Immunohistochemical Localization of T-helper 17 Cells, IL-17, and RANKL during Root Resorption Induced by Excessive Orthodontic Force in the Mouse Model of T Cell-mediated Autoimmune Disease

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Abstract

Orthodontically induced inflammatory root resorption is a common complication associated with orthodontic tooth movement. The aim of this study was to investigate how T-helper (Th) 17 cells and interleukin (IL)-17 contribute to root resorption during orthodontic tooth movement. Fifteen male 8-week-old SKG/Jclmice (rheumatoid arthritis (RA) group) and 15 male 8-week-old BALB/cAJcLmice (wild type group) 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 cathepsin K, IL-17, IL-17 receptor (IL-17R), receptor activator of nuclear factor kappa B (RANK) ligand (RANKL), and RANK proteins were determined in the resorbed lacunae of the mouse periodontal ligament (PDL) by an immunohistochemical analysis. Following the experimental tooth movement in vivo, resorption lacunae with multinucleated cells were observed in both groups. The immunoreactivities for cathepsin K, IL-17, IL-17R, RANKL, and RANK in the RA group were found to be significantly increased in the PDL tissue subjected to the orthodontic force on day 9 compared with the control group. The double-immunofluorescence analysis for IL-17/CD4 detected immunoreactivity in the PDL. The results of this study suggest that the Th17 cell response to excessive orthodontic force may lead to the progression of root resorption by increasing the expression of IL-17, RANKL, and RANK.

Introduction

External apical root resorption (EARR) is an unavoidable pathologic consequence of orthodontic tooth movement. It can be defined as an iatrogenic disorder that occurs, unpredictably, after orthodontic treatment, whereby the resorbed apical root portion is replaced with normal bone. EARR is a sterile inflammatory process that is extremely complex and involves various disparate components including mechanical forces, tooth roots, bone, cells, surrounding matrix, and certain known biologic messengers (1). Killiany (2) reported external apical root resorption of 3mm to occur at a frequency of 30%, with only 5% of treated individuals found to have 5mm of root resorption. The etiology of external apical root resorption following orthodontic treatment is not fully understood. In the last 10 years, interestingly, it was suggested that individual susceptibility (3, 4), genetics (5–7), and systemic factors (8) were risk factors for EARR.

However, the definite cause of resorption has not yet been identified. Recently, Nishioka et al. (9) found both allergy and asthma to be risk factors for orthodontic root resorption. Nigul et al. (10) also reported that allergic patients had more resorption than non-allergic patients. These data suggest that the immune system may be involved in the occurrence of EARR.

It is worth noting that an abnormal activation of the immune system leads to bone destruction in diseases such as rheumatoid arthritis (RA), and animal models deficient in immunomodulatory molecules often develop unexpected skeletal phenotypes. Takayanagi (11) concluded that accumulating evidence lends support to the theory that
interleukin (IL)-17-producing T-helper (Th) cells induce the expression of receptor activator of nuclear factor kappa B (RANK) ligand (RANKL) in synovial cells, which, together with inflammatory cytokines, stimulates the differentiation and activation of bone-resorbing osteoclasts.

The IL-17 cytokines constitute a six-member family (IL-17A-F) that is central for adaptive immune responses (12). They are products of the Th17 subset of CD4+ T lymphocytes, which have a high IL-17-dependent osteoclastogenic activity (13). IL-17A was initially found to stimulate osteoclastogenesis in mixed hematopoietic cell/osteoblasts cultures via prostaglandin synthesis and RANKL expression (14). Moreover, IL-17A mediates the activation of osteoclasts and bone destruction in joints affected by rheumatoid arthritis.

In addition, IL-17 induces RANKL production by osteoblasts, and was shown to be related to bone destruction in periodontitis (15, 16). Moreover, it has been shown that compressive force stimulates the expression of the IL-17 genes and their receptors in MC3T3-E1 cells, and also results in the induction of osteoclastogenesis (17). Hayashi et al. (18) also reported that the IL-17 induced by excessive orthodontic force stimulates odontoclastogenesis in normal rat periodontal ligament (PDL) tissues. Therefore, IL-17 may contribute to EARR during orthodontic tooth movement.

