TGF- β 1 Induces Orthodontic Root Resorption through RANKL and IL-6 Production in hPDL Cells

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Abstract

Background: Transforming growth factor- β (TGF- β) signaling is an important protein network in bone metabolism. However, the relationship between TGF- β and orthodontically induced inflammatory root resorption (OIIRR) during orthodontic treatment is not well understood. This study aimed to observe TGF- β 1 expression on the compressed side of the periodontal ligament (PDL) during orthodontic treatment, and to examine the involvement of TGF- β 1 in orthodontic root resorption.

Materials and Methods: *In vivo* experiments, 12-week-old male Wistar rats applied to 50g of heavy orthodontic force for 7 days. Then, we determined tartrate-resistant acid phosphatase (TRAP) and TGF- β 1 positive expression levels in resorbed cementum using immunohistochemical analysis. *In vitro*, the effects of a heavy compression force (4.0 g/cm²) with/without SB-431542 (an inhibitor of TGF- β 1) on TGF- β 1, receptor activator of nuclear factor kappa-B ligand (RANKL), and interleukin-6 (IL-6) production levels were measured using human PDL (hPDL) cells.

Results: *In vivo*, in the 50g group, the immunoreactivity of TRAP and TGF- β 1 with resorption lacunae was determined on day 7. *In vitro*, TGF- β 1, RANKL, and IL-6 production increased in the 4.0 g/cm² group compared to that in the control group in hPDL cells. Moreover, treatment with SB-431542 inhibited the production of these factors.

Conclusion: The results suggest that TGF- β 1 may aggravate the OIIRR by increasing the production of RANKL and IL-6 from hPDL cells.

Keywords:

root resorption, TGF- β ,
RANK ligand, interleukin-6,
periodontal ligament

Introduction

Orthodontically induced inflammatory root resorption (OIIRR) is an inevitable complication that difficult to predict before orthodontic treatment. There are two types of causes of OIIRR. One is a treatment-specific factors, including prolonged treatment (1) and heavy correctional force (2). The other one is patient-specific factors, including genetic elements (3), age (4), root abnormalities (2), a history of tooth trauma (5), and allergies (6). However, the mechanism underlying OIIRR remains unclear.

In previous studies, it is demonstrated that nuclear factor kappa-B ligand (RANKL) and interleukin-6 (IL-6) were detected in resorbed root and periodontal ligament (PDL) tissue subjected to heavy orthodontic force (7, 8). Furthermore, several studies reported that RANKL and IL-6 are induced from human PDL (hPDL) cells under the compressive force (CF) *in vitro* (9-11). Therefore, these cytokines are associated with OIIRR during orthodontic tooth movement.

Transforming growth factor- β (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 at the peri-

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odontal tissue. It is a multifunctional growth factor that produced by many cells in the bone microenvironment including osteoblasts, fibroblasts, and osteoclasts(10, 12). Itonaga *et al.*(11, 13)reported that TGF- β may indirectly induce mononuclear phagocyte osteoclast precursors differentiation to osteoclastic cells. TGF- β 1 is a locally produced bone metabolism regulator and important role in skeletal modification of the TGF- β superfamily. It is also known to be involved in alveolar bone resorption during tooth movement(12, 14). Therefore, TGF- β 1 may be involved in the occurrence of OIIRR. However, little is known about the role of TGF- β 1 in OIIRR at the time of orthodontic treatment.

We aimed to focused on the relationship between TGF- β 1 and OIIRR. For this reason, in an *in vivo* study, we examined the expressions of TGF- β 1 in areas of root resorption during orthodontic tooth movement in rats. Additionally, we investigate the effect of CF (4.0 g/cm²) which reproduce orthodontic force on TGF- β 1, RANKL and IL-6 production from hPDL cells *in vitro*.

Materials and Methods

Animals

The animal experimental protocol in this study was approved by the ethics committee of the Nihon University School of Dentistry at Matsudo(approval number AP-18MAS005-2). This study used rats(6 weeks old)of Manufactured and Distributed by Sankyo Labo Service (Tokyo, Japan). The rats are a total of 12 males and their body weight were 130.0 ± 10.4 g. The experimental rats were randomly grouped into the excessive tooth movement(TM)group(n=6)and the control group that did not TM(n = 6).

