Effect of jiggling force on Notch and Wnt signaling pathways during experimental tooth movement in rat

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Abstract

One of the adverse effects of orthodontics is orthodontic root resorption (ORR), which is induced by jiggling forces during orthodontic tooth movement. Notch and Wnt signalings are related to the differentiation of osteoblasts and osteoclasts (odontoclasts) to the root resorption in periodontal ligament cells following heavy force from one direction. However, the relationship between these signal transduction pathways and the jiggling force that causes root resorption, even with optimal orthodontic force, has not been elucidated. We aimed to investigate the relationship between the Notch and Wnt signaling pathways and jiggling. For *in vivo* experiments, we prepared rats in three groups; a control group, no intervention; an optimal force group (OF), 10 g of compression force; jiggling force group (JF), 10 g of compression force and tension force. The Jagged1 and Wnt5a were investigated by Histological immunochemical staining in the root resorption region associated with tooth movement in rats. We also evaluated the gene expressions of *Jagged1* and *Wnt5a* in the rat periodontal ligament. Based on the quantification of positive cells, the expressions of Jagged1 and Wnt5a were higher in JF than in OF. Comparisons of real-time polymerase chain reaction results revealed their gene expressions in JF than in OF. Taken together, jiggling force might activate Notch and Wnt signaling pathways and facilitate the ORR process.

Introduction

Orthodontic treatment has many advantages, such as improvement of oral function and restoration of aesthetics. However, there are some disadvantages, one of which is root resorption. Orthodontic root resorption (ORR) is difficult to predict. Once it progresses to a certain point, it cannot be repaired. Therefore, orthodontists must urgently take measures to suppress the occurrence and progression of ORR.

Several factors are considered to be involved in the occurrence of ORR. These can be roughly divided into two categories, risk factors related to treatment type, such as length of treatment [1] and heavy force [2–3], and patient-specific variables, such as genetic factors [3], age [4], abnormal root morphology [2], history of tooth trauma [5], and allergies [6], for which causes have not yet been elucidated. Among the factors that cause ORR, we focused on jiggling force as a factor related to the method of tooth movement. Root resorption occurs when jiggling during orthodontic tooth movement [7]. Tooth movement by orthodontic treatment is due to unidirectional forces. Jiggling is a movement that causes the roots to reciprocating mesial to distal or buccal to lingual during orthodontic treatment, and it is reportedly involved in ORR[7]. Moreover, jiggling itself, even with optimal orthodontic force, exacerbates root resorption compared with heavy orthodontic force from one direction[7].

In this study, we investigated the relationship between signal transduction pathways and root resorption. Previously, we report that *Notch2/Jagged1* of the Notch signaling axis results in the gene expression of inflammatory cytokines, such as *interleukin (IL)-6* and *receptor activator of nuclear factor kappa-B ligand (RANKL)* and that it is involved in root resorption in human periodontal ligament (hPDL) cells[8]. Further,

Wnt5a in the Wnt signaling pathway might be involved in osteoclast (odontoclast) cell formation in hPDL cells[9]. Notch and Wnt signaling pathways are related to the differentiation of osteoblasts and osteoclasts (odontoclasts) and induces root resorption in periodontal ligament cells (PDL) following strong corrective force application from one direction. However, the relationship between these signal transduction pathways and the jiggling force that causes root resorption, even with optimal orthodontic force, has not been elucidated. Therefore, we investigated the relationship between the optimal force of jiggling and the Notch and Wnt signaling pathways. Specifically, protein expressions of Jagged1, and Wnt5a in the root resorption region with tooth movement in rats in vivo and their gene expressions of IL-6, RANKL, Jagged1, and Wnt5a during tooth movement using hPDL cells in vivo were investigated.

