Wnt5a Stimulates the Bone Formation in Tension Side during Orthodontic Tooth Movement

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Abstract

In this study, we first investigated the expression of wnt5a in tension sites during experimental tooth movement in rats *in vivo*. We then assessed the effects of wnt5a on the expression of bone marker from human periodontal ligament (hPDL) cells.

Twelve male 6-week old Wistar rats were subjected to an orthodontic force of 10 g in order to induce the mesial tipping movement of the upper first molars for 7 days. The expression levels of wnt5a proteins in the alveolar bone were determined using an immunohistochemical analysis. Furthermore, the effects of wnt5a on the gene expressions of alkaline phosphatase (ALP) and type I collagen (COL1) were investigated using hPDL cells *in vitro*.

Under the conditions of experimental tooth movement *in vivo*, the immunoreactivity for wnt5a was detected in the tension side on day 7 in the PDL tissue of rats subjected to the orthodontic force (10 g). In the *in vitro* study, wnt5a treatment increased the gene expression of ALP and COL1 from the hPDL cells in a time-dependent manner.

These results suggest that the wnt5a stimulates the alveolar bone formation in the tension side during orthodontic tooth movement.

Introduction

Bone modeling is the uncoupled process of activation-resorption (catabolic) or activation-formation (anabolic) on the bone surfaces, which results in changes of the shape, size, or position of the bone(1). Bone remodeling or turnover, on the other hand, is a tightly coupled local process, which starts with bone resorption, followed by the reversal and bone formation phases, resulting in the replacement of old bone with new bone(2, 3). Orthodontic tooth movement is a multistep biological process characterized by the sequential reactions of periodontal tissue against biomechanical forces(4). The recruitment of osteoclast- and osteoblast-progenitor cells, and the balanced activation of these cells around and within the periodontal ligament (PDL) are essential for alveolar bone remodeling(5, 6).

Wnt proteins are soluble glycoproteins that mediate

homeostatic and developmental activity(7). The canonical wnts bind to receptor complexes composed of Lrp5/6 and Frizzled proteins and ultimately induce nuclear translocation of β -catenin. Noncanonical wnts bind to a variety of receptors, including receptor tyrosine kinases, and mediate many different activities, some of which oppose canonical wnt signaling. Wnt-mediated signaling promotes postnatal bone accrual(8) and is essential for skeletal outgrowth(9). Wnt proteins are in a large family of growth factors that are important for multiple cellular functions(10). Several wnt proteins, such as wnt1, 3a, 4, 5, 10b, and 13, are important for osteoblast formation through the activation of two main signaling pathways(11-13). Recently, Lim et al.(14, 15) reported that wnt signaling regulates the homeostasis of the periodontal ligament. Furthermore, wnt signaling enhanced bone formation and inhibited bone resorption. Rawadi et al.(16) reported that BMPs induced ALP expression in preosteoblasts through wnt-mediated activity. Hoe et al.(17) reported that wnt/ β -catenin signaling enhances ALP

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Fig.1 Experimental tooth movement. A schematic illustration showing the area of investigation (shaded box) in the distal alveolar bone aspect of the distal root of the first molar. The periodontal tissues in the tension area were a quarter of the distal area facing the distal buccal root, as determined when linked with the center of the distal buccal root and the mesial root of the first molar. The large arrow indicates the direction of the force (A). The observation of the positive cells was performed at a $300\mu\text{m}$ section (shaded box) of the alveolar bone from the bifurcation surface on the distal side, which is the tension side during tooth movement (B).

activity and matrix mineralization from hPDL cells. Therefore, it is possible that wnt signaling plays partly an important role in bone formation in the tension side during orthodontic tooth movement (OTM).

However, the role of wnt signaling during OTM is not yet fully understood. Wnt5a, a member of the noncanonical wnt proteins, is known to play important roles in the development of various organs and in postnatal cell functions. Therefore, this study focused on the relationship between OTM and wnt5a. In an *in vivo* experiment, we investigated the expressions of wnt5a in the tension side during experimental tooth movement in rats. We also investigated the effects of wnt5a on the mRNA expressions of alkalinephosphatase (ALP) and type I collagen (COL1) using human PDL (hPDL) cells *in vitro*.

