Orthodontic Root Resorption was Associated with the Secretion of IL-6 and IL-8 Stimulated by IL-17 in Dental Pulp Cells

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Abstract
Orthodontically induced inflammatory root resorption (OIIRR) is a common complication associated with orthodontic tooth movement. The aim of this study was to investigate how dental pulp contributes to root resorption during orthodontic tooth movement. Fifteen male 8-week-old BALB/c mice were subjected to an excessive orthodontic force of 25 g to induce a mesially tipping movement of the upper first molars for nine days. The expression levels of the TRAP, interleukin (IL)-17, IL-17 receptor (IL-17R), IL-6 and keratinocyte chemoattractant (KC; IL-8-related protein in rodents) proteins were determined in dental pulp (DP) by an immunohistochemical analysis. Furthermore, the effects of IL-17 on the production of IL-6 and IL-8 were investigated using human dental pulp (hDP) cells in vitro.

Resorption lacunae with multinucleated cells were observed in the 25 g group during the in vivo experimental tooth movement study. Immunoreactivity for IL-17, IL-17R, IL-6 and KC was detected in all of the DP tissues subjected to the orthodontic force on day nine. Moreover, IL-17 increased the release of IL-6 and IL-8 from hDP cells. The results of this study suggest that the IL-17 may aggravate the process of root resorption by increasing the production of IL-6 and IL-8 from hDP cells.

Introduction
Orthodontic tooth movement is induced by mechanical stimuli and facilitated by the remodeling of the periodontal ligament (PDL) and alveolar bone. Mechanical stress evokes biomechanical and structural responses in a variety of cell types in vivo and in vitro (1, 2).

Orthodontically induced inflammatory root resorption (OIIRR) is an unavoidable pathological consequence of orthodontic tooth movement. There are many studies about the cause of OIIRR by many investigators. Yamaguchi et al. (3) reported that the compressed PDL cells obtained from tissues with severe external apical root resorption produced a large amount of the receptor activator of the nuclear factor kappaB (RANK) ligand (RANKL) that up-regulated osteoclastogenesis in vitro. Nakano et al. (4) reported that the PDL in response to heavy forces induced the root resorption via RANKL/RANK. Therefore, the PDL may be deeply involved in the occurrence of OIIRR.

Considering dental pulp (DP), Yamaguchi et al. (5) reported that Substance P stimulated the production of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in human dental pulp (hDP) fibroblasts with severe orthodontic root resorption, and suggested that DP may be involved in the occurrence of OIIRR. Meanwhile, Tripuwabhrut et al. (6) reported that inflammation after extensive root resorption was confined to the compressed PDL, whereas the DP was unaffected. Recently, the immunoreactivity for Th17, IL-17, IL-6, and IL-8 was detected in the resorbed root and PDL tissue subjected to the excessive orthodontic force (7, 8). Therefore, these inflammatory cytokines in PDL may contribute to OIIRR during orthodontic tooth movement. However, little is known about the relationship between OIIRR and these

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cytokines in the DP.

In this study, the immunolocalization of IL-17, IL-17 receptor (IL-17R), IL-6 and keratinocyte chemoattractant (KC; IL-8-related protein in rodents) in mouse root resorption during experimental tooth movement were investigated using immunohistochemical analysis. Furthermore, the effects of IL-17 on the release of IL-6 and IL-8 were investigated using hDP cells in vitro.

Materials and Methods

In vivo study

Animals

Fifteen male, BALB/c mice of 8-weeks-old (Sankyo Labo Service, Inc., Tokyo, Japan, mean weight 20 ± 5g) were used for the experiments. The experimental protocol was approved by the Ethics Committee for Animal Experiments at Niho University School of Dentistry at Matsudo (approval No. AP11MD015). The mice were housed in separate cages under a 12-hour light/dark environment at a constant temperature of 23℃. The animals were provided with food and water ad libitum. The health status of each mouse was evaluated by daily body weight monitored for 1 week before the start of the experiments.

Application of orthodontic devices

The animals were anesthetized with thiamylal sodium (15 mg/kg body weight) for the application of the orthodontic devices. Experimental tooth movement was induced using the method reported by Yoshimatsu et al. (9) with a Nickel-Titanium (Ni-Ti) closed-coil spring (Tomy International, Inc., Fukushima, Japan) inserted between the upper incisors and the upper-left first molar, and fixed with a 0.008 inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan) around both teeth by means of a dental adhesive agent (Transbond XT, 3M Unitek, Monrovia, CA, USA). We used the left maxillary molar in each mouse to study the experimental tooth movement. The upper first molar was mesially moved by the closed coil spring with a force of 25 g. The force was applied for nine days.