Materials and Methods

Animals and orthodontic device

Sixty-four male Wistar rats were purchased from the Sankyo Laboratory (Sankyo Labo Service, Tokyo, Japan). Eight-week-old rats were used in all experiments. The animals were anesthetized for the application of orthodontic devices using an intraperitoneal injection of three types of mixed anesthesia, namely medetomidine hydrochloride (0.375 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg). The maxillary first molar was moved using an orthodontic device (diameter: 0.012 inches [0.3048 mm], stainless steel wire; Tomy International, Inc., Tokyo, Japan) and connected using a ligature wire (Fig. 1). Rats were assigned to the following three groups through block randomization: a control group, in which the

rats did not receive any orthodontic device treatment; an optimal force group (OF), in which the rats were subjected to 10 g of compression force for 21 days; and a jiggling force group (JF), in which the rats received 10 g of compression force for day 0 to day 7, 10 g of tension force for day 7 to day 14, and 10 g of compression force for day 14 to day 21. A force of 10 g was continuously applied to the palatal and buccal sides in JF. Our study was performed according to the method suggested by Hikida et al[8]. The experimental protocol was approved by an Ethics Committee for Animal Experiments at Nihon University School of Dentistry at Matsudo (Chiba, Japan; approval number: AP20MD002).

Tissue preparation

After anesthetizing the rats following tooth movement, the maxilla was dissected, and the specimens were demineralized with 10% disodium ethylenediamine tetraacetic acid. They were then dehydrated by ethanol washing and embedded using paraffin. Samples from each group were serially sliced horizontally to a thickness of 4 μm.

In this experiment, the cross section was observed, and the location for observation was determined based on the method of Hikida et al[8]. The observation site was the distal palatal root of the maxillary left first molar, and the observation was performed in Areas I and II. The cross section of the root in Area I had a height of 300 μm and a width of 225 μm in the direction from the upper part of the alveolar bone surface on the palatal side. In Area II, the cross section of the root had a height of 300 μm and a width of 225 μm, less than 150 μm in the direction from the alveolar bone surface on the buccal side. Area I was on the compression side in

OF, which stayed constant for 21 days, whereas Area II was on the tension side. In JF, the compression and tension sides were changed every 7 days: on the 14th day and 21st day (Fig. 2).

Histological and immunohistochemical staining

Hematoxylin and eosin staining was performed according to the usual method and then prepared for immunohistochemical staining. Each tissue section was deparaffinized and subjected to endogenous peroxidase activity. After quenching by incubation in 0.5% H_2O_2 in methanol for 30 min at room temperature and then washing with Tris-buffered saline (TBS), the sections were incubated with rabbit polyclonal anti-tartrate resistant acid phosphatase (TRAP) (sc-376875) (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-Jagged1 (ab7771) (1:100; Abcam, Cambridge, UK), and rabbit polyclonal anti-Wnt5a (sc-365370) (1:100; Santa Cruz Biotechnology) antibodies for 18 hours at 4 °C. Next, Jagged1 and Wnt5a were stained using the Histofine Simple Stain MAX-Po (G) and (R) kit (Nichirei Co., Tokyo, Japan), according to the manufacturer's protocol. Each section was washed with TBS, stained with 3,3′-diaminobenzidine tetrabasic acid (Takara, Shiga, Japan), counterstained with Mayer's hematoxylin solution, and then dehydrated in an alcohol/xylene series. Next, the sections were fixed with malinol. As a positive control Inflamed rat periodontal tissue was used as a positive control, and TBS was used instead of primary antibody as a negative control. After immunohistochemical staining, the number of positive cells on each slide was counted, and the percentage of positive cells was determined using a 200× optical microscope with the average value of five slides. Cells stained brown were considered positive. The mean values from five

slides used to calculate the ratio of positive cells/all cells. Positive staining was determined microscopically and counted by three independent observers. For the measurement of positive cells, each area (height: 300 µm × width: 225 µm) was photographed at 400x under an optical microscope in four fields of view of arbitrary hot spots.

