TNF-α induces orthodontic root resorption via the expression of RANKL

(TNF-αはRANKL存在下で歯科矯正治療中の歯根吸収を増悪させる)

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Abstract: Orthodontically-induced inflammatory root resorption (OIIRR) is one of the procedure-related adverse effects that occur during orthodontic treatment, and the incidence is related to the proinflammatory cytokine produced in response to the mechanical stress caused by the procedure. It is known that tumor necrosis factor (TNF) –α is produced following environmental insults *in vivo* at an early stage, and that it deeply affects inflammatory bone resorption. However, the relationship between the TNF-α level and root resorption is unclear. In this study, the relationship between TNF-α and root resorption was evaluated. First, we determined the levels of tumor necrosis factor (TNF) -α and receptor activator of NF-κB ligand (RANKL) in the gingival crevicular fluid (GCF) of patients with severe root resorption after orthodontic treatment using Western blot analysis. Next, we examined the relationship between TNF-α and root resorption using an experimental mouse tooth movement model and a pressure side model using human periodontal ligament (hPDL) cells, as well as an osteoclast culture system. The Western blot analysis showed that the TNF-α and soluble RANKL (sRANKL) expressions in the GCF were significantly higher in the severe root resorption group than in the control group. Nine days after the tooth movement in the

mouse model, an increase in TNF-α and RANKL positive cells were observed. *In vitro*, their levels increased in the cultures of hPDL cells exposed to the 4 g/cm^2 pressure. In addition, the differentiation of osteo/odontoclasts was promoted by TNF-α weakly, but the ability to resorb the dentin was unchanged. However, the activation of osteo/odontoclastogenesis is more potent in the presence of RANKL and TNF-α, which leads to additive activation. These results suggest that TNF-α may be an aggravating factor for root resorption during orthodontic treatment.

Key words: TNF-α, RANKL, Root resorption, Orthodontic tooth movement

Introduction

Orthodontically-induced inflammatory root resorption (OIIRR) is an undesired side effect of orthodontic treatment. It has been reported that 2-5% of orthodontic patients experience more than 5 mm of root shortening during orthodontic treatment with fixed appliances. It is therefore critical for orthodontists to anticipate this complication and prevent its occurrence. The etiology of root resorption associated with orthodontic therapy is complex; heavy force [1] lengthy treatment and abnormal roots all contribute to the resorption [2].

Various pro-inflammatory cytokines, such as receptor activator of nuclear factor-κB ligand (RANKL) and tumor necrosis factor (TNF) -α, are produced when compressive forces (CF) [3] create stress on human periodontal ligament (hPDL) cells, and a large amount of RANKL, interleukin (IL)-1β and TNF-α are produced by the hPDL cells of patients with severe root resorption [4].

TNF-α is a cytokine that is generated by a variety of cells, including macrophages and PDL cells, and is induced by exogenous stimulation, endotoxins and pathogens. It is related to inflammatory bone resorption, such as that associated with periodontal

disease and rheumatoid arthritis, is attracting attention. Üstün et al. [5] have reported that TNF-α is involved in inflammatory bone destruction in periodontal diseases and rheumatoid arthritis. TNF-α is produced intravitally at an early stage of resorption due to various stimuli, and is involved in inflammatory bone resorption [6] as well as apoptosis [7] and immune reactions [8]. There have been a number of studies conducted on the bone resorption induced by TNF-α and RANKL. However, the results have been controversial. It was recently reported that in the absence of RANKL, TNF-α induces the formation of osteoclasts in the presence of transforming growth factor (TGF) -β [9]. In contrast, it has been shown that TNF-α induces osteoclastogenesis in the presence of basal levels of RANKL [10]. The functions of osteoclast and odontoclast are closely related to physiological and pathological character [11]. There are a lot of similarities in resorption mechanism of these cells [12]. Moreover, it has been reported that excessive expression of RANKL is correlated with bone resorption, and that TNF-α increases the expression of RANKL [13]. Recent study reported that TNF-*α* and soluble RANKL (sRANKL) expressions in the gingival crevicular fluid (GCF) obtained from the severe

root resorption patients were significantly higher than the non-resorption patients [14]. These findings may imply that TNF-α is deeply involved in odontoclastogenesis.

However, there are different views on the details of its mechanism(s) of activation and relationship with RANKL in OIIRR. Therefore, this study focused on TNF-α and evaluated the relationship between TNF-α and OIIRR by (1) determination of the expressions of TNF-α and sRANKL in the GCF of patients with OIIRR, (2) examination of the release of TNF-α and RANKL in the root resorption area by an animal model, (3) investigation of the relationship between TNF-α and RANKL in the progression of OIIRR using hPDL cells and human osteoclast precursor cell (hOCPs) instead of odontoclast in vitro.