Tissue preparation

The experimental period was set at 9 days after tooth movement was initiated. The mice were deeply anesthetized by thiamylal sodium and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the maxillae were dissected out and immersed in the same fixative for overnight at 4℃. The maxillae were decalcified in a 10% disodium ethylenediamine tetraacetate acid (EDTA) solution for three weeks at 4℃. Paraffin-embedded samples were sectioned at 4μm in the vertical direction. The sections were used for hematoxylin and eosin staining (H.E.), and also for immunohistochemical staining. The area of investigation was the DP tissues in the distal buccal root of the first upper molar (Fig.2). The side that was not moved was defined as a control.

Immunohistochemistry

Immunohistochemical staining was performed as follows: The tissue sections were deparaffinized and the endogenous peroxidase activities were quenched by incubation in 3% H2O2 in methanol for 30 minutes at room temperature. After washing in Tris-buffered saline (TBS), the sections were incubated with a polyclonal anti-goat TRAP antibody (Santa Cruz Biotechnology, Inc., CA, Santa Cruz USA; working dilution, 1:100), polyclonal anti-rat IL-17 antibody (Biolegend, San Diego, CA, USA; working dilution, 1:100), polyclonal anti-rabbit IL-17R antibody (Santa Cruz Biotechnology, working dilution, 1:200), polyclonal anti-goat IL-6 antibody (Santa Cruz Biotechnology, working dilution, 1:200), or polyclonal anti-goat KC antibody (Santa Cruz Biotechnology, working dilution, 1:100) for 18 hours at 4℃.
The sections were subjected to reactions with the reagent Histofine Simple Stain MAX-Po kit (Nichirei, Co., Tokyo, Japan) at room temperature for 30 minutes. After washing with TBS and the final color reactions were performed using the 3, 3’-diaminobenzidine tetra-hydrochloride substrate reagent, they were counter-stained with hematoxylin. For immunohistochemical controls, some sections were incubated in the same way, and then incubated with 0.01 M phosphate buffered saline (PBS) instead of the primary antibody. Negative reactivity was observed for the controls.

**In vitro study**

**Human dental pulp cell culture**

HDP cells were prepared according to a modification of the method described by Hosoya et al (11). Briefly, hDP tissues were taken from the roots of premolars extracted from six healthy, young volunteers (3 males and 3 females; 14–16 years of age) during the course of orthodontic treatment. The study protocol was reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019). The hDP tissue specimens were placed in 35 mm tissue culture dishes and covered with a sterilized glass coverslip. 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, MO, USA), 50 μg/ml of gentamicin sulphate (Sigma), 0.3 μg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA), and 10% fetal calf serum (FCS) (Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO2 incubator MIP-3326, Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO2. When the cells growing from explants had reached confluence, they were detached with 0.05% trypsin (Gibco, NY, Grand Island, USA) in PBS for 10 minutes and sub-cultured in culture flasks. For the experiments, hDP cells were used at passages 6–9.

**Treatment of hDP cells with recombinant human IL-17 (rhIL-17)**

To examine the effects of rhIL-17 (R&D System, Co., Minneapolis, MN, USA) on the production of IL-6 and IL-8, approximately $1 \times 10^6$ hDP cells were transferred to six well dishes. The cells were then cultured for up to 72 hours in α-MEM containing 1% FCS with 0, 0.1, 1.0, 10, or 100 ng/ml rhIL-17. For this experiment, we used the rhIL-17 concentration described previously (8). We used the cells treated with outrhIL-17 as controls.

**Real-time polymerase chain reaction (PCR)**

The extraction of total RNA from hDP cells treated with rhIL-17 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 PrimeScript 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) in a thermal cycler (TP-800 Thermal Cycler Dice; TaKaRa). PCR primers for IL-6, IL-8 and GAPDH were purchased from Takara Co.
and were designed with reference to the respective cDNA sequences. The primers were designed as follows:

**IL-6**

5'-AAGCCAGAGCTGTGCAGATGAGTA-3'
5'-TGTCCTGCAGCCACTGGTTC-3'

**IL-8**

5'-ACACTGCGCCAACACAGAAATTA-3'
5'-TTTGCTTGAAGTTTCACTGGCATC-3'

**GAPDH**

5'-GCACCGTCAAGGCTGAGAAC-3'
5'-TGGTGAAGACGCCAGTGGA-3'.

The conditions for the real-time PCRs were denatured at 95℃ for 3 seconds, and then the primer was annealed at 60℃ for 30 seconds 40 cycles. The data were analyzed using the Smart Cycler software program (Multiplate RQ version 1.00; TaKaRa), and the expression level of each gene was normalized by the corresponding GAPDH expression level.

**Enzyme-linked immunosorbent assay (ELISA)**

IL-6 and IL-8 released into culture supernatants was measured using a Human IL-6 ELISA kit (R&D Systems) and a Human IL-8/CXCL8 ELISA kit (R&D Systems), according to the manufacturer’s protocol.

