-027-1).

Application of CF

We applied CF to simulate OTM pressure, and the cells were continuously compressed, a technique based on the

Fig. 1. Experimental tooth movement. A diagram of the method used for experimental tooth movement. The upper first molar was moved mesially by a closed-coil spring with 10 g of force (A). A schematic showing the area of investigation (shaded box) in the mesial alveolar bone aspect of the distal root of the first molar. The periodontal tissues in the pressure area accounted for one-quarter of the mesial area facing the distal buccal root (DBR), as determined when the center of the DBR was linked with the mesial root of the first molar. The large arrow indicates the direction of force (B). The number of positive cells was determined for a 300 μm section (shaded box) of the alveolar bone from the bifurcation surface on the mesial side (the side experiencing pressure during tooth movement) (C).

method by Nakajima et al. (18). Confluent hPDL cells were subjected to pressure (1.0 g/cm²) for 0–48 h. The cells were divided into two groups: control (no pressure) and experimental (with pressure).

Real-time polymerase chain reaction

We performed a real-time polymerase chain reaction (PCR) according to the method by Isogai et al. (15) using a RNeasy Mini Kit (Qiagen Co., Tokyo, Japan) and employed primer sequences designed by Isogai et al. (purchased from Takara Co., Ltd., Japan) (15). The PCR primers for Wnt5a and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) designed according to the respective cDNA sequences were as follows:

Wnt5a

Forward: 5'-TTACCACTGCAACTATTGCACCTC-3'

Reverse: 5'-CACAATGAACCTTTAGTTTCCAACC-3'

GAPDH

Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'

Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'

Enzyme-linked immunosorbent assay

The Wnt5a supernatant protein levels were measured using a human Wnt5a enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Technology LLC, Houston, TX, USA) according to the manufacturer's protocol.

Statistical analyses

The statistical analysis employed JMP software (SAS Inc., 2012), and the results were analyzed using the Mann-Whitney U test as a nonparametric method. The values in each figure represent the mean $\pm$ standard deviation (SD) for each group. P values < 0.05 were considered statistically significant.

Fig. 2. Hematoxylin and eosin and immunohistochemical staining for TRAP and Wnt5a. After an orthodontic force was applied for 7 days, alveolar bone resorption was observed in the CF group but not in the control group. (A, B) Multinucleated osteoclasts appeared on the alveolar bone surface in the CF group according to histomorphometry. TRAP immunoreactivity was observed in the multinucleated cells. (C, D) Immunoreactivity for Wnt5a was observed on the alveolar bone surface and in the PDL tissues (arrows) in the CF group. (E, F) D: dentine, C: cementum, PDL: periodontal ligament, AB: alveolar bone. Original magnification 200 $\times$, Bar: 50 μ m.

Results

The studies by using rats

Changes in weight during the experiment

In the CF group, the rats' weight temporarily decreased on day 1 after applying the orthodontic force; however, we observed subsequent weight recovery. There was no significant difference in terms of weight between the CF group and the control group (data not shown).

Histological changes of periodontal tissues in experimental tooth movement (hematoxylin and eosin staining)

PDL specimens from the control group at seven days after the tooth movement consisted of high-density connective tissue fibers and fibroblasts regularly arranged horizontally from the cementum to the alveolar bone. The alveolar bone of the PDL side surface was flat with slight mononuclear and polynuclear osteoclasts, but resorption lacunae were not observed (Fig. 2A). In the CF group, the compressed periodontal fibers and cells were arranged in a

coarse and irregular pattern (Fig. 2B). Seven days after applying orthodontic force, multinuclear osteoclasts expression in the rat alveolar bone surface and we observed numerous resorption lacunae (Fig. 2B).

TRAP-positive cells production of Immunohistochemical analysis.

We observed TRAP-positive multinuclear osteoclasts without resorption lacunae on the alveolar bone surface in control group (Fig. 2C). In contrast, we observed multinucleated TRAP-positive osteoclast with numerous resorption lacunae on the alveolar bone surface in CF group on day seven (Fig. 2D).

Protein Wnt5a production

Seven days after the tooth movement, we observed Wnt5a immunoreactivity; however, we rarely observed Wnt5a-positive cells in rat PDL tissues in the control group (Fig. 2E). In the CF group, conversely, we observed

Fig. 3. Effects of CF (1.0 g/cm²) on the mRNA production of Wnt5a by hPDL cells. The mRNA expression of Wnt5a was determined using real-time PCR after 1, 3, 6, 9, 12, and 24 h. The Wnt5a mRNA expression was significantly higher in the CF group than in the control group (*P<0.05, **P<0.01 from the Mann-Whitney U-test, indicating a significant difference compared with the corresponding control). The data are expressed as the means $\pm$ SD of six independent experiments.