Materials and Methods

Clinical studies

Experimental subjects

Ten subjects were selected from among patients seeking treatment at the Department of Orthodontics at the Nihon University School of Dentistry at Matsudo. Two groups

were established, including a control group and a root resorption group. The control group included 5 subjects (5 females, mean age: 28.0 ± 5.3 years, mean duration of treatment: 26.4 ± 3.1 months) with little radiographic evidence of root resorption (≤ 1mm). The root resorption group included 5 subjects (5 females, mean age: 28.9 ± 6.1 years, mean duration of treatment: 27.8 ± 3.3 months) with radiographic signs of severe root resorption of more than 1/3 of the original root length. Informed consent was obtained from each patient, and the project was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019). All patients providing their written informed consent.

The selection criteria for the subjects were as follows: 1. a Class I malocclusion with mild crowding (≤ 6 mm; mean 5.4 ± 0.55), 2. premolar extractions and, 3. no history or evidence of tooth injury or wear, as shown on the charts and diagnostic records.

All subjects were in good general health with healthy periodontal tissues before the orthodontic treatment; the probing depths were ≤ 3 mm, and there was no radiographic evidence of periodontal bone loss. Subjects were excluded if they received antibiotic therapy during the treatment or if they had taken anti-inflammatory medication during

the month preceding the start of the study.

GCF collection

The method used in this study has been previously described by Yamaguchi et al. [15]. GCF was collected from both the resorption and control groups following orthodontic treatment (debonding). The GCF was collected from the mesial and distal sides of the upper central and lateral incisors using filter paper strips (Periopaper, Oraflow, Smithtown, NY, USA) inserted 1-2 mm into the gingival sulcus for one minute (Fig. 1-A). After one minute, the second collection was performed. Care was taken to prevent mechanical injury to the soft tissue. The contents were eluted into 1× phosphate buffer saline (PBS) containing a protease inhibitor (0.1 mM phenylmethylsulphonylfluoride) and stored at -30°C until a further analysis.

For the evaluation of the cytokine expression, the paper strips were placed individually in 100 μl of PBS and then subjected to vortexing 3 times over a 30 minute period. The strip were then removed and the eluate was centrifuged for 5 minutes at 3,000 x g. The protein concentration in the extract was estimated using bovine serum albumin as a

standard.

Western blotting analysis

The TNF-α and sRANKL expressions in the GCF samples were determined using a Western blotting analysis. The protein content of the samples was measured using the Bradford reagent (BIO-RAD, Tokyo, Japan) according to the manufacturer's protocol. The samples were boiled for 3 minutes with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 3.3% SDS, 30% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue) and the samples (10 μl) were then resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 150 V for 1 hour (h). The proteins were electrotransferred from the SDS gels onto an Hybond ECL (GE Healthcare UK Ltd Amersham Place, Little Chalfont, Buckinghamshire, UK) for the immunoblot analyses. Blocking of nonspecific antigen-binding sites was performed with 5% nonfat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween 20 (TBST) buffer (Sigma Chemical Co., St.Lois, MO, USA). The membrane was incubated for 24 h with anti-TNF-α mouse monoclonal antibodies (MAB610: R&D Systems Inc.,

Minneapolis, MN, USA) diluted at 1:500 and anti-RANKL rabbit monoclonal antibodies (ab124797: abcam PLC., Tokyo, Japan) diluted 1:1000 in 5% nonfat dry milk-TBST. Subsequently, the blots were incubated for 2 h with goat anti-mouse IgG (H+L)-HRP conjugate (BIO-RAD) diluted at 1:2500 and goat anti-rabbit IgG (H+L)-HRP conjugate (BIO-RAD) diluted at 1:2000 in 5% nonfat dry milk-TBST, then detected using an ECL system (GE Healthcare Limited). Quantification of the band intensity was performed using the Image J Software program (NIH, Bethesda, MD, USA).

In vivo studies

Animals

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. AP11MD015). A total of 15 male (control = 7, orthodontic force (OF) = 8) 8-week-old wild-type BALB/c mice (body weight 20 ± 5 g; Sankyo Labo Service, Inc., Tokyo, Japan) were used for 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. [16] 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 the device was 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 upper-left first molar in each mouse to study the experimental tooth movement. The upper-left first molar was mesially moved by the closed-coil spring with a force of 25 g, which was selected based on the report by Al-Qawasmi et al. [17] (Figs. 1-B, C). The force was applied for nine days.