Implementing tooth movement using orthodontic equipment

Tooth movement was performed as described by Asano *et al.*(13, 15). The rats were injected intraperitoneally with three anesthetic agents(medetomidine hydrochloride, 0.375 mg/kg; midazolam, 2.0 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 coil was ligated during to the first molar to incisor by wire(wire size, 0.005 in; diameter, 1/12 in; Accurate Sales Co., Tokyo, Ja-

pan). The experiment lasted 7 days, during which time the maxillary first molar was moved in the mesial direction by the closed-coil spring with a force of 50 g

Tissue preparation

At the end of the experimental period, the rats were deeply anesthetized with the mixture of anesthetics used during attachment of the device. The maxillary bone was dissected immediately after perfusing the heart of each rat with a fixative comprising saline, 4.0% paraformaldehyde, and 0.1 M phosphate buffer. The removed maxillary bone was soaked in this fixative for 18 hours(h)at 4°C. The obtained samples were decalcified for 4 weeks at room temperature in 10% disodium ethylenediaminetetraacetic acid solution(pH 7.4). The decalcified specimens were then dehydrated through a graded series of ethanol washes and embedded in paraffin using the usual methods for preparation. Each block was sliced horizontally into consecutive sections(4 μ m thick)and prepared for hematoxylin and eosin(H.E)and immunohistochemical staining(Fig. 1).

Immunohistochemical staining

The tissue sections were deparaffinized, and endogenous peroxidase activity was allowed to continue for 30 min at room temperature before quenching by incubation in 3.0% H₂O₂ and methanol. The deparaffinized sections were washed with Tris-buffered saline(pH7.4 : TBS), and incubated with polyclonal anti-rabbit tartrate-resistant acid phosphatase(TRAP)antibody(Santa Cruz Biotechnology, Inc., Dallas, TX, USA)or polyclonal anti-rabbit TGF- β receptor 1 antibody(ab31013, Abcam, Cambridge, UK)for 18 h at 4°C. Then, their sections were stained using Histofine[®] Simple Stain MAX-Po(G)and(R)kits (Nichirei, Co., Tokyo, Japan)in accordance with the manufacturer's protocol. After washing with TBS and staining with 3,3'-diaminobenzidine tetrabasic acid(Takara Co., Shiga, Japan), sections were immersed in Mayer's hematoxylin solution for contrast staining. The observation area was determined as described by Kikuta *et al* (14, 11). The periodontal tissue on the compression side of the buccal root of the first maxillary right molar was examined under a light microscope at 200 $\times$ magnification. Cells that stained brown were considered as a positive for either TRAP or TGF- β 1. The control group sections exhibited negative reactions.

Fig. 1. Experimental tooth movement model and observation site

A: Experimental tooth movement model

Mesial movement of the maxillary right first molar was achieved with a closed coil spring ligated to the maxillary first molar using a 0.08-inch stainless steel ligation wire (wire size: 0.005 inch, diameter: 1/12 inch). The other side of the coil spring was ligated using the same ligation wire to a hole drilled with a 1/4 round bur in the maxillary incisor, just above the gingival papilla. The molar was moved mesially with 50 g forces for 7 days.

B: Observation site

The mesial part of the region formed by the line connecting the center of the mesial buccal root (MBR) and the center of the distal buccal root (DBR) and the perpendicular line of the maxillary right first molar. A quarter of periodontal tissue was observed. The arrow indicates the force direction. The positive cells of each staining and root resorption were examined in the mesial plane of the DBR root on the compression side in the region located in the 300 μ m apical direction from the bifurcation.

Culture of hPDL cells

The hPDL cells were cultured according to a modification of the method described by Somerman *et al.* (15, 16). Briefly, hPDL tissue was obtained from the roots of premolars extracted from 4 periodontally healthy patients (2 men and 2 women, aged 18–29 years) during orthodontic treatment. The study protocol was reviewed by the Ethics Committee of the Nihon University School of Dentistry at Matsudo (EC18-17-021-1), and informed consent was obtained from each individual.