Quantitative polymerase chain reaction (qPCR)

Periodontal tissues were procured from the roots of the buccal and palatal portions of the left maxillary first molar. The total RNA was extracted from the periodontal tissue using a RNeasy® Mini Kit (Qiagen Co., Tokyo, Japan) according to the manufacturer's protocol. Reverse transcription of mRNA into cDNA was performed using a PrimeScript™ RT Reagent Kit (Takara). qRT-PCR was performed using a TB Green® Premix Ex Taq™ (Takara) in a thermal cycler (TP-800 Thermal Cycler Dice®, Takara). The PCR conditions were as follows: initial activation at 95 ˚C for 30 sec, then 40 amplification cycles of denaturation at 95 ˚C for 5 sec, followed by annealing and extension at 60 ˚C for 30 sec. The sequences of the PCR primers for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *Wnt5a*, *Jagged1*, *RANK*, and *IL-6* were as follows: *GAPDH* 5′-AGGGCTGCCTTCTCTTGTGA -3′ and 5′- AACTTGCCGTGGGTAGAGTCA -3′; *Wnt5a* 5′-CGAAGACGGGCATCAAAGA -3′ and 5′- TGCATCACCCTGCCAAAGA -3′; *Jagged1* 5′-TCCAGCCTCCAGCCAGTGAA -3′ and 5′- GGAAGGCTCACAGGCTATGT -3′; *RANKL* 5′-ACGCCAACATTTGCTTTCGG -3′ and 5′-ATTGCCCGACCAGTTTTTCGTG -3′; and *IL-6* 5′-ACCCCAATTTCCAATGCTCTCC -3′ and 5′- AGGCATAACGCACTAGGTTTGC -3′.

Data analyzed performed using a Multiplate RQ（Ver. 5.11B, Takara); the quantified value of each sample was normalized with that of the *GAPDH* value of the same sample, which was amplified simultaneously with the target gene. Then, relative quantity was calculated as the expression level (Fold Difference) of the unknown sample is normalized to the expression level of the control sample (the control expression set to 1.0).

Statistical analysis

The results were analyzed using the Mann-Whitney U test to compare the means of each group, and each date shows the means ± standard deviation for each group, with $P < 0.05$ and $P < 0.01$ indicating significance.

Results

Body weight of rats during the experiment

During the experiment, there was no significant difference in the weight change in rats among the experimental groups.

Histological findings in HE staining

The specimen in control group had a relatively smooth root surface and consisted with connective tissue fibers and fibroblasts aligned horizontally from the root cement (data not shown). On days 7, 14 and 21, the irregular with fibers running, and no root resorption compressed in Areas I and II of OF. Root resorption was

not found on the palatal root surface but a few multinucleated cells. On day 7, the irregular with fibers running, and no root resorption observed in Areas I and II of JF. On day 14, no root resorption was observed in Area I of JF, and root resorption lacunae was observed in Area II of JF. On day 21, many root resorption lacunae were observed on root surfaces in Areas I and II of JF (Fig. 3).

Immunohistochemical findings

Immunohistochemical staining for Jagged1, Jagged1-positive cells were not confirmed in Areas I and II of OF. In JF, some Jagged1-positive cells were confirmed on day 14 in Areas I and II. and many positive cells were identified on day 21 in Areas I and II. Immunohistochemical staining for Wnt5a, Wnt5a-positive cells were not confirmed in Areas I and II on days 7 and 14 of OF, On day 21, a few Wnt5a-positive cells were confirmed of the Area I. In JF, a few Wnt5a-positive cells were confirmed on day 7 in Areas I and II. After days 14, lots of Wnt5a-positive cells were not confirmed in Areas I and II. The quantitative assessment of immunohistochemistry positive cells, the number of Jagged1-positive cells were increased on day 21 compared to day 14 of JF, and there was a significant time-dependent increase, in particular, there was a significant increase on day 21 in Area I. On Wnt5a, the number of the positive cells were increased in JF in a time-dependent manner in both Areas I and II, however, no significant difference was observed between both. A few TRAP-positive cells were observed in both regions. The number of cells increased in a time-dependent manner. (Fig. 7).

