Original Article

Effects of anti-mouse RANKL antibody on orthodontic tooth movement in mice

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Abstract Background/purpose: Orthodontic tooth movement is achieved by alveolar bone remodeling, and therefore the balance of bone resorption and formation is important. Receptor activator of nuclear factor-κB ligand (RANKL) plays a crucial role in bone resorption. We previously reported that tumor necrosis factor-α (TNF-α) is also important in bone resorption during tooth movement. In this study, we focused on bone and root resorption during orthodontic tooth movement in mice using anti-mouse RANKL antibody (anti-mRANKL ab).

Materials and methods: Anti-mRANKL ab was administered intraperitoneally to mice that subsequently underwent orthodontic tooth movement. After 10 days, tissues around the moved teeth were histologically evaluated. To confirm the effects of anti-mRANKL ab on TNF-α induced bone resorption, TNF-α was administered with and without anti-mRANKL ab into the supracalvaria and the sutures of the calvaria were histologically evaluated.

Results: Orthodontic tooth movement was suppressed in mice treated with anti-mRANKL ab. Root resorption was observed after orthodontic tooth movement, but not in mice treated with anti-mRANKL ab. In the calvarial experiment, the number of TRAP-positive cells in the calvarial sutures was lower in mice administered TNF-α with anti-mRANKL ab than in mice administered TNF-α alone.

Conclusion: Our findings suggest that anti-mRANKL ab suppressed orthodontic tooth movement. This needs to be considered when orthodontic tooth movement is required in patients using anti-RANKL antibody.

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Introduction

Skeletal mass is maintained through bone resorption and formation. The effective activity of osteoclasts and osteoblasts is important for maintaining bone homeostasis. Loss of this balance results in various diseases processes. Post-menopausal osteoporosis is a typical bone resorption disease. Estrogen deficiency in women leads to increased production of the receptor activator of nuclear factor-κB (RANK) ligand (RANKL), which is a key cytokine in osteoclastogenesis. Patients regularly treated with glucocorticoids for conditions such as autoimmune diseases and allergies often develop secondary osteoporosis. Glucocorticoids decrease sex steroid levels and increase parathyroid hormone levels, promoting the expression of RANKL. Metastatic cells in bone osteolytic tumor lesions also increase the RANKL level and induce hyperactivation of osteoclastogenesis. Thus, RANKL plays a central role not only in physiological bone resorption, but also in the disease processes described above. RANKL, which is mainly expressed by osteoclastogenesis-supporting cells such as osteoblastic cells and bone marrow stromal cells, binds to RANK on the surface of osteoclast precursors, and then induces osteoclastogenesis. Recently, Nakashima et al. reported that osteocytes also play a crucial role in RANKL expression.

Denosumab is a molecular-targeted drug consisting of a fully human monoclonal antibody for RANKL. It has a beneficial effect on inhibition of the RANKL-RANK interaction, and consequently reduces pathological bone resorption. Recently, the inhibitory effect of anti-mouse RANKL antibody (anti-mRANKL ab) on bone resorption in mice has been confirmed. A single intraperitoneal shot of the antibody contributed to increasing the trabecular bone mass in mice even after 4 weeks. Therefore, we hypothesized that an injection of anti-mRANKL ab would suppress orthodontic tooth movement in mice. We previously described several aspects of experimental tooth movement using a mouse model. Orthodontic tooth movement occurs as a result of remodeling of bone as loading-induced alveolar bone resorbs and forms around the tooth root. We have shown that tumor necrosis factor-α (TNF-α) plays an important role in osteoclastogenesis during orthodontic tooth movement. Garlet et al. reported that RNA levels of TNF-α and RANKL were more highly expressed in human periodontal ligament cells on the pressure side than on the tension side after orthodontic tooth movement. In a rat model, RANKL gene transfer to the periodontal tissue enhanced orthodontic tooth movement. Thus, RANKL also plays an important role in orthodontic tooth movement. The aim of this study was to investigate the effects of anti-mRANKL ab on orthodontic tooth movement in mice.