Application of the CF

To reproduce the CF of orthodontic tooth movement (OTM) in the hPDL, the following *in vitro* experiments were conducted based on procedures described by Nakajima *et al.* (10). The cells were seeded onto a 100-mm dish and cultured in α -minimum essential medium (MEM) containing 10.0% fetal calf serum (FCS) at 37°C in 5.0% CO₂. After overnight incubation, the medium was changed to α -MEM containing 1.0% FCS. When the cells were confluent, the cell layer was placed in a dish, which was covered with a cover glass. The experimental groups were subjected to 4.0 g/cm² of CF for 1, 3, 6, 9, 12, or 24 h. The control groups using a cover glass plate were subjected to 0.032 g/cm² (Fig. 2). In TGF- β Inhibition experi-

ment, a TGF- β Inhibitor (SB-431542; Tocris, Bristol, UK) was added to the culture medium at a concentration of 10⁻⁵ M to inhibit cleavage of the TGF- β intracellular domain as reported by Kawahara *et al.* (16, 17).

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from hPDL cells using the RNeasy Mini kit. Aliquots containing equal amounts of mRNA were subjected to real time PCR. The total RNA was converted to cDNA using a prime script RT reagent kit (Takara Co., Shiga, Japan). Real-time PCR amplification was performed using a SYBR Premix Ex Taq (Takara Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara) according to the manufacturer's primer. The PCR primers were purchased designed with reference to the respective cDNA sequences as follows:

<i>TGF-β1</i>	Forward: 5'-TGGACCGCAACAACGCCATC-TATGAGA-3'
	Reverse: 5'-TGGAGCTGAAGCAATAGTTG-GTATCCA-3'
<i>RANKL</i>	Forward: 5'-TGGATGCCTTGAATAATA-AGCAGGA-3'
	Reverse: 5'-AATTTGCGGCACTTGTG-GAA-3'
<i>IL-6</i>	Forward: 5'-AAGCCAGAGCTGTGCAGAT-

Fig. 2. Applications of compression force (CF)

A compression side model was prepared by placing a weight with 4.0 g/cm^2 as the heavy CF, targeted at 0, 1, 3, 6, 9, 12, 24, or 48 h. The PDL cells in the control group were treated with a thin micro-glass plate without weight, producing a CF of 0.032 g/cm^2 .

GACTTA-3'

Reverse: 5'-TGTCCTGCAGCCACTGGTTC-3'

GAPDH Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'

Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'

Enzyme-linked immunosorbent assay (ELISA)

The culture medium was recovered for ELISA. The levels of TGF- β 1, soluble RANKL (sRANKL) and IL-6 released into the culture supernatants were measured using a human TGF- β 1 ELISA kit, human IL-6 ELISA kit (R&D Systems Co., Minneapolis, MN) and human sRANKL ELISA kit (Biomedica Co., Vienna, Austria), according to each manufacturer's protocol. The TGF- β 1 kit measured TGF- β 1 releases in the control group and TM group, and sRANKL and IL-6 also measured levels with control group, TM group and TGF- β 1 inhibitor group.

Statistical analyses

Each value represents the average $\pm$ standard deviation (SD) of each group. The Mann-Whitney *U*-test was used to compare group averages. *P*-values of <0.05 and <0.01 were considered as statistically significant.

Results

Animal weight change during the experiments

The body weights of the rats were $130.0 \pm 10.4 \text{ g}$ before the experiments and $127.0 \pm 11.2 \text{ g}$ after tooth movement. The weights did not change significantly during the experiments.

Changes to periodontal tissue during OTM determined by HE staining

After the 7-day experiment, PDL tissue from rats in

the control group (0 g) comprised fibroblasts aligned horizontally from the root cement that were associated with relatively dense connective tissue fibers. There was no resorption on the root surface or alveolar bone, which was relatively smooth and had no nearby osteoclasts (Fig. 3-a). Conversely, in the 50 g group, the root surfaces showed a coarse arrangement of fibers and root absorption lacunae and multinucleated odontoclasts were identified. These lacunae appeared to invade the dentin from the cement (Fig. 3-b).

Immunohistochemical findings of TRAP staining

No root resorption lacunae with TRAP-positive multinucleated were detected in the control group (0 g) on day 7 (Fig. 3-c). Conversely, in the 50 g group, many resorption lacunae with multinuclear TRAP-positive odontoclasts were found on the root surfaces and alveolar bones on day 7 (Fig. 3-d).

