Notch Signaling Response to Heavy Compression Force Induces Orthodontic Root Resorption via RANKL and IL-6 from Cementoblasts

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Introduction

Orthodontic root resorption (ORR) occurs in tooth movements during orthodontic treatment, although the degree varies depending on the patient. Many ORRs recorded in orthodontic clinics are not severe and are localized in the root surface and apex. Eventually, the repair mechanism of the absorption site occurs by cementogenesis, without any clinical problem. However, in orthodontic practice, cases of tooth roots that are absorbed extensively and cause tooth sway, which greatly affects tooth function and safety, are rarely reported.

Kaley et al. (1) showed that ORR occurred in most patients who received orthodontic treatment, of which 3% had severe ORR (absorption of more than a quarter of the root length) in the maxillary central incisors. Many factors are involved in the process of ORR.

Risk factors in orthodontic treatment include prolonged treatment and abnormal root morphology (2), strong orthodontic power (3) and tooth movement (4). Some of the patient’s risk factors include genetic factors (5), history of dental trauma (6), and allergies (7), but the cause is still unclear.

In the study of Yamaguchi et al. (8), receptor activator of NF-Kappa B ligand (RANKL) was received when compression force (CF) was added to human periodontal ligament (hPDL) cells derived from patients with severe ORR. RANKL, as well as inflammatory cytokine expressions, may play a crucial role in ORR.
sion in the periodontal ligament, is reportedly involved in the development of ORR. In addition, Kikuta et al. (9) induced RANKL and interleukin (IL)-6 through Notch signaling of Jagged1 and Notch2 in hPDL cells with strong orthodontic force, suggesting the possibility of worsened ORR by promoting odontogenic differentiation.

Therefore, the relationship between Notch signal and RANKL is deeply related to ORR in tooth movement in orthodontic treatment. Cementum is the tissue that covers the surface of the tooth root and is classified into cellular cementum and acellular cementum. Cementoblast have a round or polygonal cell morphology and are adjacent to the cementum. These cells have tubules and communicate with adjacent cement and bone cells (10, 11). In addition, cementoblasts are similar to bone cells in matrix proteins such as type I collagen, osteopontin, and osteocalcin (12). Considering the biological similarity between cementum and bone, the same situation can occur in the cementoblasts on the compression side. Huynh et al. (13) demonstrated that cementoblasts have the capacity to induce osteoclastogenesis. Furthermore, Yao et al. (14) suggest that inflammatory cytokines may impair the cementum remodeling under mechanical stimulation. However, the effect of Notch signal in the cementoblasts on the compression side of tooth during orthodontic treatment has not been elucidated.

In this study, we focused on Notch signals of Jagged1 and Notch2 in cementoblasts. Jagged1, Notch2, RANKL, and IL-6 were immunohistochemically stained using a rat experimental tooth movement model in vivo. We also examined the gene expression levels of Jagged1, Notch2, RANKL, and IL-6 when CF was added using cultured human cementoblast-like cell (HCEM) line in vitro.

Materials and Methods

In vivo studies

1 Experimental animals

The animal experiments conformed to the guideline by the ethics committee of Matsudo School of Dentistry at Nihon University (Approval No. AP17MD 007-2).

Wistar male rats (Sankyo Labo Service, Tokyo, Japan) were carried at 5 weeks of age, and at 6-weeks-old Wistar male rats (n=18, body weight, 120.1 ± 10.5 g), they were used for 1 week of preliminary breeding. The rats were randomly assigned to the following three groups: control group, where no force was applied (n=6); 10 g group, where an optimum force of 10 g was applied (n=6); and 50 g group, where a heavy force of 50 g was applied (n=6).

2 Experimental tooth movement model

The rats were intraperitoneally injected with three anesthetics agents (medetomidine hydrochloride, 0.375 mg/kg; mitazolam, 2 mg/kg; butorphanol tartrate, 2.5 mg/kg). The experimental tooth movement model was created as per the method of Asano et al. (15) After deep anesthesia, mesial movement of the maxillary right first molar was achieved with a closed coil spring ligated to the maxillary first molar using a 0.008-inch stainless steel wire. Forces of 10 g and 50 g were applied for 7 days.

3 Tissue preparation

The rats applied with orthodontic force underwent deep anesthesia by using the abovementioned three types of mixed anesthesia. Moreover, perfusion fixation was...
performed with saline and 10% neutral buffered formalin. Immediately after the maxilla was dissected and immersed in the same fixative for 18 h at 4°C, the sample was decalcified with a 10% disodium ethylenediaminetetraacetic acid (pH 7.4) solution for 4 weeks at room temperature. Then, we washed the decalcified sample and generated a paraffin-embedded block. Each sample was continuously sliced horizontally at 4 µm thickness and prepared for hematoxylin and eosin (H.E.) and immunohistochemical staining procedures.