However, little is known about the relationship between the immune system and EARR in the PDL tissues. In this study, the immunolocalization of IL-17, IL-17R, RANKL, and RANK in a mouse model of T cell-mediated autoimmune disease (RA mouse) was investigated with regard to its role in root resorption during experimental tooth movement due to the application of a heavy force.

Materials and Methods

Animal studies

The animal experimental protocol used in this study was approved by the Ethics Committee for Animal Experiments at the Nihon University School of Dentistry at Matsudo (approval No. ECA-08-0039). Fifteen male 8-week-old BALB/cAJcL and 15 male 8-week-old SKG/Jcl mice (CLEA Japan, Inc., Tokyo, Japan, body weight 20 ± 5g) were used for the experiments. For each strain, mice were randomly assigned into two groups; control group, where mice received no appliances, and orthodontic force (OF) group, where mice subjected to the excessive orthodontic force.

Mice per strain in each group were as follows: BALB/cAJcL (control=7, OF=8), and SKG/Jcl (control=7, OF=8). Animals were maintained at the animal center of Nihon University School of Dentistry at Matsudo in separate cages in a 12-hour light/dark environment at a constant temperature of 23°C, and were provided with food and water ad libitum. The health status of each mouse was evaluated by daily body weight monitoring for one week before the start of the experiments.

Application of orthodontic devices

Animals were anesthetized with thiamylal sodium (15 mg/kg body weight) during the application of orthodontic devices. Experimental tooth movement was induced using the method reported by Yoshimatsu et al. (19), with a Ni-Ti closed-coil spring (Tomy International, Inc., Fukushima, Japan) inserted between the upper incisor and the upper-right first molar, and the device was fixed with a 0.008 inch stainless steel ligature wire around both teeth by means of a dental adhesive agent (Transbond XT, 3M Unitek, Monrovia, CA, USA). The upper first molar was moved mesially by the closed coil spring with a force of 25g. The force was applied for 9 days (Fig.1).

Tissue preparation

The experimental period was set at nine days after tooth movement was initiated. The animals were deeply anesthetized using a thiamylal sodium and then were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution, after which, the maxilla was immediately

![Fig.1 Experimental tooth movement was induced by a Ni-Ti closed-coil spring inserted between the upper incisor and the upper-right first molar, which was fixed with a 0.008 inch stainless steel ligature wire around both teeth by means of a dental adhesive agent. The upper first molar was moved mesially by the closed coil spring with a force of 25g. The force was applied for 9 days.](image_url)
dissected and immersed in the same fixative for 18 hours at 4°C. The specimens were decalcified in a 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for four weeks at room temperature (RT), and the decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin using the usual methods for preparation. Each sample was sliced into 4μm serial sections in the horizontal direction, and then was prepared for hematoxylin and eosin (H.E.) staining, and also for immunohistochemical staining. The periodontal tissues in the mesial part of the distal buccal root of a first upper molar were observed (Fig. 2). The sites of observation were based on the report by Yoshimatsu et al. (19). The root length between the bifurcation surface and the apical end of a mouse first molar approximately 600μm. There suggests that approximately 300μm of the root from the bifurcation surface at the mesial side is the pressure side during tooth movement. In addition, the alveolar crest is about 80μm away from the bifurcation surface. Thus, horizontal sections from five levels of the root: 100, 140, 180, 220, and 260μm away from the bifurcation surface were prepared. These sections were used to count the number of each immunohistochemical staining. The ratio of positive cells/all cells were expressed as the mean from the five sections obtained from the five levels of the root.

**Immunohistochemistry**

Immunohistochemical staining was performed as follows: The sections were deparaffinized, and the endogenous peroxidase activities were quenched by incubation in 3% H₂O₂ in methanol solution for 30 minutes at room temperature. After washing in tris-buffered saline (TBS) solution, the sections were incubated with a polyclonal anti-cathepsin K antibody, polyclonal anti-IL-17 antibody, polyclonal anti-IL-17R antibody, polyclonal anti-RANKL antibody, or polyclonal anti-RANK antibody (Santa Cruz Biotechnology, Santa Cruz, CA).
Cruz, CA, USA; working dilution, 1:100) for 18 hours at 4°C. The Histofine Simple Stain MAX-Po (G) kit was used for the staining of cathepsin-K and RANKL and the MAX-Po (G) kit was used for the staining of IL-17, IL-17R and RANK. The sections were rinsed with TBS and the final color reactions were performed using the 3,3'-diaminobenzidine tetra-hydrochloride substrate reagent, and the sections were then counter-stained with hematoxylin.