TGF- β protein expression levels determined by immunohistochemical staining

Immune reactivity with regard to TGF- β was examined on day 7 of OTM. The control group (Fig. 3-e) contained very few TGF- β -positive cells in the PDL tissues, whereas there were many TGF- β -positive cells in the 50 g group (Fig. 3-f). The control group sections exhibited negative reactions.

Expression of TGF- β 1, RANKL and IL-6 mRNA determined by real-time PCR

The expression levels of TGF- β 1 mRNA increased in comparison to those in the control over a 12-h period, reaching a peak at 3 h. The expression of TGF- β 1 was in-

Fig. 3. Light microscope images for H.E, TRAP and TGF- β 1.

It demonstrating the effects of various orthodontic forces on multinucleated osteoclasts subjected to hematoxylin and eosin (H.E) and immunohistochemical staining to reveal the presence of TRAP and TGF- β 1. The orthodontic force was applied for 7 days. There was root resorption in the 50g group (b), but not in the control group (a). TRAP immunoreactivity was observed in the multinucleated cells in the 50 g group on Day 7 (d), but did not appear in the control group (c). TGF- β 1 immunoreactivity was observed on the root surfaces (arrows) in the 50 g group on Day 7 (f), but not in the control group (e). AB: alveolar bone; D: dentin; C: cementum; PDL: periodontal ligament; original magnification = $200\times$; bar = $50\text{ }\mu\text{m}$.

creased in the 4.0 g/cm^2 group compared to in the control group (Fig. 4). The gene expression levels of RANKL were lower in the CF group treated with SB-431542 than in the CF control group and decreased by approximately 53% on 12 h. The gene expression levels of IL-6 were also lower in the CF group following incubation with SB-431542 than in the CF control group and were decreased by approximately 23% over 12 h. The gene expression levels of RANKL and IL-6 in the CF group with SB-431542 were lower than in the 4.0 g/cm^2 CF group (Fig. 5).

Effects of CF on release of TGF- β 1, RANKL and IL-6 from hPDL cells, as determined by ELISA

In CF group, TGF- β 1 released from the hPDL cells was increased in a time-dependent manner compared with that observed in the control group (Fig. 6). Both RANKL and IL-6 released from hPDL cells in the CF group (4.0 g/cm^2) were increased in a time-dependent manner

following compression for 24 h compared to that from the control group cells (Fig. 7).

Discussion

In this study, to determine whether TGF- β 1 is involved in the occurrence of root resorption during orthodontic treatment, we induced root resorption by applying heavy orthodontic force in animal models, and examined for cytokine that is expressed in the resorption site.

First, we induced root resorption by applying a heavy orthodontic force in an animal model. In the 50 g group, TGF- β 1 immunoreactivity was detected in rat PDL tissues subjected to force on day 7. Previous studies reported that TGF- β expression increased on the compression side of root during OTM (17, 18, 19). Also, Shimazaki *et al.* (18, 20) demonstrated that TGF- β and RANKL were expressed surrounding the roots of deciduous teeth during physiological root resorption. In addition, TGF- β induced the differentiation of osteoblast and osteoclast,

Fig. 4. Effects of various compression forces on TGF- β 1 gene expression over 24 h. When hPDL cells were cultured in the presence or absence of a force of 4.0 g for up to 24 h, TGF- β 1 mRNA expression increased significantly compared to TGF- β 1 mRNA expression in the control. The data are expressed as the means $\pm$ SDs of four independent experiments. * $P < 0.01$ significantly different from the control

Fig. 5. Effects of compression forces (4.0g/cm²) on the gene expression levels of RANKL and IL-6 in the presence or absence of a TGF- β inhibitor (SB-431542) over 24 h.

When hPDL cells were cultured with or without SB-431542 for up to 24 h, the expression levels of RANKL and IL-6 mRNA increased significantly for 12 h, compared to the corresponding expression levels in the control group. The mRNA expression level in the CF with the inhibitor (SB-431542) was lower than the CF without the inhibitor. The data are expressed as the means $\pm$ SDs of four independent experiments.