Materials and methods

Mice and reagents

Male C57BL6/J mice (aged 7–8 weeks) were purchased from CLEA Japan (Tokyo, Japan) and maintained in the animal facility of Nagasaki University. The animals were provided with solid food (MF diet; Oriental Yeast, Tokyo, Japan) and tap water. The protocols for all animal procedures were in accordance with regulations of the Animal Care and Use Committee of Nagasaki University. The experiments were approved by the President of the University (Approval Number: 1911071576, 07 November 2019). The neutralizing antibody against mouse RANKL was obtained from Oriental Yeast and recombinant mouse TNF-α was obtained from R&D Systems (Minneapolis, MN, USA).

Orthodontic tooth movement

The experimental protocol was based on previous studies and slightly modified. Briefly, the mice were anesthetized with a mixture of medetomidine, midazolam, and butorphanol, and a nickel titanium closed coil spring (Tomy, Fukushima, Japan) was placed between the incisors and the upper left first molar to move the first molar in a mesial direction (Fig. 1). The force level of spring was approximately 10 g. The upper right side was observed as a non-loaded control. Anti-mRANKL ab (100 μg) was injected intraperitoneally and tooth movement was started 21 days later. The anti-mRANKL ab non-injected group was injected with phosphate-buffered saline (PBS).

Observation and measurement of tooth movement

After tooth movement, tissues from the maxillae were fixed in 4% formaldehyde overnight, and then observed using μ-CT (R_mCt; Rigaku, Tokyo, Japan). The image acquisition conditions were as follows: radiograph source voltage, 90 kV; current, 150 μA; scanning time, 2 min; resolution, 20 μm/pixel. Tooth movement was evaluated by measuring the shortest distance between the first and second molars in the impression under a stereoscopic microscope (VH-7000; Keyence, Osaka, Japan) as in previous reports (n = 7).

Figure 1 Photograph of orthodontic tooth movement. A nickel titanium closed coil spring was placed between the upper incisors (U1) and the upper left first molar (M1) to move the molar in a mesial direction. The arrow indicates the direction of orthodontic tooth movement of M1.
Preparation for histological observation

After fixation, the samples were decalcified in 10% EDTA for 10 days at 4 °C, and embedded in paraffin. Horizontal 4 μm sections of the root of the upper first molar region were prepared as previously described. The sections were deparaffinized, stained for tartrate-resistant acid phosphatase (TRAP) activity and counterstained with hematoxylin. For TRAP staining, the sections were incubated in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma, St. Louis, MO, USA), Fast Red Violet LB Salt (Sigma) and 50 mM sodium tartrate. TRAP-positive cells on the mesial side of the distobuccal root of the upper first molar were counted in four sections per mouse and the mean values were calculated (n = 4).

Measurement of the root resorption area and observation of odontoclasts

The root resorption observed on the mesial side of the distobuccal root surface of the upper first molar was evaluated using TRAP-stained paraffin sections. The length of the root resorption area and the mesial root surface were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and ratio of resorption area/root surface area was calculated for four sections per mouse and the mean values were determined (n = 4). TRAP-positive cells adjacent to the root surface on the mesial side of the distobuccal root of the upper first molar were counted as odontoclasts in four sections per mouse and the mean values were calculated (n = 4).

TNF-α-induced osteoclastogenesis in calvaria

After being anesthetized with isoflurane inhalation, the mice were injected with anti-mRANKL ab (45 μg) in the vertex of the head. Two days after injection, 1.5 μg of TNF-α was injected in same place for 5 days. Anti-mRANKL ab or TNF-α non-injected groups were injected with PBS. After the TNF-α injection, tissues from the calvaria were fixed overnight with 4% paraformaldehyde at 4 °C, decalcified in 10% EDTA for 4 days at 4 °C, and 4 μm paraffin-embedded sections were prepared. The sections were deparaffinized, stained for TRAP activity and counterstained with hematoxylin. TRAP-positive cells in the calvarial sutures were counted in four sections per mouse and the mean values were calculated (n = 6).

Statistical analysis

All data are expressed as means ± SD. Statistical analyses were performed using Scheffe’s F tests. Values of P < 0.05 were considered to indicate statistically significant differences.

