TGF- β 1 Stimulates Bone Resorption during Orthodontic Tooth Movement

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Abstract

BACKGROUND: Transforming growth factor (TGF)- β , which controls bone metabolism, is an important protein network. However, the relationship TGF- β and bone metabolism during orthodontic treatment of tooth movement is not well elucidated.

PURPOSE: This study aimed to observe TGF- β 1 expression on the compressed side of the periodontal tissue by moving rat teeth with orthodontic force in an animal experiment. In vitro, the change in TGF- β 1 expression level when compression force (CF) was applied to human periodontal ligament (hPDL) cells was examined.

MATERIAL AND METHODS: *In vivo*, 20 male rats were subjected to 10 g of orthodontic force for 7 days to move maxillary first molars in the mesial direction. Then, immunohistochemical analysis was performed to observe the expression of tartrate-resistant acid phosphatase (TRAP) and TGF- β 1 protein in rat alveolar bone. In vitro, hPDL cells were used to study the effect of CF on TGF- β 1 mRNA and protein expression.

RESULTS: *In vivo* study, after 7 days, multinucleated cells and resorption lacunae were found on the surfaces of the alveolar bones in the 10-g group. Additionally, TGF- β 1 immunoreactivity with orthodontic force was observed in the periodontal ligament (PDL) tissue on the 7th day. *In vitro*, TGF- β 1 expression increased in the 1.0 g/cm² group compared to that in the control group in hPDL cells.

CONCLUSION: Taken together, these results reveal that TGF- β 1 expression is activated in the periodontal tissue when optimal orthodontic force is applied. Moreover, it is possible that the TGF- β controls alveolar bone resorption during OTM.

Keywords :

TGF- β 1, tooth movement, compression force, periodontal ligament

Introduction

The orthodontic tooth movement (OTM) is the consequence of biological reactions due to the response of mechanical force on the periodontal tissue (1). This phenomenon is caused by the alveolar bone remodeling in the periodontal tissue. The periodontal ligament (PDL) plays a critical role in this phenomenon. In this process, harmonious interaction between osteoblasts and osteoclasts surrounding the PDL is required (2, 3). When orthodontic force is applied, PDL corresponding to the direction of or-

thodontic force is compressed, and the alveolar bone in that area is resorbed. When OTM is achieved with light force, alveolar bone is resorbed directly. In contrast, heavy force leads to local ischemia, vitreous degeneration, and cell death of the PDL tissue (4).

Transforming growth factor (TGF)- β signaling controls development and homeostasis of various somatic tissues. TGF- β is responsible for cell differentiation, proliferation, adhesion, apoptosis, and extracellular substrate production in fibroblasts and alveolar bone in the periodontal tissue. Recent studies have focused on the involvement of TGF- β signaling in bone metabolism. TGF- β receptor 1 (TGF- β 1) is a locally produced bone metabolism regulator (5, 6) and has been shown to regulate osteoblast prolifera-

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tion(7) and culture and osteoclast formation(8). Kaneda et al.(9) demonstrated that TGF- β signaling by TGF- β 1 regulated osteoclast differentiation. Furthermore, TGF- β 1 expression is caused by a compression force(CF) and involved in the regulation of bone metabolism by mechanical stress(10, 11). Therefore, TGF- β 1 may partly have a critical role in OTM. However, the involvement of TGF- β 1 in OTM at the time of orthodontic treatment is not well elucidated.

We aimed to investigate how TGF- β 1 contributes to OTM. For this reason, we examined that the expression of TGF- β 1 in compression side during OTM *in vivo* by immunohistochemistry, and that the effect of CF(1.0 g/cm²) which reproduce OTM on TGF- β 1 production from hPDL cells *in vitro*.

Materials and Methods

• Studies using rats

Animals

The animal experiments were conducted in accordance with a protocol certified by the ethics committee of Nihon University School of Dentistry at Matsudo(No. AP-18MAS005-2). In this study, we utilized 12 male rats(6 weeks old, Sankyo Labo Service, Sapporo, Japan). The rats' mean body weight was 130 $\pm$ 10.4 g. Six randomly selected rats were allotted to the CF group adapting heavy CF, and six to the control group, which did not. The CF group was subjected to 10 g optimum CF.