Materials and Methods

In vivo studies

Animals

The animal experimental protocol used in this study was approved by the Ethics Committee for Animal Experiments at the Nihon University School of Dentistry at Matsudo (approval No. AP12MD020). A total of 12 male 6-week old Wistar rats (body weight $180 \pm 10\text{g}$; Sankyo Labo Service, Tokyo, Japan) were used for the experiments.

Rats were randomly assigned into two groups: the control group, where rats received no application of force, and the tension force (TF) 10g group, where rats were subjected to TF (control = 6, TF = 6).

Application of the orthodontic devices

Kohno et al.(18) reported that the optimum force for the movement of the rats' upper molars may be 10g. Therefore, the experimental tooth movement was induced using the method of Asano et al.(19), with a closed-coil spring (wire size: 0.005-inch, diameter: 1/12 inch; Accurate, Inc., Tokyo, Japan) ligated to the maxillary right first molar with a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan). The other side of the coil spring was also ligated, with holes in the maxillary incisors that were drilled laterally just above the gingival papilla using a 1/4 round bur, applying the same ligature wire. The upper first molar was moved mesially by the closed-coil spring with a force of 10g. The period of the experiment was 7 days (Fig. 1).

Tissue preparation

The animals were deeply anesthetized using thiamylal sodium and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the maxilla was immediately dissected and immersed in the same fixative for 18h at 4°C . The specimens were decalcified in 10% disodium ethylenediaminetetraacetic acid (EDTA, pH 7.4) solution for four weeks, and the decalcified specimens were subsequently dehydrated using a graded ethanol series and embedded in paraffin. Each sample was sliced continuously into $4\text{-}\mu\text{m}$ sections in the horizontal direction and prepared for hematoxylin and eosin (HE) and immunohistochemical staining. The animals in which the tooth did not move were assigned to the control group.

Immunohistochemistry

The tissue sections were deparaffinized, and the endogenous peroxidase activity was quenched via incubation in 3% H₂O₂ in methanol for 30 min at room temperature.

After washing in Tris-buffered saline, the sections were incubated with polyclonal anti-goat wnt5a (R&D Systems Co., MN, USA) for 18 hrs at 4°C. Wnt5a was stained using the Histofine Simple Stain MAX-Po (G) kit (Nichirei, Co., Tokyo, Japan). Negative reactivity was observed in the control samples.

In vitro studies

hPDL cell culture

hPDL cells were prepared according to a modification of the method reported by Somerman et al. (20). Briefly, hPDL tissues were collected from the roots of premolars extracted from six healthy young volunteers [male (n=3), female (n=3); age: 14-16 years] during the course of orthodontic treatment. The study was performed after obtaining informed consent from the donors and the hPDL tissues were used according to a protocol reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019).

Stimulation of hPDL cells with wnt5a in the supernatant

To examine the effects of wnt5a (R&D Systems) on the mRNA expressions of ALP and OC, approximately 7×10^6 hPDL cells were transferred to 100 mm cell culture dishes. Confluent-stage cells were treated with wnt5a (100 ng/ml), which was added to the culture medium. For this experiment, we used the wnt5a concentration described previously (21, 22). To determine the time-course effects, the hPDL cells were treated with 100 ng/ml wnt5a in the supernatant for the indicated times (0-24 h). All of the above experiments were conducted in duplicate and in three different cell lines. After 24 h of incubation, total RNA was extracted for evaluation by a real-time polymerase chain reaction (PCR) (21). We used the cells treated without wnt5a as controls.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from the hPDL cells using an RNeasy Mini kit (Qiagen Co., Tokyo, Japan), and aliquots containing equal amounts of mRNA were subjected to real-time PCR. The mRNA was reverse transcribed to cDNA using the Prime Script RT Reagent Kit (Takara Co., Shiga,