mRNA expression profilings

IL-6 was significantly increased on day 7, 14 and 21 compared to the control in Area I and II of both OF and JF. Comparison between OF and JF, JF had higher expression than OF in both Area I and II. Significant increases were shown in Area I of day 14 and 21 in JF (Fig. 8A). *RANKL* expression profile were similar to that of *IL-6*. Compared to the control, *RANKL* was significantly increased in both areas of OF and JF. JF showed significant increases in a time-dependent manner in Area I. (Fig. 8B). *Jagged1* was significantly increased in both areas of OF and JF compared to the control. There were no significant time-dependent changes in their expression levels. On day 21, their expression levels had higher than that of day 14. Especially, a significant increase was observed on day 21 in Area I of JF (Fig. 8C). In OF, no tendency of *Wnt5a* expression was observed in each Area. JF increase their expressions in a time-dependent manner in both Area I and II. However, there were no significant difference in both Areas (Fig. 8D).

Discussion

Previous studies have shown that ORR occurs in association with inflammatory cytokines[11–15] and that jiggling causes root resorption in association with inflammatory cytokines during orthodontic treatment[16–20]. Therefore, we focused on the relationship between inflammatory cytokines and ORR caused by jiggling force. We investigated the cause of root resorption from the perspective of signal transduction related to inflammatory cytokines by employing both histochemical and molecular biological processes in an experimental jiggling model of rats. HE staining showed that no root resorption was observed during an

experiment period in OF. However, it was observed in Area II on day 14 in JF. TRAP-positive cells in JF were significantly more abundant than those in OF, and they were observed more in Area I than in Area II. These results suggest that the jiggling force causes root resorption more than OF on one side. Matsuda et al[19]. report that the number of osteoclasts and the area of root resorption were significantly increased when jiggling force was applied to the teeth of rats by alternating buccal and palatal movements, consistent with the results of the present study. Hikida et al. report that jiggling force increase the gene expressions of RANKL and IL-6, which induce osteoclasts and aggravate root resorption. In the present study, *RANKL* and *IL-6* showed significantly higher expression in JF than in OF, suggesting that the jiggling force enhance gene expression of inflammatory cytokines, and then formation of osteoclasts that induce root resorption.

Recently, signal transduction has been reported to be involved in the expression of RANKL and IL-6, and a high orthodontic pressure induce root resorption through Notch signaling response to RANKL and IL-6 expressions[9]. and Wnt5a induces RANK expression in osteoclastic progenitor cells and promotes osteoclast formation[21–22]. Therefore, we investigated the possibility that Notch and Wnt signaling pathways may be involved in the increase in the expressions of inflammatory cytokines due to jiggling. On day 21, the expression level of Jagged1 had higher than that of day 14. Especially, a significant increase was observed on day 21 in Area I of JF. In JF, Wnt5a was increase their expressions in a time-dependent manner in both Area I and II. In JF, the number of Jagged1- and Wnt5a-positive cells and their gene expressions were the highest in both Areas on day 21 compared with those in OF. These results suggested that jiggling force might promote the expressions of *Jagged1* and *Wnt5a*, which increase *RANKL* and *IL-6* expressions and induce

root resorption. The gene expressions of *Jagged1, RANKL,* and *IL-6* increased in Area I compared with that in Area II on day 21 in JF. PDL tissue was subjected to compression forces two times and tension force one time in Area I, whereas it was subjected to compression force one time and tension force two times in Area II. These results suggested that more compression force might enhance the expressions of *Jagged1*, *Wnt5a*, *RANKL*, and *IL-6*.

In our study, *Jagged1* and *Wnt5a* expressions promoted inflammatory cytokines and aggravated root resorption. Several recent studies contradict our results. The canonical Wnt signaling pathway induces the expressions of bone formation markers when Wnt signaling is activated, activates the expression of genes related to bone formation, and promotes bone formation [22–25]. Furthermore, Notch2 is involved in Jagged1-induced bone formation and the differentiation of hPDL cells, and Notch signaling has been shown to increase bone formation [26-28]. Further research is still necessary to understand the relationship between IL-6 and RANKL expressions and Wnt signaling activation in jiggling using Wnt and/or Notch signaling knocked-out mice.