4 Immunohistochemical staining

We deparaffinized each tissue section and quenched the endogenous peroxidase activity via incubation in 0.5% H₃O₂ in methanol for 30 min at room temperature. After being washed in Tris-buffered saline (TBS), the sections were incubated with rabbit polyclonal anti tartrate-resistant acid phosphatase (TRAP), rabbit polyclonal anti Jagged1, rabbit polyclonal anti Notch2, goat polyclonal anti RANKL, and goat polyclonal anti IL-6 (individual working dilution, 1:100; Santa Cruz Biotechnology, Inc., CA, USA) antibodies for 18 h at 4°C. Then, TRAP, Jagged1, Notch2, RANKL, and IL-6 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 Co., Shiga, Japan), counterstained with Mayer’s hematoxylin solution, and dehydrated in alcohol xylene series. Then, they were sealed with marineor. The observation site was referred to the method of Kikuta et al. (9) (Fig. 2). Furthermore, the periodontal tissue around the compression side of the efferent distal buccal root of the maxillary right first molar was observed with a light microscope at 200 ×, the brown-stained cells were considered positive.

In vitro studies

1 Culture of HCEM

HCEMs were acquired from Professor Takata Takashi (Hiroshima University).

HCEMs were cultured according to the method described by Kitagawa et al. (16) The medium used was alpha minimum essential medium (α-MEM; Wako, Osaka, Japan) supplemented with 100 µg/ml of penicillin G (Sigma Chemical Co., St. Louis), 0.3 µg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA), 10% fetal calf serum (Cell Culture Laboratories, OH, USA), and 50 µg/ml of gentamicin sulfate (Sigma Chemical Co., St. Louis). The culture solution was then stored at 37°C in a humidified incubator (Forma CO₂ incubator MIP-3326; Sanyo Electric Medical System Co., Tokyo, Japan) under 95% air and 5% CO₂ conditions.
We reproduced continuous CF at orthodontic tooth movements in HCEM, according to the method described by Kikuta et al. (9) (Fig. 3).

At first, HCEMs were seeded in 100-mm cell culture dishes with 10% FCS. After overnight incubation, the medium of approximately confluent cells was changed to 1% FCS and cover glass plate was placed on top. Furthermore, we prepared a compression model by placing a weight of 1.0 g/cm² as the optimum CF and 4.0 g/cm² as the heavy CF harvest at 0, 1, 3, 6, 9, 12 and 24 h. Cells in the control group were treated with a thin cover glass plate without weight, producing a CF of 0.032 g/cm².

3 Notch inhibitor addition

GSI (R&D Systems Co., MN, USA) was added to the medium at 5.0 µM concentration to inhibit Notch before CF application according to the method reported by Osathanon et al. (17) and Fukushima et al. (Fig. 3).

4 Real-time polymerase chain reaction (PCR)

We extracted the total RNA from HCEMs by using the RNeasy Mini Kit (Qiagen Co., Tokyo, Japan) and performed real-time PCR on aliquots containing equal mRNA amounts. The Prime Script RT Reagent Kit (Takara Co. Shiga, Japan) was used to reverse-transcribe the mRNA into cDNA according to the manufacturer’s protocol. Real-time PCR was amplified using the SYBR Premix Ex Taq (Takara Co., Shiga, Japan) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara Co., Shiga, Japan). PCR primers for Jagged1, Notch2, RANKL, IL-6, and GAPDH were purchased from Takara Co. and designed with reference to the respective cDNA sequences.

5 Statistical processing

Each value represents the mean ± standard deviation of each group. Group means were compared using Mann-Whitney U test. P values of < 0.05 and < 0.01 were considered statistically significant.

Results

In vivo studies

1 Changes in body weight of experimental animals

No significant difference was observed in the body weight changes in the experimental animals during the experiment. (data not shown).

2 Change of periodontal tissue in H.E. staining of experimental tooth movement model

The PDL tissue of the control rats consisted of root cementum and fibroblasts. There were no resorption pits on the root surface, it was almost smooth and no osteoclasts were found around it. (Fig. 4a). In the 10 g group after 7 days, the root cementum almost did not change, but many resorption pits were observed on the alveolar bone surface (Fig. 4b). In the 50 g group after 7 days, coarse arrangement of fibers with dilated vessels was observed, and many root resorption pits with multinucleated osteoclasts were identified on the root surface (Fig. 4c).
Findings for TRAP

After 7 days, TRAP-positive multinucleated cells were not found on the root surface and alveolar bone of the control group (Fig. 4d). Conversely, in the 10 and 50 g groups such cells were found in the bone resorption pit on the alveolar bone surface (Fig. 4e) and in the root resorption pit, respectively (Fig. 4f).

Findings for Jagged1, Notch2, RANKL, and IL-6

Immunoreactivity for Jagged1, Notch2, RANKL, IL-6 was examined on day 7 after tooth movement. In the control group, bone resorption and root resorption were not observed, and Jagged1, Notch2, RANKL, IL-6 positive cells were diffusely observed in the PDL tissue (Fig. 5g, j, m, p). Conversely, in the 10 g groups, Jagged1, Notch2, RANKL, and IL-6 positive cells were observed around osteoclasts in bone resorption pits on the alveolar bone surface (Fig. 5h, k, n, q). In the 50 g group, Jagged1, Notch2, RANKL, and IL-6 positive cells were observed around odontoclasts in the root resorption pit (Fig. 5i, l, o, r).