Japan) according to the manufacturer's protocol. Real-time PCR amplification was performed using SYBR Premix Ex Taq (Takara Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara). The PCR primers for Jagged, and GAPDH were purchased from Takara Co. (Takara Co.) and designed with reference to the respective cDNA sequences (23), as follows:

ALP

Fw: 5' -CTCGTTGACACCTGGAAGAGCTTCAAACCG-3'

Rv: 5' -GGTCCGTCACGTTGTTCTCCTGTTTCAGC-3'

COL1

Fw: 5' -AGGGCTCCAACGAGATCGAGATCCG-3'

Rv: 5' -TACAGGAAGCAGACAGGGCCAACGTCG-3'

GAPDH

Fw: 5' -GCACCGTCAAGGCTGAGAAC-3'

Rv: 5' -TGGTGAAGACGCCAGTGGA-3'

The data are expressed as the means $\pm$ s.d. of six independent experiments.

Statistical methods

The values in each figure represent the mean $\pm$ standard deviation (s.d.) for each group. The Mann-Whitney U-test was used to compare the means of groups with values of $P < 0.05$ and $P < 0.01$, which was considered to indicate a significant difference from the corresponding control.

Results

In vivo studies

Histological changes in periodontal tissues during tooth movement (HE staining)

For the control group (0g) on day 7 after tooth movement, PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that regularly ran in a horizontal direction from the root cementum towards the alveolar bone. Blood capillaries were mainly recognized near the alveolar bone in the PDL. The alveolar bone and root surfaces were relatively smooth. In the experimental group (10g) on day 7 after tooth movement, the arrangement of the fibers and fibroblasts became coarse and irregular (Fig. 2A, B).

The protein expression levels of wnt5a

Immunoreactivity for wnt5a was examined on day 7 after tooth movement. In the control group, wnt5a-positive cells were rarely observed in the PDL tissues (Fig. 2C). In contrast, in the TF (10g) group, many wnt5a-positive cells

Fig.2 HE and immunohistochemical staining for wnt5a. After an orthodontic force was applied for 7 days, the arrangement of the fibers and fibroblasts was observed. It became coarse and irregular in the TF (10g) group (arrow heads), but not in the control group (A, B). Immunoreactivity for wnt5a was observed on the alveolar bone surface and in the PDL tissues (arrow heads) in the TF (10g) group (C, D). D: dentine, C: cementum, PDL: periodontal ligament, AB: alveolar bone. Original magnification $200\times$, Scale bar: $50\mu\text{m}$

were observed in the PDL tissues (Fig. 2D). There was a greater level of increase in the number of wnt5a-positive cells in the TF group than in the control group (Fig. 3).

In vitro studies

The effects of wnt5a on the mRNA expressions of ALP and COL1 in the hPDL cells, as determined by a real-time PCR

To evaluate the effects of wnt5a on the mRNA levels of ALP and COL1, hPDL cells were stimulated with 100ng/ml wnt5a for 0-24h. The mRNA expressions of ALP and COL1 were significantly increased comparison to the control cells, in a time-dependent manner until 24h (Figs. 4, 5). Significant increases in ALP and COL1 were detected at 6h after the initiation of wnt5a stimulation, and the stimulatory

effects of wnt5a were maintained for the whole stimulation period of 24h. These data indicate that wnt5a increases the expressions of ALP and COL1 by hPDL cells.

Discussion

When one considers the method of tooth movement, it can be stated that 10g of light force application produced tooth movement without root resorption over a period of seven days in rats. Gonzales et al.(24) showed that, in comparison to when a heavier force was applied, 10g of light force application produced significantly larger tooth movement with significantly less root resorption in rats over a period of 28 days. The optimum force for the movement of the rats' upper molars may be less than 10g as previously sug-

Fig.3 The number of Wnt5a-positive cells. The number of Wnt5a-positive cells was significantly different from the control (* $p < 0.01$).

gested(18). Therefore, the model in this study was supported as a method of efficient tooth movement.

