Original article

Effect of interleukin-4 on orthodontic tooth movement and associated root resorption

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Summary

Objectives: Interleukin-4 (IL-4) is a recognized immunomodulatory cytokine that regulates bone homeostasis. However, the influence of IL-4 on orthodontic tooth movement (OTM) and subsequent root resorption is still unknown. Therefore, the purpose of this study was to investigate the effect of IL-4 on tooth movement and its associated root resorption in a mouse model.

Materials and methods: The maxillary first molars of four male mice for each experimental group were subjected to mesial force by a nickel titanium coil spring for 12 days. Control mice were not given appliances and injections. Varying doses of IL-4 were injected locally, adjacent to the first molar. Two sets of experiments were designed. The first set was composed of three groups: the control, treatment with phosphate-buffered saline (PBS), or 1.5 µg/day of IL-4. The second set was composed of five groups: the control, treatment with 0 (PBS only), 0.015, 0.15, or 1.5 µg/day of IL-4. The distance of OTM was measured and tartrate-resistant acid phosphatase positive cells along the loaded alveolar bone and root surface were identified. The root resorption associated with OTM was evaluated by a scanning electron microscope.

Results: The amount of OTM and the number of osteoclasts were significantly decreased in the IL-4-treated mice. Moreover, IL-4 significantly suppressed force-induced odontoclasts and root resorption.

Conclusion: IL-4 inhibits tooth movement and prevents root resorption in the mouse model. These results suggest that IL-4 could be used as a useful adjunct to regulate the extent of OTM and also to control root resorption.

Introduction

Orthodontic tooth movement (OTM) is dependent on remodelling of the periodontal ligament (PDL) and alveolar bone upon the application of an external force. These force-induced strains activate numerous molecules, such as neurotransmitters, cytokines, growth factors, and bone matrix constituents. Some of these molecules mediate the differentiation and function of osteoclasts and osteoblasts, resulting in bone resorption and apposition, respectively (1–5).

Osteoclasts are multinucleated cells that are derived from haematopoietic stem cells. Two cytokines are required for osteoclast formation: macrophage colony-stimulating factor and the receptor activator of nuclear factor kappa-B ligand (RANKL) (6). Tumour necrosis factor-α (TNF-α) is another cytokine that impacts osteoclastogenesis and is capable of the direct differentiation of osteoclast precursors to osteoclasts in vitro (7–9) and in vivo (10,11). It has been demonstrated that orthodontic force induces the expression of TNF-α (12). Moreover, it has been reported that TNF-α plays a significant role in the mechanical loading-induced OTM by using TNF receptor-deficient mice, which exhibited less tooth movement compared with that observed in the wild-type mice (13–15).
Several lymphocyte-derived cytokines play critical roles during bone metabolism under physiological and pathological conditions (16). Interleukin-4 (IL-4) is a pleiotropic cytokine secreted by activated T lymphocytes, mast cells, eosinophils, and basophils. It is a key regulator in humoral and adaptive immunity that regulates the function of lymphocytes and macrophages (17). IL-4, secreted by T helper type 2 (TH2) cells, is a potent inhibitor of the RANKL-induced osteoclastogenic process (18). The effect of IL-4 in osteoclastogenesis is supported by the finding that over-expression of IL-4 in vivo prevents bone erosion in animal models of inflammatory arthritis (19,20). Furthermore, the administration of IL-4 to the mice in vivo inhibits hypercalcaemia induced by parathyroid hormone-related peptide (21) and bone loss caused by ovariectomy (22). Additionally, we reported that IL-4 inhibited TNF-α-mediated osteoclast formation in vitro (23) and in vivo via both TNF-α-activated stromal cells and TNF-α-activated osteoclast precursors (24). However, there is a scarcity of information with regard to the effect of IL-4 on osteoclast formation and bone resorption associated with OTM.

Therefore, in this study, we examined the influence of IL-4 on OTM in a mouse model. Moreover, we investigated the effect of IL-4 on root resorption, which is an unavoidable pathologic consequence of OTM.

Materials and methods

Experimental animals

The protocols for all animal procedures were in accordance with Tohoku University regulations. Ten- to twelve-week-old male C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) for use in this study. During the experiment, the mice were kept in cages in a room maintained at 21–24°C with a 12-hour/12-hour light/dark cycle and were fed a granular diet (Oriental Yeast, Tokyo, Japan) to prevent them from exercising an excessive chewing force.