Conclusions

In this study, jiggling force might have activated Wnt and Notch signaling pathways and induced *RANKL* and *IL-6* expressions, which could induce the root resorption process. Therefore, Wnt and Notch signaling pathways which stimulated by jiggling forces play an important role in root resorption process, although there are other pathways.

Author Contributions

K. Takagi contributed to study conception, design, data acquisition, analysis, and interpretation, manuscript drafting and critically revised the manuscript; M.Shimizu and S. Negishi contributed to study conception, design, data interpretation, and manuscript drafting and critically revised the manuscript.

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Figure legends

Fig. 1 Experimental design of tooth movement

Rat maxillary first molars were activated palatal or buccal with a force of 10 g by activating the orthodontic device. The orthodontic device was connected by ligature wires. Rats were divided into three groups; control group without any treatment, an optimal force group (OF) that received 10 g of compression force (CF) from day 0 to day 21, and a jiggling force group (JF) that received 10 g of compression force from day 0 to day 7, 10 g of tension force from day 7 to day 14, and then 10 g of compression force from day 14 to day 21.

Fig. 2 Schematic illustration showing the area of the location for histological observation

Distal palatal root of the maxillary left first molar (M1) was observed and divided into Areas I and II.; the Area I had a height of 300 µm and width of 225 µm from the upper part of the alveolar bone surface on the palatal side, Otherwise, the Area II had a height of 300 µm and width of 225 µm, less than 150 µm from the alveolar bone surface on the buccal side. DP: distal palatal root, DB: distal buccal root, MP: Mesial palatal root, M: mesial root, MB: mesial buccal root, PDL: periodontal ligament, bar; 500 µm

Fig. 3 Histological findings in hematoxylin and eosin staining (×200)

After experimental tooth movement in rats, the samples were sliced horizontally in succession and observed with hematoxylin and eosin staining in cross section at 7, 14, and 21 days in each group. The

observation site was the distal palatal root of the maxillary left first molar, and performed were made in Area I and Area II. PDL: periodontal ligament, C: cementum, D: dentin, bar = 50 μm.

Fig. 4 Immunohistochemical staining for tartrate- resistant acid phosphatase (TRAP) (×200)

After experimental tooth movement in rats, *TRAP* staining was performed and observed at 7, 14, and 21 days in each group. PDL: periodontal ligament, C: cementum, D: dentin, bar = 50 μm. Narrow: positive cell

Fig. 5 Immunohistochemical staining for jagged1 (×200)

After experimental tooth movement in rats, *Jaggd1* staining was performed and observed at 7, 14, and 21 days in each group. PDL: periodontal ligament, C: cementum, D: dentin, bar = 50 μm. Narrow: positive cell.

Fig. 6 Immunohistochemical staining for Wnt5a (×200)

After experimental tooth movement in rats, *Wnt5a* staining was performed and observed at 7, 14, and 21 days in each group. PDL: periodontal ligament, C: cementum, D: dentin, bar = 50 μm. Narrow: positive cell.

Fig. 7 The quantitative assessment of immunohistochemistry positive cells

The number of positive cells on each slide was counted, and the percentage of positive cells was determined using a 200× optical microscope with the average value of five slides. Cells stained brown were considered positive. The mean values from five slides used to calculate the ratio of positive cells/all cells. Significant differences were determined at *P value < 0.05 and **P value < 0.01.

Fig. 8 Gene expression profilings

Rats were assigned to the following three groups; a control group (no orthodontic device treatment), an optimal force group (OF; 10 g of compression force), a jiggling force group (JF; 10 g of compression force for day 0 to day 7, 10 g of tension for day 7 to day 14, and 10 g of compression force for day 14 to day 21). RNA was extracted from the cross section of the root in Area I on the palatal side and in Area II on the buccal side and then gene expression was performed by qPCR. Relative quantity was calculated as the expression level of the each sample is normalized to the expression level of the control sample (the control expression set to 1.0). All data were showed as a mean ± S.D. (n = 5). $^{+}$P value < 0.05, indicating a significant difference from the control. *P value < 0.05, **P value < 0.01, indicating a significant difference from the corresponding group.