Experimental tooth movement methods

We moved each rat's first molar following to the experimental method of Asano et al.(12). The rats were injected intraperitoneally with three anesthetic agents(midazolam, 2.0 mg/kg; medetomidine hydrochloride, 0.375 mg/kg; and butorphanol tartrate, 0.25 mg/kg of body weight). After anesthesia, the experimental tooth was moved in the mesial direction using a closed-coil spring(length, 0.005 in; diameter, 1/12 in; Accurate Inc., Tokyo, Japan). The first molar was ligated to the incisor using a wire coil(thickness: 0.005 inch, diameter: 1/12 inch; Accurate, Inc., Tokyo, Japan). The other end of the spring was ligated with an identical ligature wire to a maxillary incision with a lateral holes formed just above the gingival papilla with a 1/4 diameter round bar. The experiment lasted 7 days, during which time the maxillary molar was applied a force of 10 g by a closed-coil spring and was

pulled to mesial direction(Fig. 1).

Tissue preparation

At the end of for experimental period, the rats were deeply anesthetized using a mixture of anesthetics used during device attachment. The maxillary bone was immediately dissected after perfusing the heart of each rat with a fixative comprising saline, 4% paraformaldehyde, and 0.1 M phosphate buffer. And the removed maxillary bone had soaking for 18 h in the same solution at 4°C. The obtained samples were decalcified for 4 weeks at room temperature in 10% disodium ethylenediaminetetraacetic acid solution(pH 7.4). Next, the decalcified specimen was dehydrated by a series of stepwise ethanol washes and embedded in paraffin using conventional preparation methods. Each block was sliced horizontally into consecutive sections(4 μ m thick)for hematoxylin and eosin(H & E)staining and immunohistochemical staining.

Immunohistochemical staining

The tissue sections prepared by the above treatment were deparaffinized, heat-treated in a microwave oven, immersed in citric acid buffer, and incubated overnight at a low temperature(4°C). Endogenous peroxidase activity was then continued in 3% H₂O₂ at room temperature for 30 minutes. Deparaffinized sections were washed with Tris-buffered saline(TBS)and then incubated with polyclonal anti-rabbit tartrate-resistant acid phosphatase (TRAP; Santa Cruz Biotechnology, Inc.)and polyclonal anti-rabbit TGF- β 1(ab31013, Abcam)antibodies for 18 hours at a low temperature(4°C). Thereafter, TRAP and TGF- β 1 had staining with Histofine® Simple Stain MAX PO(G)and(R)kits(Nichirei, Tokyo, Japan)following to the protocol of manufacturer. After washing with TBS and staining with 3,3'-diaminobenzidine tetrabasic acid(Takara Co., Shiga, Japan), each section was immersed in Mayer's hematoxylin solution for contrast staining and dehydrated with an alcoholic xylene series. Then, the samples were washed with TBS and sealed with mariners and cover glasses. The observation area was determined by the method demonstrated by Kikuta et al.(13). The periodontal tissue on the compression side of adjacent to the maxillary right primary molar's root of buccal was observed with a light microscope($\times$ 200 magnification). Cells that stained brown were determined positive for ei-

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), determined when the center of the DBR was associated 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- μ m section (shaded box) of the alveolar bone from the bifurcation surface on the mesial side (the side with pressure during tooth movement) (C).

ther TRAP or TGF- β 1. The control group sections exhibited negative reactions.

• *In vitro* studies hPDL cell culture

We modified the experimental method demonstrated by Somerman et al. (14) and performed hPDL cell culture. The hPDL tissue used in the experiment was collected from the roots of extracted premolars of young and healthy patient (Six persons; 3 males and 3 females, 15–20 years old) undergoing orthodontic treatment. Informed consent was obtained from the subjects according to organizational procurement, and protocol certified by the ethics committee of Nihon University School of Dentistry at Matsudo (EC18–17–021–1).