**Double immunofluorescence staining**

For double immunofluorescence staining, the sections were deparaffinized and washed in TBS, then the sections were incubated with a monoclonal anti-mouse CD4 antibody (Biolegend, San Diego, CA, USA; working dilution, 1:100) for 18 hour at 4°C. Subsequently, CD4 staining was performed using Alexa fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA; working dilution, 1:100) for 1 hour at RT. The sections were then incubated with a polyclonal anti-rabbit IL-17 IgG (Abcam, Tokyo, Japan; working dilution, 1:100) for 1 hour at RT. and IL-17 staining was performed using Alexa fluor 568 goat anti-rabbit IgG (Invitrogen). After washing the sections with TBS, all were counter-stained with 4'6-diamindino-2-phenylindole (DAPI) (Invitrogen) and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Statistical methods**

The values in each figure represent the means ± standard deviation (S.D.) for each group. Intergroup comparisons of average values were performed by a one-way analysis of variance (ANOVA), followed by Tukey’s test, with values of p < 0.05 considered to indicate a significant difference.

**Results**

**Body weights during the experimental period**

The body weights of the mice in both groups subjected to the 25g force decreased transiently on day 1 and then recovered. No significant differences between the two groups were observed (data not shown).

**Histological changes in periodontal tissues during tooth movement (H.E. staining)**

In the control (0g) wild type and RA groups, the mouse PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that were horizontally aligned from the root cement. The root surface was relatively smooth, with few mononuclear and multinucleated osteoclasts (Figs.2a and 2c). In the wild type group subjected to the excessive orthodontic force (25g), there was a coarse arrangement of fibers and compressed blood capillaries. On day 9, several root resorption lacunae with multinucleated cells were recognized on the surface of the root (Figs.2b and 2d).

**Immunohistochemical findings of Cathepsin K**

In the control (0g) wild type and RA groups, resorption lacunae with cathepsin K-positive cells were rarely observed (Figs.3a and 3c). In the wild type mice subjected to the excessive orthodontic force (25g), several cathepsin K-positive cells were observed in the PDL on day 9 (Fig.3b). In the RA group subjected to the excessive orthodontic force (25g), the number of cathepsin K-positive cells were higher than that in the wild type mice (Fig.3d).

A quantitative evaluation showed that the ratio of cathepsin K-positive cells/all cells was significantly increased in the RA group on day 9 compared with the wild type mice (p < 0.01) (Fig.3e).

**Protein expression levels of IL-17, IL-17R, RANKL, RANK**

The immunoreactivity for IL-17, IL-17R, RANKL, and RANK was examined on day 9 after tooth movement. IL-17-positive cells was rarely observed in the control (0g) wild type mice (Fig.4a), while the levels in this group were increased by excessive orthodontic force (Fig.4b). In the control (0g) RA mice, some IL-17-positive cells were observed in the PDL tissues (Fig.4c), and the levels in the RA group were also increased by the application of the excessive orthodontic force (Fig.4d). In the RA group subjected to the excessive orthodontic force (25g), the number of IL-17-positive cells increased in comparison to that of the wild type group exposed to the force (p < 0.01) (Fig.4e).

IL-17R-positive cells was observed in all groups (Figs.5a-d). In comparison to the wild type groups (Figs.5a and 5b), the RA groups (Figs.5c and 5d) showed significantly increased expression. Both the wild type groups (Figs.5a and 5b) and the RA groups (Figs.5c and 5d) showed a tendency to increased by the application of the excessive orthodontic force, but there was no significant difference between the groups (Fig.5e).

RANKL-positive cells was observed in all groups (Figs. 6a-d). In comparison to the wild type groups (Figs.6a and
6b), the RA groups (Figs. 6c and 6d) showed significantly increased expression. Both the wild type groups (Fig. 6a and 6b) and the RA groups (Fig. 6c and 6d) showed a tendency to increased by the application of the excessive orthodontic force, but there was no significant difference between the groups (Fig. 6e).