Results

Inhibitory effects of anti-mRANKL ab on the distance of orthodontic tooth movement

Ten days after tooth movement, the upper left first molar moved mesially and a space between the first and the second molar was observed (Fig. 2A). In the group treated with anti-mRANKL ab before orthodontic tooth movement, the space between the first and the second molar was significantly reduced (Fig. 2B).

Inhibitory effects of anti-mRANKL ab on TRAP-positive cells

In the TRAP-stained paraffin sections, TRAP-positive cells were observed on the mesial side of the distobuccal root of the upper first molar after tooth movement (Fig. 3A). Additionally, the number of TRAP-positive cells was significantly lower in the anti-mRANKL ab treated group compared with the untreated group (Fig. 3B).

Inhibitory effects of anti-mRANKL ab on root resorption

Root resorption of the upper first molar was assessed on TRAP-stained sections. After 10 days of tooth movement, root resorption was observed on the mesial surface of the distobuccal root of the upper first molar (Fig. 4A). However, in the same region in the anti-mRANKL ab treated group, no root resorption was observed after tooth movement (Fig. 4B). TRAP-positive odontoclasts was observed on the mesial side of the distobuccal root of the upper first molar in the tooth movement groups (Fig. 4C). The number of odontoclasts was significantly lower in the anti-mRANKL ab treated group compared with the untreated group.

Inhibitory effects of anti-mRANKL ab on TNF-α-induced osteoclastogenesis

To assess the effects of anti-mRANKL ab on TNF-α-induced osteoclastogenesis in the mouse calvaria, TRAP-stained sections were used. In the TNF-α treated groups, TRAP-positive cells were observed in the calvarial sutures (Fig. 5A). However, the number of TRAP-positive cells was significantly lower in the group treated with both anti-mRANKL ab and TNF-α than the group treated with TNF-α alone (Fig. 5B).

Discussion

In this study, the effects of anti-mRANKL ab on orthodontic tooth movement were investigated in mice. The distance of tooth movement and the number of TRAP-positive cells on the pressure side of the tooth root were reduced in mice treated with anti-mRANKL ab. Tohyama et al. showed that anti-mRANKL ab reduced the number of TRAP-positive cells in mouse calvaria.
Effects of anti-mRANKL ab on orthodontic tooth movement. (A) \(\mu\)-CT images and photographs of upper molars. The left \(\mu\)-CT images show sagittal sections of the upper molars. The middle images show horizontal sections. The photographs on the right show an occlusal view of the upper molars. The arrow indicates the direction of orthodontic tooth movement of the upper left first molar. The distance between the arrowheads shows the amount of tooth movement of the upper first molar. M1: upper first molar; M2: second molar; M3: third molar. Scale bars = 500 \(\mu\)m. (B) Distance of orthodontic tooth movement. The data are expressed as means ± SD \((n = 7; *P < 0.05, **P < 0.01)\). Differences were detected using Scheffe’s F tests.
Figure 3  Effects of anti-mRANKL ab on the expression of TRAP-positive cells. (A) TRAP-stained horizontal sections of the distobuccal root of the upper first molar. The lower panels are high magnification images of the boxed areas in the upper panels. The arrow indicates the direction of orthodontic tooth movement of the upper left first molar. The arrow heads indicate TRAP-positive cells. M: mesial side of the root; D: distal side of the root; R: root; P: periodontal ligament; A: alveolar bone. Scale bars = 100 μm. (B) Number of TRAP-positive cells after orthodontic tooth movement. The data are expressed as means ± SD (n = 4; *P < 0.05, **P < 0.01). Differences were detected using Scheffe’s F tests.
osteoclasts in a cancer-associated mandibular bone destruction model. Kuritani et al. demonstrated that anti-mRANKL ab inhibited periodontal bone destruction and lipopolysaccharide-induced calvarial osteoclastogenesis. Consistent with these previous reports, the results of our study suggest that orthodontic tooth movement was decreased by a reduction in the number of osteoclasts as an effect of anti-mRANKL ab.