Tissue preparation

The animals were deeply anesthetized using thiamylal sodium, and then were transcardially perfused with 4% paraformaldehyde. The maxilla was then immediately dissected and immersed in the same fixative for 18 h at 4°C. The specimens were decalcified in a 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for 3 weeks, 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 4 μm sections in the horizontal direction, and then was prepared for hematoxylin and eosin staining (H&E), and also for immunohistochemical staining. The PDL tissues in the mesial part of the distal buccal root of upper-left first molar were observed. The sites of observation were based on the report by Yoshimatsu et al. [16].

Immunohistochemistry

Immunohistochemical staining was performed as follows: The tissue sections were deparaffinized, and then the endogenous peroxidase activities were quenched by incubation in 3% H_2O_2 in methanol for 30 minutes (min) at room temperature. After being washed in tris-buffered saline (TBS), the sections were incubated with a

polyclonal anti-TNF-α antibody (AF510NA: R&D Systems, Inc., Minneapolis, MN, USA; working dilution, 1:100) and polyclonal anti-RANKL antibody (AF462: R&D; working dilution: 1:100) for 18 hours (h) at 4 °C. The secondary antibodies were conjugated with Histofine Simple Stain MAX-Po (G) kit (Nichirei, Co., Tokyo, Japan) according to the manufacturer's protocols. The sections were rinsed with TBS, and the final color reactions were performed using the 3, 3'-diaminobenzidine tetra-hydrochloride substrate reagent, then the sections were then counterstained with hematoxylin. As immunohistochemical controls, several sections were incubated with 0.01 M phosphate-buffered saline (PBS) instead of the primary antibody.

In vitro studies

Human periodontal ligament (hPDL) cell culture

The hPDL cells were prepared according to a modification of the method reported by Somerman et al. [18]. Briefly, hPDL tissues were collected from the roots of premolars extracted from 6 healthy young volunteers (3 males, 3 females; 14-16 years of age), and were used according to a protocol reviewed by the Ethics Committee of Nihon

University School of Dentistry at Matsudo (EC 10-019). The hPDL tissue specimens were placed in 35 mm tissue culture dishes and covered with a sterilized glass coverslip. The cells were maintained in α-MEM medium (Wako, Osaka, Japan) supplemented with 10 unit/ml of penicillin (Sigma Chemical Co., St. Louis, MO, USA), 50 μg/ml of gentamicin sulfate (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 in 5% CO_2 and 95% air.

Application of compression force

In order to reproduce the conditions of pressure during orthodontic tooth movement, we performed the following *in vitro* experiments in accordance with the method reported by Yamaguchi et al. [4] (Fig. 1-D). Briefly, cells were seeded in 10 cm cell culture dishes (inside diameter: 83 mm) with medium (1% FCS). After overnight incubation, the medium for the nearly confluent cells was changed, and the cells were subjected to 4.0 g/cm^2 of compressive forces (CF) for 1, 3, 6, 9, 12, 24 or 48 h. Control

cells were covered with thin glass plates without lead weights, which produced 0.032 g/cm^2 of CF. In this manner, hPDL cells were continuously compressed using a uniform compression method as a model of pressure at the site of orthodontic tooth movement.

Human osteoclast precursor cells (hOCP) culture

hOCPs were purchased from Lonza Walkersville, Inc. The cells were seeded onto 16-well Lab-Tek chamber slides (Nunc, Naperville, IL, USA) at a density of 1×10^4 cells/100 μl with hPDL culture medium. We prepared 2 cell culture groups in which cells were cultured with hOCP culture medium with RANKL (66 ng/ml), and hOCP culture medium without RANKL. In each culture group, we added following supernatant and/or reagents as additional treatment groups,

1) Culture supernatant obtained from CF treated hPDL cell culture (CFM)

2) CFM and anti-TNF-α antibody (10 μg/ml)

3) rhTNF-α (10 ng/ml)

In addition, we added osteoprotegerin (OPG) in the groups of hOCP culture medium without RANKL. OPG inhibits residual RANKL within CFM.

We followed the hOCP culture protocol. The culture group of hOCP culture medium with RANKL without any supernatant and/or reagents was used as a control group (Table 1).