** $P < 0.01$, * $P < 0.05$ significantly different from the control

†† $P < 0.01$, † $P < 0.05$ significantly different from the 4.0 g group.

and the formation and resorption of bone (19, 21-24). Therefore, TGF- β may involve with bone metabolism through participate in the activation of the differentiation of osteoclasts and odontoclasts.

Next, in order to determine the mechanisms responsi-

ble for the alterations induced OIIRR by heavy orthodontic force, we measured the mRNA and protein production levels of TGF- β 1, RANKL, and IL-6 in hPDL cells subjected to a CF (4.0 g/cm²). The results indicated that the CF increased the mRNA expression of TGF- β 1. Mean-

Fig. 6. Effects of compression forces (4.0g/cm²) on TGF-β1 release over 24 h. When hPDL cells were cultured in the presence or absence of a force of 4.0 g for up to 24 h, TGF-β1 release was significantly increased compared to the release of TGF-β1 in the control. The data are expressed as the means ± SDs of four independent experiments. **P* < 0.01 significantly different from the control

Fig. 7. Effects of various compression forces on the release of RANKL and IL-6 in the presence or absence of a TGF-β inhibitor (SB-431542) over 24 h.

When hPDL cells were cultured with or without SB-431542 for up to 24 h, the levels of RANKL and IL-6 increased significantly in a dose dependent manner, compared to the corresponding levels in the control. The data are expressed as the means ± SDs of four independent experiments.

***P* < 0.01, **P* < 0.05 significantly different from the control

††*P* < 0.01, †*P* < 0.05 significantly different from the 4 g group.

while, RANKL and IL-6 mRNA expression levels reached a peak after the peak of TGF-β1 expression. Furthermore, treatment with SB-431542 inhibited RANKL and IL-6 mRNA levels, respectively. The protein levels of TGF-β1, RANKL and IL-6 increased at 24 h in a time-dependent manner. Furthermore, treatment with SB-431542 inhibited the increases in RANKL and IL-6 mRNA levels, respectively. These results suggest that the TGF-β1 re-

sponse to heavy orthodontic forces partly stimulates the production of RANKL and IL-6 in hPDL cells.

TGF-β stimulated RANKL-dependent osteoclast formation *in vitro* (25–27) and increased RANK expression in monocytic murine cells, which stimulate osteoclast differentiation (28). Fuller *et al.* (29) and Kaneda *et al.* (20, 30) reported that TGF-β and RANKL are necessary for osteoclast formation. Moreover, TGF-β-activated kinase 1

mediates mechanical stress-induced IL-6 expression in osteoblasts(21, 31). Therefore, TGF- β may induce osteoclastogenesis via RANKL and IL-6 in PDL cells. Moreover, TGF- β has been related to several pathological bone conditions in which pathological bone resorption occurs. Lettesjö *et al.*(22, 32)reported that TGF- β was detected in large amounts in the synovial fluid of patients with rheumatoid arthritis. TGF- β production by tumor cells may also stimulate tumor osteolysis and hypercalcemia (23, 33). RANKL-stimulated TGF- β induction may play a role in these osteolytic changes in human osteoclasts, which are associated with increased cytokine production. Therefore, TGF- β may be involved in OIIRR and pathological tooth conditions. Taken together, these findings our present results suggest that TGF- β 1 induced OIIRR via RANKL and IL-6 in hPDL cells subjected to heavy orthodontic force.

Interestingly, TGF- β expression was found to be increased during OTM on both the tension and compression sides(18, 19, 34, 35). TGF- β has anabolic functions, including by inducing the proliferation of PDL cells and up-regulation of the expression of type I collagen(24–27, 36–39). TGF- β also presented anabolic functions on bone tissue by inducing the differentiation of osteoblast precursors and enhanced the production of bone matrix proteins (28, 40, 41). Garlet *et al.*(29, 42)reported that osteoprotegerin and osteocalcin showed higher expression on the tension side than on the compression side during OTM. Therefore, TGF- β has conflicting functions in bone formation on the tension and resorption on the compression sides during OTM. Further studies are necessary to investigate these interesting functions of TGF- β during orthodontic movement and OIIRR.

Conclusions

We demonstrated that the response of TGF- β 1 to heavy orthodontic force may stimulate root resorption via RANKL and IL-6 production from hPDL cells.

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Conflicts of Interest

The authors declare no conflicts of interest associated

with this manuscript.

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