CF application

To reproduce the CF of OTM in the PDL, the accord-

ing *in vitro* experiments were conducted reference to the procedures described by Nakajima et al. (15). The human periodontal ligament (hPDL) cells were compressed with uniform continuously at the mechanics of orthodontic movement. First, the cells were seeded onto a 100-mm dish and cultured in α -MEM (minimum essential medium comprising 10% fetal calf serum [FCS]) at 37°C in 5% CO₂. After overnight incubation, the medium was changed to α -MEM containing 1% FCS. The cells were substantially confluent; the cell layer was placed in a dish, which was covered with a glass plate. Then, the cells were applied to 1.0 g/cm² of CF for 1–24 h. A thin micro glass plate was placed on top of the control group cells, resulting in a CF of 0.032 g/cm².

Real-time polymerase chain reaction

Reverse transcription PCR was performed Total RNA containing an equal amount of mRNA was extracted

from cultured hPDL cells using RNeasy Mini Kit (Qiagen Co., Tokyo, 18 Japan). Total RNA containing an equal amount of mRNA was extracted from cultured hPDL cells. Then, using the PrimeScript RT Reagent Kit (Takara Printing, Shiga, Japan), reverse transcription of cDNA from mRNA was performed following to the protocol of manufacturer. Real-time PCR amplification was performed using SYBR Premix Ex Taq (Takara) and a thermal cycler (TP-800 thermal cycler dice, Takara). We purchased PCR primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TGF- β 1 from Takara Co. and designed them based on the following cDNA sequences.

TGF- β 1

Forward: 5'-TGGACCGCAACAACGCCATCTATGA-GA-3'

Reverse: 5'-TGGAGCTGAAGCAATAGTTGGTATC-CA-3'

GAPDH

Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'

Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'

Enzyme-linked immunosorbent assay (ELISA)

After collecting the supernatant from the cell culture medium for ELISA, the amount of TGF- β 1 protein released from hPDL cells was measured using the TGF- β 1 ELISA kit (R&D Systems Co.).

Statistical analyses

Each value represents the average $\pm$ standard deviation for experimental group. Using the Mann-Whitney U test, we compared the average of the group. P-values < 0.05 and < 0.01 were indicated statistically significant differences.

Results

• Studies using rats

Body weight change during the experimental period

In the CF group, the rats' mean gross body weight decreased on the first day of experimentation; however, this decrease was transient, inasmuch as the rats body weight recovered, and remained in good health from the second day onward (data not shown). Body weight did not differ between the CF and control groups, and the rats' weight remained stable throughout the experiment (data not shown).

Histological changes in periodontal tissues in experimental tooth movement (H&E staining)

After the 7-day experiment, the PDL tissue from rats in the control group (CF: 0 g) comprised fibroblasts aligned horizontally from the root cement to the alveolar bone. There were no absorption pits on the root surface or alveolar bone, which was relatively smooth and had no nearby osteoclasts (Fig. 2A). In the CF group, the arrangement of the fibers and fibroblasts was coarse and irregular (Fig. 2B). Numerous multinuclear osteoclasts were expressed on the alveolar bone surface after 7 days of force application, and in these areas, the alveolar bone comprised irregular osteoclast-like cells localized on resorption lacunae (Fig. 2B).

TRAP-positive cell production of immunohistochemical analysis

No root resorption lacunae were found in the control group after 7 days, but multi-transparent TRAP-positive cells (odontoclasts or osteoclasts) were present (Fig. 2C). Conversely, in the CF group, large resorption lacunae with multinuclear TRAP-positive cells (odontoclasts or osteoclasts) were found on the surfaces of the alveolar bones after 7 days (Fig. 2D).

Protein of TGF- β 1 production

Immune reactivity with regard to TGF- β 1 was examined after 7 days of OTM. There were extremely few TGF- β positive cells around the PDL tissue in the control group (Fig. 2E). Conversely, a numerous TGF- β positive cells were confirmed around the PDL tissue of the CF group, and multinucleated cells including osteoclasts in the resorption lacunae were also observed (Fig. 2F).

• Studies using hPDL cells

Observation of TGF- β 1 mRNA expression using real-time PCR

Comparing the increase in TGF- β 1 mRNA expression level in each group, the CF (1.0 g/cm²) group was larger than the control group. The TGF- β expression levels were increased over 6 h and peaked at 3 h in CF (1.0 g/cm²) group (Fig. 3).