RANK-positive cells was rarely observed in the control (0 g) wild type mice (Fig. 7a), but the expression levels were increased by the exposure to the excessive orthodontic force (Fig. 7b). In the control (0 g) RA group, some RANK-positive cells were observed in the PDL tissues (Fig. 7c), and their expression was also increased by the application of the excessive orthodontic force (Fig. 7d). In the RA group subjected to the excessive orthodontic force (25 g), the number of RANK-positive cells increased in comparison to that in the wild type mice (p < 0.01) (Fig. 7e).

Double immunofluorescence findings of Th17 cells

In the control (0 g) mice in both groups, CD4- and IL-17-positive (Th17) cells were rarely observed on day 9 (Figs. 8a and 8c). In the wild type group subjected to the excessive orthodontic force (25 g), several CD4- and IL-17-positive cells appeared in the PDL tissues (Fig. 8b). In the RA group subjected to the excessive orthodontic force (25 g), however, many CD4- and IL-17-positive cells were observed in the PDL tissue (Fig. 8d).
Discussion

The SKG/Jcl mouse is suggested to be an appropriate animal model for human RA (20). These mice spontaneously develop T cell-mediated chronic autoimmune arthritis as a consequence of a mutation in the gene encoding the Src homology 2 domain of the ζ-associated protein of 70kDa (ZAP-70), a key signal transduction molecule in T cells (21-23). Clinically, mice demonstrate swelling of the joints, and histologically severe synovitis with joint destruction consistent with the cytokine imbalance seen in human RA (24). Komatsu et al. (25) have shown that CD4+ T cells, especially IL-17-producing Th17 cells, play an important role in RA development. Furthermore, recent studies reported that Th17 cells and IL-17 might play a role in the pathogenesis of periodontitis (26, 27). Therefore, studying orthodontic root resorption using the SKG/Jcl mouse will provide important information about the involvement of Th17 cells and IL-17 in EARR. In the present study, we investigated whether Th17 cells and IL-17 were involved in root resorption by applying an excessive orthodontic force in SKG/Jcl mice. Al-Qawasmi et al. (28) demonstrated the osteoclastic resorption of roots on the pressure side surfaces of teeth subjected to heavy orthodontic force (25g). Therefore, in the present study, 25g was used as the

Fig.4 A comparison of the IL-17-positive cells in the RA model and the wild type mice exposed to the excessive orthodontic force by immunohistochemistry (original magnification 200×). The IL-17-positive cells appeared in the PDL for all groups (a-d). IL-17-positive cells were rarely observed in the control (0g) wild type mice (a), but the number of cells was increased by the application of the heavy force (b). In the control (0g) RA group, some IL-17-positive cells were observed in the PDL tissues (c), and the number in the RA mice was also increased by the heavy force (d). In the RA+OF group, the number of IL-17-positive cells increased in comparison to that in the wild type mice exposed to the force (b and d). A quantitative evaluation showed that the ratio of IL-17-positive cells/all cells was significantly increased in the RA+OF group compared with the wild type mice (p < 0.01) (e).

The results of our study showed that immunoreactivity for cathepsin K was detected in PDL tissues on day 9 after the initial application of the force, and the immunoreactivity in the RA group was higher than that in the wild type mice (3). Cathepsin K is a protease that is primarily responsible for the degradation of the bone matrix by osteoclasts (29). Domon et al. (30) demonstrated that cathepsin K mRNA was detected in root resorption during tooth movement in rats. Yoshimatsu et al. (19) reported that the expression level of cathepsin K mRNA greatly increased for application of excessive OF.

This study also showed a similar result in RA group which were given excessive orthodontic force. However wild type group showed a tendency to increase, but there was no significant difference between the groups. In addition, RA association with root resorption was assumed from the presence of a number of cathepsin K-positive cells in the RA group.