**Statistical methods**

The values in each figure represent the mean ± standard deviation (S.D.) for each group. Values are shown as the means ± S. D. A Mann-Whitney’s U-test was used to compare the means of the groups. The values of \( p < 0.05 \) were considered to indicate a significant difference.

**Results**

**In vivo study**

**Body weights during the experimental period**

The body weights of the mice in both force groups decreased transiently on day 1 and then recovered. No significant differences between the two groups were observed (data not shown).

**Histological changes in dental pulp during tooth movement (H.E. staining)**

In the control group (0g), the mouse DP specimens were composed of relatively dense connective tissue fibers and fibroblasts that were sagittally aligned from the root dentin. The root surface was relatively smooth (Fig.3a). In the 25g group, there was a coarse arrangement of fibers and compressed blood capillaries. On day 9, several root resorption lacunae with multinucleated odontoclasts were recognized on the surface of the root (Fig.3b).

**Immunohistochemical findings of TRAP**

In the control group, resorption lacunae with TRAP-positive multinucleated odontoclasts were not observed on the surfaces of the root (Fig.3c). In the 25g group, several resorption lacunae with multinucleated TRAP-positive odontoclasts were observed on the surface of the root (Fig.3d).

**Protein localization of IL-17, IL-17R, IL-6 and KC**

The immunoreactivity for IL-17, IL-17R, IL-6 and KC was assessed nine days after tooth movement. IL-17, IL-17R, IL-6 and KC-positive cells were rarely observed in the control group (Figs.4A, B, b, e). In the 25g group, many IL-17, IL-17R, IL-6 and KC-positive cells were observed (Figs.4A, B, c, f).

**In vitro study**

**Effects of rhIL-17 on the mRNA expression of IL-6 and IL-8 by hDP cells, as determined by real-time PCR**

To evaluate the effects of rhIL-17 on the mRNA levels of IL-6 and IL-8 hDP cells were stimulated with rhIL-17 for 24 hour. The expression of IL-6 and IL-8 mRNA increased in comparison to the control in a dose-dependent manner at 3 hours after treatment (Figs.5a, b). The mRNA expression levels of IL-6 and IL-8 were significantly increased in a time-dependent manner, and peaked at 3 hours after the initiation of rhIL-17 stimulation (Figs.6a, b).

**The effects of rhIL-17 on the release of IL-6 and IL-8 by hDP cells, as determined by an ELISA**

The release of IL-6 and IL-8 hDP cells in response to rhIL-17 treatment was evaluated by ELISA. When hDP cells were subjected to rhIL-17 treatment for 72 hours, the releases of IL-6 and IL-8 were increased compared with the control cells, and this occurred in a time-dependent manner (Figs.6c, d).

**Discussion**

To investigate whether IL-17, IL-17R, IL-6 and IL-8 are involved in root resorption during orthodontic treatment, we induced root resorption by applying excessive orthodontic force in an animal model. Hartsfield JK Jr. et al. (12) reported that internal root resorption was induced by excessive orthodontic force. Internal root resorption is
caused by transformation of normal pulp tissue into inflammation pulp tissue with odontoclasts, which resorb dentin (13). The inflammation pulp induced expression of these cytokine (14). In the 25g group, immunoreactivity for IL-17 and IL-17R was detected in mouse DP tissues (Fig. 4A). Although IL-17 can be produced by various types of immune cells, including neutrophils and eosinophils, it is produced predominantly by CD4+ T cells (Th17 cells) (15). In fact, it was reported in a recent study that important acquired immune responses in periodontal tissue are specifically caused by CD4+ T cells (16). Therefore, this production of IL-17 has been considered to be the result of immune cells migrating from peripheral vessels. Force-induced tooth movement may lead to a reaction associated with early wound-healing events, such as macrophage invasion, cell proliferation and angiogenesis (17). Xiong et al. (18) demonstrated that the immunohistochemical localization of IL-17 was observed during the development of periapical lesions in rats. Oseko et al. (19) also reported that the expression of IL-17A mRNA was significantly induced in periapical lesions of wild-type mice after infection. Therefore, it is thought that the immune cells are the cause of the increased IL-17 production.

Immunoreactivity for IL-6 and KC was also detected in mouse DP tissues and odontoclasts in the 25g group (Fig. 4B). IL-6 is a multifunctional cytokine that plays an important role in osteoclastic bone resorption (20). Kurihara et al. (21) demonstrated that IL-6 stimulated the formation of early osteoclast precursors. Adebanjo et al. (22) reported that IL-6 activated mature osteoclasts. Furthermore, IL-6 stimulates osteoclastogenesis through stromal/osteoblastic cells (23).