Experimental tooth movement

OTM was performed as described previously (25). In brief, the mice were anaesthetized with pentobarbital sodium and a nickel titanium closed coil spring (Tomy, Fukushima, Japan) was placed between the upper anterior alveolar bone and the left first molar to move the molar in a mesial direction. The appliance was fixed with a stainless steel wire (0.01 mm diameter) to a hole drilled through each of the two incisors at the alveolar bone level and tied to the first molar at the posterior end (Figure 1A). According to the manufacturer, the force level of the coil spring after activation was approximately 10 g. Recombinant mouse IL-4 (R&D Systems, Minneapolis, Minnesota, USA) dissolved in phosphate-buffered saline (PBS; 20 μl) was injected into the buccal site around the upper left first molar by using a 0.5 ml syringe with a 30 G 10 mm needle (Nipro, Osaka, Japan). For histological analysis of the effect of IL-4 on tooth movement, three groups were created: mice without an appliance (control group) and mice with activated coil springs (experimental group) treated with PBS or with 1.5 μg/day of IL-4 every other day for 12 days. For evaluation of the dose-dependent effect of IL-4 on tooth movement and the subsequent root resorption, five groups were created: mice without appliances (control group) and mice with activated coil springs (experimental group) treated every other day for 12 days with different doses of IL-4: 0 (PBS only), 0.015, 0.15 or 1.5 μg/day. Four mice were used for each group.

Measurement of tooth movement

After 12 days of tooth movement, the animals were deeply anaesthetized and perfused with 0.02 M PBS (pH 7.2) and then with 4 per cent paraformaldehyde in 0.1 M PBS. After dissection of the maxillary region, impressions of the teeth and maxillae were obtained with the use of individual trays containing hydrophilic vinyl polysiloxane impression material (EXAFAST Injection Type, GC Co., Tokyo, Japan). The samples were fixed in 4 per cent paraformaldehyde after the impressions were obtained. The amount of tooth movement was evaluated by measuring the closest distance between the first and the second molars in the impression under a stereoscopic microscope (VH-7000; Keyence, Osaka, Japan; Figure 1B). The left side of the maxillae of four mice was used for evaluation in each of the groups. For each mouse, the measurement was taken four times, and the mean value was used.

Three-dimensional reconstruction images

Microfocal computed tomography (ScanXmate-E090; Comscan, Kanagawa, Japan) was used in all experimental animal groups to clarify the changes in their tooth movement. The TRI/3D-BON64 software (RATOCS System Engineering, Tokyo, Japan) was used to generate three-dimensional (3D) reconstruction images of the maxilla.

Preparation for histological observation

The fixed teeth and maxillae were decalcified in 14 per cent ethylenediaminetetraacetic acid for 21 days at room temperature. After dehydration, the samples were embedded in paraffin and sectioned in the horizontal plane at 4 μm thickness. Apical to the first molar bifurcation area, we prepared horizontal sections from the distobuccal root at five levels: 100, 140, 180, 220, and 260 mm. These sections were deparaffinized, stained for tartrate-resistant acid phosphatase (TRAP) activity, and counterstained with haematoxylin. For the TRAP staining, the sections were incubated in an acetate buffer (pH 5.0) containing naphthol-ASMX-phosphate (Sigma, St Louis, Missouri, USA), Fast Red Violet LB Salt (Sigma), and 50 mM sodium tartrate. By using light microscopy, the TRAP-positive multinuclear cells, located in lacunae in the resorbed alveolar bone surface, were counted as osteoclasts. Conversely, we considered the TRAP-positive multinuclear cells located in lacunae in the resorbed root surface as odontoclasts. In all the five sections, the number of TRAP-positive cells was identified on the surface of the alveolar bone and the mesial side of the distobuccal root, and the mean values were calculated. Four mice were used for evaluation in each group.