Observation of TGF- β 1 protein expression using ELISA

Comparing the increase in TGF- β 1 Protein released level in each group, the CF (1.0 g/cm²) group was larger

Fig. 2. Hematoxylin and eosin and immunohistochemical staining for TRAP and TGF- β 1. 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 (arrowheads) appeared on the alveolar bone surface in the CF group according to histomorphometry. TRAP immunoreactivity (arrowheads) was observed in multinucleated cells. (C, D) Immunoreactivity for TGF- β 1 (arrowheads) was observed on the alveolar bone surface and in PDL tissues in the CF group. (E, F) D, dentine; C, cementum; PDL, periodontal ligament; AB, alveolar bone. Original magnification 200 $\times$, bar, 50 μ m.

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

Fig. 4. Effects of CF (1.0 g/cm²) on the release of TGF- β 1 by hPDL cells. TGF- β 1 levels were determined using an ELISA after 1, 3, 6, 9, 12, and 24 h. TGF- β 1 levels were 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 means $\pm$ SDs of six independent experiments.

than the control group. The TGF- β 1 level released in CF (1.0 g/cm²) group, increased in a time-dependent manner following compression for 1–24 h (Fig. 4).

Discussion

Regarding to an optimal force for OTM, rats were able to reproduce tooth movement with a light force of 10 g

without root resorption. Gonzales et al.(16) compared movement of a rat's teeth for 28 days with heavy force and light force of 10 g and concluded that light force causes more tooth movement and less root resorption. As demonstrated in previous studies, the optimal force for moving a rat's upper molars may be <10 g(17). Therefore, we believe that the method used in this animal experiment was accurate for replicating the movement of teeth in orthodontic treatment without root resorption.

Recent studies have shown that various signal transductions are involved in OTM. Odaira-Yamazaki et al.(18) demonstrated that Notch signaling by Jagged1 response of optimum orthodontic force stimulates the process of bone remodeling in PDL cells during OTM. Moreover, Goto et al. reported that Wnt signaling by Wnt5a response of heavy orthodontic force activates RANKL expression and causes orthodontic root resorption(19). Shimazaki et al.(20) have suggested that TGF- β and RANKL were expressed in the tissues surrounding the roots of deciduous teeth during physiological root resorption. Furthermore, TGF- β 1 is an important cytokine that regulates bone metabolism and has been reported to be expressed in bone tissue due to mechanical stress(21). The results of these studies indicate that TGF- β 1 is an important factor of bone remodeling. Therefore, it may be involved in bone metabolism during OTM.

To investigate the involvement of TGF- β in alveolar bone remodeling in OTM, we first confirmed the expression level of TGF- β 1 by applying optimal orthodontic force in this rat model. In the CF group, as a result of orthodontic force(10 g), the protein of TGF- β 1 expressed in the compressed PDL tissue on day 7(Fig. 2F). The results of this experiment demonstrated that TGF- β 1 produced by tooth movement may stimulate bone resorption at compression side. Thereafter, CF(1.0 g/cm²) was added to hPDL cells, expression level changes of TGF- β 1 mRNA and protein were measured, and the mechanism induced by optimal orthodontic force was evaluated. As a result, the mRNA expression level of TGF- β 1 in the CF group increased significantly at 6 h in a time-dependent manner in comparison with the control group(Fig. 3). In addition, the release of TGF- β 1 protein increased in a time-dependent manner for 24 h in comparison with the control group(Fig. 4). These results suggest that the expression of TGF- β 1 mRNA and protein may be induced with optimal orthodontic force and may stimulate bone

resorption in the hPDL tissue.

Quinn et al.(22) reported that TGF- β mediated osteoclastogenesis with RANKL and tumor necrosis factor α . Furthermore, Rachelle et al.(23) demonstrated that TGF- β has a stimulating effect on osteoclastogenesis of hematopoietic cells treated with macrophage colony-stimulating factor and RANKL in bovine cortical bone. TGF- β increases RANK expression in monocytic murine cells, which stimulate osteoclast differentiation(24).

Our data suggest that the applying orthodontic force to hPDL cells increase in TGF- β 1 mRNA and protein expression and may activate osteoclastogenesis. However, further study is needed to elucidate the detailed relationship between TGF- β signaling.

In summary, we demonstrated that increased expression of TGF- β 1 response to orthodontic force may stimulate alveolar bone resorption in hPDL cells. In other words, TGF- β may play a critical role in bone metabolism during OTM.

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