In vitro studies

1 Changes in the gene expression of Jagged1, Notch2, RANKL and IL-6 by CF in HCEM cells

The gene expression of Jagged1 had a higher tendency in the CF group (1.0 and 4.0 g/cm²) than in the control group. Furthermore, in the CF group, it increased in the 4.0 g/cm² group compared with that in the 1.0 g/cm² group, showing its peak at 6 h after the compression began. The gene expression of Notch2 also increased in the CF 4.0 g/cm² group, showing its peak at 9 h after the start of compression. Similarly, RANKL and IL-6 gene expression increased in the CF 4.0 g/cm² group, showing its peak at 12 h after the start of compression (Fig. 6).

2 Changes in RANKL and IL-6 gene expression addition of Notch inhibitor in HCEM cells

For inhibition experiments, the Notch inhibitor GSI was added to HCEM cells for 24 h. At that time, the gene expression of RANKL decreased by approximately 58% at
12 h in the CF 4.0 g/cm² group with GSI addition compared with that in the group without GSI addition. The gene expression of IL-6 also decreased by approximately 42% at 12 h in the CF 4.0 g/cm² group with GSI addition compared with that in the group without GSI addition (Fig. 7).

Discussion

In this study, we investigated the effect of Notch signal of Jagged1 and Notch2 by two different orthodontic forces, namely, optimal force and heavy force on cementoblasts and examined whether such effect is involved in ORR during orthodontic treatment.

Huynh et al. (13) demonstrated that cementoblasts can induce osteoclastogenesis in relation to ORR and cementum. Furthermore, Liao et al. (18) stated that cementoblasts differentiate from periodontal progenitor cells and share many common features with PDL and osteoblasts. In addition, Kikuta et al. (9) induced RANKL and IL-6 through Notch signaling of Jagged1 and Notch2 in hPDL cells with strong orthodontic force, suggesting possible worsening of ORR by promoting odontogenic differentiation. Therefore, we investigated how Notch signal affects ORR in cementoblasts.

In vivo, an experimental tooth movement model using rats was used to investigate the periodontal tissue response when two different orthodontic forces (10 g and 50 g) were applied to rats for 7 days. In the 10 g group after 7 days, the root cementum slightly changed, but many resorption pits were observed on the alveolar bone surface. In the 50 g group, ORR occurred, reaching the cementum and dentin, additionally, immunoreactivity of Jagged1 and Notch2 was detected in the PDL tissue, and osteoclasts were observed. In addition, RANKL and IL-6 immunoreactivity was detected in the root resorption pits. Duan et al. (19) suggested that Notch signaling may be involved in pathological bone remodeling. Therefore, ORR may be involved with PDL, as well as cementum. Thereafter, to elucidate the mechanism of ORR occurrence of in the cementum, we utilized HCEM-like cells with two different CF (CF 1.0 and 4.0 g/cm²) in vitro, and examined the gene expression levels of Jagged1, Notch2, RANKL, and IL-6. Results showed that the peak of Jagged1 was at 6 h, which increased in the CF 4.0 g/cm² group compared with that in the control group and CF 1.0 g/cm² group. Meanwhile, the peak of Notch2
was at 9 h, which increased in the CF 4.0 g/cm² group.
Furthermore, the peaks of RANKL and IL-6 were at 12 h,
which increased significantly in the CF 4.0 g/cm² group.
In addition, in the inhibition experiment using GSI (Notch inhibitor), RANKL and IL-6 decreased in the CF 4.0 g/cm² group with GSI addition than in the CF 4.0 g/cm² group without GSI addition. At 12 h, RANKL decreased by approximately 58%, and IL-6 decreased by approximately 42%.

These results suggest that Notch signal may promote production of RANKL and IL-6 and cause ORR by applying excessive CF to cementoblasts.

For future research, it is necessary to investigate the relationship between Notch signal and ORR using Notch knockout mouse. Sethi et al. (20) reported that Jagged1 advances tumor growth by stimulating IL-6 release from osteoblasts and directly activating osteoclast differentiation. Furthermore, Nakao et al. (21) reported that Jagged1 advances RANKL-induced osteoclastogenesis, whereas Fukushima et al. (22) reported that RANKL induces the production of Jagged1 and Notch2 in bone marrow macrophages during osteoclastogenesis. Taken together, the increased Jagged1 expression observed in HCEM cells treated with excessive CF activates osteo/odontoclastogenesis.

However, there are studies in recent years that contradict this study. For example, Li et al. (23) reported that Notch signaling enhances the osteogenic differentiation of periodontal ligament stem cells in osteoporotic rats, and Ugarte et al. (24) documented that Notch signaling enhances osteogenic differentiation in primary human bone marrow stromal cells. The result of Notch signal changes may be related to the difference in cell type and the stimulus applied to the cell.

In conclusion, applying excessive CF to HCEM may activate Notch signaling, promote production of RANKL and IL-6, and stimulate the process of ORR. Therefore, there are several pathways related to ORR, but the Notch signal in cementum plays an important role in the process of ORR. We suggested that Notch signaling may be important for the development of ORR in cementum although the involvement of other pathways cannot be
ruled out.

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