Measurement of the root resorption area

After tooth movement, the first molars were carefully extracted by removing the parodontal tissues, including the soft tissue and alveolar bone around the first molars, by immerging in 2 per cent sodium hypochlorite for 10–30 minutes. After the surface of the distobuccal root was completely clear, the mesial side of the root was observed under a scanning electron microscope (TM-1000; Hitachi, Tokyo, Japan). For standardizing the orientation of the samples, the tooth was rotated distopalatally until it was placed on the distal roots. The mesial side of the distobuccal root was used for evaluation. As described previously (26,27), the ratio of the root resorption area was calculated using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).
Furthermore, we evaluated the root resorption area by the percentage of the resorbed surface on the pressure side of the distobuccal root of the first molar after OTM.

**Statistical analysis**

All data are presented as mean ± standard deviation values of independent replicates. We performed the Kruskal–Wallis test, followed by the Scheffe’s *F* test. Any *P* values less than 0.05 were considered significant. All analyses were conducted with Statcel3 software (The Publisher OMS Ltd, Saitama, Japan).

**Results**

**Effect of IL-4 on the amount of tooth movement**

After 12 days of mechanical loading, the distance of tooth movement in the mice that received PBS was 0.177 ± 0.055 mm. The distance of tooth movement was significantly reduced to 0.045 ± 0.012 mm in those injected with 1.5 µg of IL-4. No significant difference was observed between the control and the 1.5 µg IL-4-injected groups (Figure 1C and 1D).

**Effect of IL-4 on the number of TRAP-positive cells along the mechanically loaded alveolar bone**

TRAP staining was performed on tissue sections from the distobuccal root of the first molar before and after experimental tooth movement and with and without IL-4 injections. In the control group, the TRAP-positive cells were not located along the alveolar bone on the mesial side of the root. After 12 days of tooth movement, osteoclastic cells were observed abundantly on the mesial side of the alveolar bone in the mice that received PBS (11.95 ± 3.37 cells/section). The mice injected with 1.5 µg of IL-4 demonstrated a significantly reduced number of osteoclastic cells.

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**Figure 1.** Orthodontic appliance and effect of interleukin-4 (IL-4) on amount of tooth movement after loading. (A) Intra-oral photograph of the appliance placed between the anterior alveolar bone and left first molar. (B) Photograph of the impression of the upper left molars under stereoscopic microscope taken after tooth movement. The distance of tooth movement (black double arrow) was measured between the distal marginal ridge of the first molar and mesial marginal ridge of the second molar at the level connecting the central fossae of the first and second molars (dash line). (C) Intra-oral photographs of the upper left molars after 12 days of experimental loading with administration of phosphate-buffered saline (PBS) or 1.5 µg of IL-4, and the control (unloaded). Representative micro-computed tomography images clarifying the experimental tooth movement for each group. Occlusal (left) and sagittal (right) views were obtained. (D) Graphs illustrating the distance of tooth movement after 12 days of experimental loading with administration of PBS or 1.5 µg of IL-4, and the control (unloaded). *n* = 4 for each group. **P < 0.01**
(6.85 ± 1.77 cells/section) compared with the PBS-administered mice (Figure 2A and 2B).

Histological evaluation of the effect of IL-4 on mechanical load-induced root resorption

We evaluated odontoclast formation after 12 days of tooth movement. Odontoclasts were found on the root surface of the mesial side of the distobuccal root on the PBS-administered mice (2.13 ± 1.32 cells/section) and significantly reduced in those that received 1.5 μg of IL-4 (0.45 ± 0.13 cells/section). No significant difference was observed between the 1.5 μg IL-4 group and the control (Figure 3A and 3B). Furthermore, we assessed the root resorption area in the histological sections under a stereoscopic microscope. The transverse histological sections revealed the root resorption surface area to be significantly larger in the PBS-administered mice than in 1.5 μg IL-4-administered ones (26.62 ± 12 and 5.54 ± 4.25 per cent, respectively). The root resorption in the control mice was not significantly different to the 1.5 μg IL-4-administered mice (Figure 3C and 3D).

Effect of dose-dependent IL-4 on the amount of tooth movement

After 12 days of mechanical loading, the amount of tooth movement was observed to have decreased significantly once IL-4 reached a 0.15 μg level (Figure 4A). The mice administered with 1.5 μg of IL-4 showed the least distance (0.065 ± 0.009 mm) and this was significantly less than other examined groups. There was no substantial difference observed between the PBS and 0.015 μg IL-4-administered mice (0.172 ± 0.023 and 0.165 ± 0.017 mm, respectively), while both groups were significantly greater than the 0.15 μg IL-4-administered group (0.102 ± 0.008 mm; Figure 4B).