On day 9, immunoreactivity for IL-17 and IL-17R were detected in the PDL tissues exposed to the force, and the immunoreactivities in the RA group were higher than those in the wild type group (Figs. 4 and 5). RANKL and RANK immunoreactivities were also strongly detected in the PDL tissues in the RA group (Figs. 6 and 7). Oshiro et al. (31) suggested that osteoblast/stromal cells and PDL fibroblasts were involved in supporting osteoclast differentiation during tooth movement. Odontoclasts, which were responsible for

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**Fig. 5** An immunohistochemical comparison of the IL-17R-positive cells in the RA model and wild type mice subjected to the excessive orthodontic force (original magnification 200×). The IL-17R-positive cells appeared in the PDL for all groups (a-d). In the wild type + OF group, the number of IL-17R-positive cells was increased compared with that of the control (0g) mice (a and b). Similarly, in the RA + OF group, the number of IL-17R-positive cells was increased compared with that in the control (0g) group (c and d). A quantitative evaluation showed that the ratio of IL-17-positive cells/all cells was significantly increased in the wild type + OF and RA + OF groups compared to the control (0g) groups (p < 0.01) (e).

the resorption of teeth, are thought to be derived from mononuclear precursors of the monocyte/macrophage lineage, and demonstrate characteristics similar to osteoclasts, which are responsible for bone resorption (32). Cathepsin K-positive cells on the root surface or in the root resorption lacunae were judged to be odontoclasts. Zhang et al. (17) reported that compressive force induced the expression of IL-17 and its receptor in osteoblast-like cells, and that the IL-17 and IL-17R produced in response to compressive force might affect osteoclastogenesis.

Considering the relationship between IL-17 and RANKL/RANK, Okamoto et al. (33) concluded from their review that, with regard to the mechanisms underlying the bone destruction found in RA, accumulating evidence lends support to the theory that IL-17-producing Th17 cells induce the expression of RANKL in synovial cells, which in turn stimulates the differentiation and activation of osteoclasts, together with inflammatory cytokines. IL-17 was shown to upregulate RANK on human osteoclast precursors in vitro, leading to increased sensitivity to RANKL signaling, osteoclast differentiation and bone loss (34). Nakano et al. (35) reported that RANKL/RANK may facilitate the process of root resorption caused by excessive orthodontic force. In addition, Hayashi et al. (18) reported that the IL-17 induced by excessive orthodontic force stimulates odontoclastogenesis through IL-6 production. Taken together, these findings and our present results suggest that the increased IL-17 released from Th17 cells and the RANKL/
RANK secretion in PDL tissues induced by excessive orthodontic force may activate odonto/osteoclastogenesis. Therefore, IL-17 and RANKL/RANK may stimulate the differentiation of odonotoclasts from osteoclast progenitor cells.

Finally, in the wild type mice, several CD4- and IL-17-positive (Th17) cells were detected in the compressed PDL tissues on day 9 (Fig.8b). In the RA group, however, many of these cells were observed in the compressed PDL tissue outside of the area of the resorbed root surface (Fig.8d). Th17 cells constitute a third subset of T helper cells that are important in the development of autoimmune diseases and the immune response against infections. IL-17 is produced predominantly by CD4+ T cells (Th17 cells). In a recent study, it was reported that important acquired immune responses in periodontal tissue are specifically caused by CD4+ T cells (36). However, IL-17 can also be produced by other immune cells, such as neutrophils and eosinophils (37). Zhang et al. (38) reported that compressive force induces the expression of IL-17 and its receptor in osteoblast-like cells, and that the IL-17 and IL-17R produced in response to compressive force may affect osteoclastogenesis. Thus, this production of IL-17 has been considered to be the result of immune cells migrating from peripheral vessels. When an orthodontic force is applied, local tissue ischemia and the microenvironment enhance cell damage, and necrotic tissue appears in the compressed root surface adjacent to the PDL (39). Subsequently, immune cells accumulate to remove the necrotic tissue (40). It is thought that these immune cells are the cause of the increased IL-17 production.

In conclusion, T-helper 17 cells response to excessive orthodontic force may progress the process of root

Fig.7 A comparison of the RANK-positive cells in the RA model and wild type mice subjected to the excessive orthodontic force by immunohistochemistry (original magnification 200×). The RANK-positive cells appeared in the PDL for all groups (a-d). In the RA + OF group, the number of RANK-positive cells increased in comparison to that of the wild type mice (b and d). A quantitative evaluation showed that the ratio of RANK-positive cells/all cells was significantly increased in the RA + OF group compared with the normal group (p < 0.01) (e).

resorption via IL-17, RANKL, and RANK expressions.

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References