Fig. 4. Effects of CF (1.0 g/cm²) on the release of Wnt5a by hPDL cells. Wnt5a levels were determined using an ELISA after 1, 3, 6, 9, 12, 24, and 48 h. Wnt5a levels were significantly higher in the CF group than in the control group (*P<0.01 from the Mann-Whitney U test, indicating a significant difference compared with the corresponding control). The data are expressed as the means $\pm$ SD of six independent experiments.

numerous Wnt5a-positive cells in the PDL tissues, and also detected multinucleated cells considered osteoclasts in resorption lacunae (Fig. 2F).

The studies by using hPDL cells

Observation of Wnt5a mRNA expression using real-time PCR

The cells were compressed by weight for 24 hours, and Wnt5a mRNA expression in the hPDL cells was measured

with real-time PCR at each time. As a result, in the CF group, expression of Wnt5a mRNA was increased in a time-dependent manner and significantly difference compared with the control group at 6h, 9h, 12h after compression adaptation (Fig. 3).

Observation of Wnt5a proteins expression using ELISA

We performed an ELISA to assess the release of Wnt5a proteins from hPDL cells reaction by added compression force. The release of Wnt5a from the CF group were increased in a time-dependent manner and significantly difference compared with the control group at 9h, 12h, 24h after compression adaptation (Fig. 4).

Discussion

Given the OTM method, we can achieve tooth movement without root resorption by moving the tooth by applying 10 g of light force in rats. Gonzalez et al. (19) reported that the application of 10 g of light force resulted in significantly greater tooth movement with significantly less root resorption compared with the application of a larger force when moving rat teeth for 28 days. The optimum force for moving a rat's upper molar could be less than 10 g, as demonstrated in past studies (20). We therefore considered that the animal model used in this experiment was suitable for reproducing efficient tooth movement.

Previous *in vivo* studies have suggested that Wnt signaling could be a potent bone-stimulating signal and important regulator of bone mass (21, 22). Other studies have also reported that osteogenic nature of PDL is dependent by Wnt signaling (13). Isogai et al. (15) demonstrated that Wnt5a expression was observed in PDL tissue on the tension side in OTM subjected to orthodontic force of 10 g, suggesting that Wnt5a may be involved in alveolar bone formation during OTM.

To investigate whether Wnt signaling is related to the process of alveolar bone resorption in the compression side during OTM, we applied an optimum force to move the tooth in an animal model. On day seven, tooth movement was achieved without root resorption by applying 10 g of light force. We detected Wnt5a immunoreactivity and osteoclasts in the rat PDL tissues in the compression side in the CF group (Fig. 2F). This phenomenon showed that Wnt5a stimulated not only bone formation but also bone resorption.

To determine the systems responsible for bone resorption

in Wnt5a production by orthodontic force, we evaluated Wnt5a mRNA and protein expression in hPDL cells subjected to compression in an *in vitro* model (16). The compression was found to significantly increase Wnt5a mRNA expression at 12 h in a time-dependent manner (Fig. 3), whereas the Wnt5a protein levels were increased at 24 h in a time-dependent manner (Fig. 4). These results suggest that Wnt5a expression by optimal orthodontic forces can induce bone resorption in hPDL cells.

By considering the association of mechanical stress and the Wnt signaling in bone resorption, Nakashima et al. (23) showed that increased RANK expression by Wnt5a is important for osteoclastogenesis in response to RANKL secreted from osteoblast lineage cells including bone cells. According to Maeda et al., Wnt5a/ROR2 signaling promotes osteoclast formation by enhancing osteoblasts and osteoclast precursors osteoclast differentiation (24). In summary, these findings and those of our study suggest that osteoclastogenesis is activated by the expression of Wnt5a occurring in hPDL cells with added optimal orthodontic force.

Some findings obtained from recent research, however, contradict the results of our research. Xu et al. (25) suggested that the canonical Wnt signaling pathway promotes bone formation by activating the expression of osteogenesis-related target genes (Runx2, osterix, osteopontin). In another study, Zhong et al. (26) suggested that expression of osteogenic markers increase as Wnt signaling is upregulated. The conflicting findings between these studies and our study could be due to the type of cell used and differences in the stimuli applied to the cells. Further research is still necessary to examine the relationship between the RANK and RANKL expression and Wnt signaling in OTM using mice with knocked-out Wnt signaling.

In conclusion, our findings suggested that the expression of Wnt5a on orthodontic forces in hPDL cells may induce bone resorption. Furthermore, Wnt5a in Wnt signaling may play a valuable role in bone metabolism during OTM.

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