Effect of IL-4 on mechanical load-induced root resorption

We evaluated the orthodontic force-induced root resorption on the mesial side of the distobuccal root under a scanning electron microscope. After 12 days of tooth movement, the PBS-administered mice showed a broad root resorbed area along the pressure side of the distobuccal root (42.13 ± 2.73 per cent). The root resorbed area was significantly reduced in those injected with 1.5 μg of IL-4 (3.92 ± 2.61 per cent). The mice that received 0.15 or 0.015 μg of IL-4 showed root resorption (33.35 ± 6.98 and 33.15 ± 3.5 per cent, respectively) with no significant difference compared with the PBS group, but it was substantially greater than that in the 1.5 μg IL-4-administered mice (Figure 5A–5C).

Discussion

IL-4 is an important immune cytokine that regulates osteoclastogenesis and bone resorption. Despite that, its role on OTM remains unknown. In this study, for the first time, the effect of IL-4 on OTM and root resorption was investigated. The results showed that the administration of IL-4 significantly inhibited OTM and osteoclastogenesis when compared with the PBS-administered mice. Moreover, the mice treated with IL-4 showed significantly inhibited root resorption and odontoclastogenesis in contrast with the PBS-injected mice.

Osteoclast activation in response to mechanical stress is an integral mechanism in tooth movement. In this study, local administration of IL-4 decreased the number of TRAP-positive cells along the alveolar bone on the pressure side. This indicated a reduction in osteoclast formation, which consequently downregulated the tooth displacement rate in the mice treated with IL-4. We evaluated whether there is a systemic effect of IL-4 on osteoclastogenesis. We, therefore, analysed the histological sections of the side opposite to the IL-4-injection site. There was no difference in the TRAP activity of the mice injected with PBS and IL-4. Thus, IL-4 had no systemic effect on osteoclastogenesis on the side opposite to IL-4 injection in vivo (data not shown). Moreover, the present findings demonstrated that IL-4 of doses higher than 0.015 μg led to variations in the tooth movement rate with a significant inhibitory effect. In this study, we attempted to use microfocal computed tomography for the measurement of tooth movement. However, it was difficult to locate the reference points for reliably controlling the rotation angle of the 3D images. Therefore, the distance of tooth movement was measured directly from the impression under the stereoscopic microscope.

Oshiro et al. (28) have demonstrated the substantial need for RANKL signalling for OTM as shown by an increase in RANKL induction and osteoclastic resorption-induced tooth movement in the osteoprotegerin (OPG)-deficient mice. OPG is an inhibitory decoy receptor of RANKL. In addition, studies using TNF receptor-deficient mice have revealed a possible important role of TNF-α in inducing bone resorption during OTM (14). It is known that IL-4 suppresses RANKL and TNF-α-mediated osteoclast formation in vitro (18,23).

Figure 2. Histology of the horizontal sections of alveolar bone in the maxillary left first molar area. (A) Tartrate-resistant acid phosphatase (TRAP)-stained histological sections of the distobuccal root of the maxillary left first molar before (left panel) and after 12 days of experimental tooth movement with administration of phosphate-buffered saline (PBS; middle panel) or 1.5 μg of interleukin-4 (IL-4; right panel). Arrows indicate the direction of orthodontic force. (B) Graph demonstrating the numbers of TRAP-positive cells along the alveolar bone on the pressure (mesial) side before and after 12 days of tooth movement with administration of PBS or 1.5 μg of IL-4. n = 4 for each group (*P < 0.05 and **P < 0.01).
Figure 3. Evaluation of root resorption on the transverse histological sections. (A) Tartrate-resistant acid phosphatase (TRAP)-stained histological sections of the distobuccal root of the maxillary left first molar of unloaded (left panel) and loaded groups for 12 days with injection of phosphate-buffered saline (PBS; middle panel) or 1.5 μg of interleukin-4 (IL-4; right panel). Arrows indicate the direction of orthodontic force. (B) The numbers of TRAP-positive cells along the root surface on the pressure (mesial) side before and after 12 days of mechanical loading with injection of PBS or 1.5 μg of IL-4. n = 4 for each group (*P < 0.05). (C) Diagram demonstrating the evaluation of the root resorption surface on the transverse histological sections. Solid line represents the root surface and the interrupted line represents the resorption surface. The root resorption surface was measured by the percentage of interrupted line/solid line. (D) Ratio of the root resorption surface on the histological sections of unloaded (control) and loaded groups for 12 days with injection of PBS or 1.5 μg of IL-4, n = 4 for each group (***P < 0.01).