In recent years, an anti-human RANKL ab known as denosumab has been widely used to increase bone mineral density. As a routine treatment, denosumab is subcutaneously injected at a dose of 60 mg every 6 months for osteoporosis and 120 mg every 4 weeks for bone tumor metastasis. In this study, we confirmed the effect of 10 days’ tooth movement after a single intraperitoneal administration of anti-mRANKL ab. Few previous studies have used anti-mRANKL ab during orthodontic tooth movement. Only one study, which conducted daily local administration of anti-mRANKL ab during orthodontic tooth movement, was found. However, it is different from our method, which more closely simulated the conditions of actual clinical practice. Denosumab is known to be stable in the blood stream, so its serum half-life is long after a single injection. In a mouse model study of osteopetrosis that also used a single injection of anti-mRANKL ab, serum TRAP-5b levels were almost non-existent even after 28 days. Our simplified single injection for orthodontic tooth movement is considered to be a viable method option.

We occasionally encounter patients using bone-modifying agents such as denosumab and bisphosphonate. Some reports have shown that bisphosphonate suppresses orthodontic tooth movement. Clinical reports about the effect of denosumab on tooth movement could not be confirmed. In this study, a single injection of anti-mRANKL antibody reduced orthodontic tooth movement in mice. Patients may actually continue using denosumab for a long time. It is important to be prepared for adverse outcomes in orthodontic treatment because the teeth may not move as planned.

Mechanical loading on a tooth induces the production of various cytokines and results in differentiation of osteoclasts and odontoclasts on the pressure side of the periodontium. Odontoclasts, similar to osteoclasts in their morphological and functional characteristics, play a key role in root resorption. It is speculated that the strong or shorter period of exertion force in orthodontic treatment increases root resorption, but this has not been clearly revealed. Root resorption is undesirable symptom, and care should be taken to limit its progression. It is often observed in mouse models of orthodontic tooth movement. In this study, root resorption was observed in the anti-mRANKL ab untreated mice, but was not observed in the treated mice. These results suggest that anti-mRANKL ab suppressed not only osteoclastogenesis but also odontoclastogenesis.

**Figure 4** Effects of anti-mRANKL ab on root resorption. (A) Root resorption after orthodontic tooth movement. The distance between the black arrowheads shows the area of root resorption of the distobuccal root of the upper first molar, and open arrowheads indicate TRAP-positive odontoclasts. The arrow indicates the direction of orthodontic tooth movement of the upper left first molar. The vertical line indicates the border of the mesial (M) and distal side (D) of the root. (B) The percentage of root resorption area on the distobuccal root of the upper first molar. The data are expressed as means ± SD (n = 4; *P < 0.05). Differences were detected using Scheffe’s F tests. (C) Number of TRAP-positive odontoclasts after orthodontic tooth movement. The data are expressed as means ± SD (n = 4; **P < 0.01). Differences were detected using Scheffe’s F tests.

**TNF-α** is an inflammatory cytokine related to osteoclastogenesis. Our previous reports have shown that TNF-α plays a crucial role during orthodontic tooth movement. We administered TNF-α with anti-mRANKL ab to the supra-
Figure 5  Effects of anti-mRANKL ab on TNF-α-induced osteoclastogenesis in calvaria. (A) TRAP-stained histological sections of calvaria. The right panels are high magnification images of the boxed areas in the left panels. The arrowheads indicate TRAP-positive cells. Scale bars = 50 μm. (B) Number of TRAP-positive cells in the calvarial suture. The data are expressed as means ± SD (n = 6; **P < 0.01). Differences were detected using Scheffe’s F tests.
of TNF-α administration to the supracaivalvaria was based on previous studies.\textsuperscript{35,36} The results indicated that anti-mRANK ab suppressed calvarial osteoclastogenesis induced by TNF-α. This finding raises the possibility that anti-mRANK ab may suppress TNF-α related bone resorption in orthodontic tooth movement in mice. Our results suggest that anti-mRANK ab decreases orthodontic tooth movement by reducing osteoclastogenesis on the pressure side of the tooth root. The inhibitory effect of anti-mRANK ab on alveolar bone resorption could be considered during clinical orthodontic treatment.

Declaration of competing interest

All authors have no conflict of interest relevant to this article.

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