In this study, in order to investigate whether the wnt signal is involved in the alveolar bone remodeling during orthodontic tooth movement (in an animal model), we moved the tooth by applying an optimum orthodontic force. On day 7, the immunoreactivity for wnt5a was detected in the stretched PDL tissues subjected to the TF (10 g) group (Figs. 2C, D and 3). Kim et al.(25) reported that wnt signaling is regarded as a potent bone-stimulating signal. Baron et al.(26) reported that wnt signaling is a key regulator of bone mass. Furthermore, Takada et al.(27) reported that mice with the deletion of a single allele of the *wnt5a* gene (*wnt5a*^{+/-} mice) exhibited a low bone mass phenotype with increased adipogenesis. Lim et al.(15) reported that the normally osteogenic nature of the PDL depends on wnt signaling. The expression of osteogenic markers is high in an environment where wnt signaling is upregulated(28). Taken together, the above findings indicate that wnt5a is involved in PDL metabolism and alveolar bone remodeling during orthodontic tooth movement.

In order to investigate the mechanisms responsible for the alterations in wnt5a induced by the orthodontic force during orthodontic tooth movement, the expression levels of ALP and COL1 mRNA were examined. The results indicated that the treatment of wnt5a significantly increased the mRNA expressions of ALP and COL1 from 6h to 24h in a time-

dependent manner (Figs. 4, 5).

Considering the relationship between wnt signaling and mechanical stress, Robinson et al.(29) demonstrated that wnt/b-catenin signaling is required for mechanotransduction in bone. Premaraj et al.(30) reported that the wnt/b-catenin signaling pathway components are functional and activated by mechanical strain in hPDL cells. Shi et al.(31) reported that the uniaxial mechanical tension enhanced ALP and COL1 mRNA expressions of rat tendon-derived stem cells via the Wnt5a. These results suggest that the wnt5a response to orthodontic forces may induce the process of bone formation in hPDL cells. Interestingly, Fig2B shows that the number of PDL fibroblasts seemed to increase in response to TF. Fig. 4 shows that the expression of ALP did not increase until 9h (after which there was a subsequent increase). Furthermore, Vuga et al.(32) reported that wnt5a enhanced the proliferation of human lung fibroblast. Therefore, wnt5a may also enhance the proliferation of PDL cells and promote collagen synthesis. Further studies are necessary to investigate the relationships between the expression of COL1 and the proliferation of PDL cells subjected to orthodontic tooth movement.

In contrast, recent pathological and *in vitro* studies contradict the findings of the present study. The upregulation of RANK expression by wnt5a may be critical for osteoclastogenesis in response to RANKL secreted from osteoblast-lineage cells including osteocytes(33, 34). Maeda et al.(21) reported that wnt5a was highly expressed in synovial tissues from mice with type II collagen-induced arthritis. These findings suggest that wnt5a secreted from the synovial tissue is involved in bone destruction in rheumatoid arthritis (RA) (21, 35). The discrepancies between these previous and the present results may be due to differences in the cell types used and the stimuli applied to the cells.

In conclusion, we herein demonstrated that the wnt5a response to an orthodontic force may stimulate the process of bone formation from hPDL cells. Our results suggest that wnt5a, which is involved in wnt signaling, may play an important role in bone remodeling during orthodontic tooth movement.

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Fig.4 The time-course effect of wnt5a (100ng/ml) on the mRNA expression of ALP in hPDL cells. The hPDL cells were treated with wnt5a, then the mRNA expression of ALP was observed to increase in a time-dependent manner for up to 24 h (Mann-Whitney U-test, * $P < 0.05$, ** $P < 0.01$). The data shown are representative of four independent experiments using samples from six different patients.

Fig.5 The time-course effect of wnt5a (100ng/ml) on the mRNA expression of COL1 in hPDL cells. The hPDL cells were treated with wnt5a, then the mRNA expression of COL1 was observed to increase in a time-dependent manner for up to 24 h (Mann-Whitney U-test, * $P < 0.05$, ** $P < 0.01$). The data shown are representative of four independent experiments using samples from six different patients.

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