Figure 4. Dose-dependent effect of interleukin-4 (IL-4) on the amount of tooth movement. (A) Intra-oral photographs of the maxillary left molar areas after 12 days of experimental tooth movement with injection of phosphate-buffered saline (PBS) or different doses of IL-4 (0.015, 0.15, or 1.5 μg). (B) Distance between first and second molars after 12 days of tooth movement with injection of PBS or different doses of IL-4 (0.015, 0.15, or 1.5 μg). n = 4 for each group (*P < 0.05 and **P < 0.01).
Taking all these findings together with the present results, it is suggested that IL-4 might have inhibited osteoclast formation and tooth movement by disruption of the mechanisms of RANKL and TNF-α-induced osteoclastogenesis on the pressure side. Nevertheless, it is not clearly known whether IL-4 inhibits TNF-α and/or RANKL expression during OTM. Therefore, future studies are required to evaluate the expression of TNF-α and RANKL.

Root resorption is an unavoidable iatrogenic sequela of orthodontic treatment. Excessive force magnitude is a critical factor for root resorption (29,30). In this study, a force of 10g aggravated root resorption, and resorption lacunae with odontoclasts appeared on the root surface during OTM, in accordance with the findings of previous reports (25–27,31). These results suggest that the orthodontic force used in this study was excessive. Odontoclast-mediated root

![Image](image_url)
resorption possesses general morphology similar to osteoclasts. It is thought that the cellular mechanisms of bone resorption are quite similar to those of root resorption during tooth movement (32–35). In this study, TRAP-positive cells observed were not only located on the root surface but were also present in the PDL tissues. This is consistent with the reports of Brudvik and Rygh (32,36,37) where they further proposed that these TRAP-positive cells present in the PDL might participate in root resorption after the removal of hyalinized tissue. In this study, we found that local administration of IL-4 to the mice over 12 days of tooth movement inhibited root resorption and significantly reduced the number of odontoclasts when compared with the PBS-injected mice. This study showed that osteoclastogenesis and odontoclastogenesis might be regulated by a common osteo-immunological mechanism, as both bone and root resorption were inhibited simultaneously via IL-4.

The osteoclastogenic process underlying the periodontium is regulated by immune cell-derived cytokines. IL-4, a TH2 cytokine, is known to suppress synthesis of a number of proinflammatory cytokines. It has been suggested that localized lack of IL-4 in periodontal tissues could predispose the individual to periodontal disease development and progression (38). Indeed, IL-4 was observed in the healthy gingival tissue and was undetectable in periodontal disease (39,40). Furthermore, the detected levels of IL-4 did not differ between orthodontically treated young individuals and the untreated control group (41). Therefore, the orthodontic force in teeth with periodontal disease might aggravate osteoclastogenesis, owing to the absence of IL-4. Conversely, in another study, IL-4 was below the limit of detection at the compression and tension sides of canines before and after application of retraction force (42). These contradictory results could have been because of the different sensitivity of the techniques used for the identification of IL-4 molecules.

It has been suggested that the therapeutic use of IL-4 in some chronic inflammation, such as the transfer of IL-4, has been shown to be protective against bone erosion induced by collagen arthritis (19). In this study, local administration of IL-4 inhibited tooth movement and root resorption by suppression of underlying osteoclastogenesis and odontoclastogenesis, respectively. These results suggest a potential therapeutic role of IL-4 in controlling OTM and preventing orthodontic treatment-related root resorption. Within the limitations of this study, IL-4 might be useful for preventing mechanical stress-induced root resorption. However, because IL-4 simultaneously inhibits tooth movement, additional studies are needed.

In conclusion, the present findings demonstrate the inhibitory effect of IL-4 on tooth movement and its associated root resorption through suppression of osteoclastogenesis and odontoclastogenesis, respectively. Further studies are required to elaborate on the role of IL-4 during OTM.

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