IL-8 was detected in the gingival crevicular fluid from adult periodontitis (24) and orthodontic tooth movement (25). Recombinant human IL-8 directly stimulated human bone marrow mononuclear cells (HBMCs) to differentiate...
Fig. 4  The effects of orthodontic force on the expression of IL-17, IL-17R, IL-6 and KC, as determined by immunohistochemistry. Immunoreactivity for IL-17, IL-17R, IL-6 and KC was observed on the root surface and DP tissues (arrows) in the 25 g group (A, B; c, f).

Fig. 5  The effects of different concentrations of rhIL-17 on the IL-6 and IL-8 mRNA expression by DP cells. HDP cells were cultured with rhIL-17 (0-100 ng/ml) for 3 hours. The mRNA expression of IL-6 (a) and IL-8 (b) were significantly increased in a dose-dependent manner (*p < 0.05, **p < 0.01 Mann-Whitney U-test). The data are expressed as the means ± SD of four independent experiments.

Fig. 6  The effects of rhIL-17 on the production of IL-6 and IL-8 by hDP cells. The time course effects on the rhIL-17-induced mRNA expression of IL-6 (a) and IL-8 (b) were examined by real-time PCR. When hDP cells were cultured with rhIL-17 for up to 24 hours, the IL-6 and IL-8 mRNA expression was significantly increased compared with that in the control (*p < 0.01 Mann-Whitney U-test). The data are expressed as the means ± SD of four independent experiments.

The time course effect of the rhIL-17-induced IL-6 (c) and IL-8 (d) release, as determined by ELISA. When hDP cells were exposed to IL-17 for 24 h, the release of IL-6 and IL-8 was increased compared with that in the control cells, in a time-dependent manner (*p < 0.01 Mann-Whitney U-test). The data are expressed as the means ± SD of four independent experiments.
into osteoclast-like cells (26). Therefore, it has been suggested that IL-6 and IL-8 play important roles in bone resorption.

To investigate the mechanism responsible for the alterations in IL-6 and IL-8 induced by IL-17, the expression levels of IL-6 and IL-8 mRNA from hDP cells and the levels of IL-6 and IL-8 secretion stimulated by IL-17 were measured. The results indicated that IL-17 significantly increased the mRNA expression levels of IL-6 and IL-8 within 3 hours in a dose-dependent manner (Fig.5). The release of both IL-6 and IL-8 also increased in a time-dependent manner (Fig.6). Hot et al. (27) reported that IL-17 induced the expression of the IL-6 and IL-8 genes in human endothelial cells. Kotake and Kamatani (28) reported that IL-17 stimulates epithelial, endothelial and fibroblastic stromal cells to secrete IL-6 and IL-8. Zehnder et al. (29) reported that IL-6 and IL-8 were expressed at significantly higher levels in symptomatic vital teeth with severe caries lesions compared to clinically healthy pulp, and a concordance test for independence revealed a significant correlation between the IL-6 and IL-8 mRNA levels. Regarding PDL tissues, Asano et al. (7) reported that IL-8 (CINC-1) and MCP-1 may facilitate the process of root resorption arising due to excessive orthodontic force. In addition, Hayashi et al. (8) reported that the IL-17 induced by excessive orthodontic force stimulates odontoclastogenesis through IL-6 production. It is therefore possible that the incidence of root resorption may involve DP cells, as well as PDL tissues.

Uchiyama et al. (30) reported that dental pulp and periodontal ligament cells are involved in regulating the differentiation and function of osteoclasts via their regulation of the expression of RANKL and OPG. Zhu et al. (31) indicated that deciduous dental pulp stem cells influence osteoclastogenesis during the physiological root resorption process by changing the RANKL/OPG expression ratio. A previous study (5) showed that HDPF obtained from patients with severe OIIRR produced large amounts of IL-1β, IL-6, and TNF-α after neuropeptide stimulation and also stimulated osteoclast formation. Kotake et al. (32) reported that IL-6 stimulated osteoclast formation. Moreover, Hashizume and Mihara (33) reported that IL-6 causes bone resorption by inducing osteoclast formation via the induction of RANKL in synovial cells. Bendre et al. (34) similarly demonstrated that IL-8 stimulated osteoclast differentiation and activity via RANKL mRNA expression. Taken together, these findings and our present results suggest that the increased IL-6 and IL-8 secretions from DP cells induced by IL-17 may partially activate osteo/odontoclastogenesis via the induction of RANKL.

In this study, IL-6, IL-8 and IL-17 in DP tissues were induced by heavy orthodontic force. Furthermore, IL-17 stimulated the production of IL-6 and IL-8 from HDP cells in vitro.

Conclusions

The results of this study suggest that the induction of IL-17 may aggravate the process of root resorption via the production of IL-6 and IL-8 from DP in the presence of excessive orthodontic force.

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