Enzyme-linked immunosorbent assay (ELISA)

The culture medium was collected for an ELISA. And samples were concentrated by Centrifugal filter devices (Merck KGaA, Darmstadt, Germany). The protein concentrations in the culture medium were determined from standard curves generated for TNF-α and soluble RANKL (sRANKL) using ELISA kits (TNF-α kit; R&D Systems Co.) (sRANKL kit; BIOMEDICA Co., Wien, Austria). The absorbance at 450 and 540 nm was recorded.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured hPDL cells by means of the RNeasy Mini kit (Qiagen Co., Venlo, Netherlands). Aliquots containing equal amounts of mRNA were subjected to real-time PCR. RNA was amplified with a RT-PCR kit, 40 μl of purified

total RNA was obtained, and the total RNA was converted to cDNA using a PrimeScript RT reagent kit (TaKaRa Co., Shiga, Japan). Real-time PCR amplification was performed using SYBR Premix Ex TaqII (TaKaRa Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; TaKaRa) following the manufacturer's protocol. The real-time PCR conditions were 95°C for 3 seconds and 60°C for 30 seconds for 40 cycles after a hot start, and measurements were taken at the end of the annealing step at 60°C during each cycle. PCR primers for TNF-α, RANKL and GAPDH were purchased from (Takara Co.) and designed with reference to the respective cDNA sequences. The primers used were as follows:

GAPDH

Fw: 5'-GCACCGTCAAGGCTGAGAAC-3'

Rv: 5'-TGGTGAAGACGCCAGTGGA-3'

TNF-α

Fw: 5'-GACAAGCCTGTAGCCCATGTTGTA-3'

Rv: 5'-CAGCCTTGGCCCTTGAAGA-3'

RANKL

Fw: 5’-ATCGTTGGATCACAGCACATCAG-3’

Rv: 5’-GGAT GTCGGTGGCATTAATAGTGAG-3’

TRAP staining

TRAP staining was performed after hOCPs had been cultured for seven days (TRAP staining kit; TaKaRa Co.). TRAP-positive and multinucleated cells that contained three or more nuclei were judged to be osteoclasts.

Pit formation assay

The resorptive activity of osteoclasts formed *in vitro* was evaluated by the ability of the cells to form resorption pits on dentin slices (diameter, 6 mm; thickness, 0.15 mm). When the planned incubation was completed, the samples were washed three times with PBS. The slices were placed for 30 min in 1 M NH_4OH and cleaned by ultrasonication to remove adherent cells, and were then washed and dried. After drying, the dentin slices were mounted onto stubs and were sputter-coated with platinum for electron microscopy or were placed on glass slides for light microscopic examination.

The entire surface of each dentin slice was examined using a scanning electron microscope (JSM-6340F; JEOL, Tokyo, Japan). The dentin slices were continuously stained with Lucifer Yellow CH dilithium salt (MP Biomedicals, Inc., Tokyo, Japan). The area of resorption pits was measured in four randomly selected areas of each dentin slice by a laser scanning microscope (LSM 510 META ConfoCor 3; Carl Zeiss Microscopy Co.,), and the images were analyzed by the analytical software program (Imaris; Bioplane Co., CT, USA) (Fig. 1-E).

Statistical methods

The values in each figure represent the means ± standard deviation (s.d.) for each group. A Mann-Whitney U-test was used to compare the means of the groups with values of $P < 0.05$ considered to indicate a significant difference.

Results

Clinical studies

In all patients, the degree of plaque accumulation throughout the study was minimal,

and the subjects' gingival health was excellent. Furthermore, the probing depths remained less than 3 mm at all times throughout the experimental period, and there was no bleeding on probing.

The mean volumes of GCF obtained from the paper strips were compared. There were no significant differences in the mean volumes of GCF between the root resortion group (mean: 4.10 ± 0.05 μl) and the control group (mean: 4.30 ± 0.05 μl).

Determination of the TNF-α and RANKL expressions using a Western blot analysis

Western blot analysis was performed to detect the sRANKL and TNF-α expression in the control and resorption groups. Immunoblotting against TNF-α was detected in both group samples. The intensity of band in resorption group showed higher than that observed in the control group (Fig. 2-A). Immunoblotting against sRANKL was detected in the resorption group. The control group had less intense bands than the resorption group (Fig. 2-B).

In vivo studies

Body weights during the experimental period

The body weights of the mice in the experiment group decreased transiently on day 1 after the application of the orthodontic devices and then recovered. No significant differences between the two groups were observed (data not shown).

Histological changes in the periodontal tissues after tooth movement (H.E. staining)

In the control group (0 g), 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, and resorption lacunae were not observed (Fig. 3-A). In the experiment group, there was a coarse arrangement of fibers. On day 9 after the application of the orthodontic devices, many root resorption lacunae were recognized on the surface of the root (Fig. 3-B).

Protein expression levels of TNF-α and RANKL

The immunoreactivity for TNF-α and RANKL was examined on day 9 after tooth movement. TNF-α and RANKL positive cells were rarely observed from the control group. In the experiment group, many TNF-α and RANKL-positive cells were observed in the PDL tissues. (Fig. 3-C-F).

In vitro studies

The effects of CF on the release of TNF-α and RANKL by hPDL cells

When hPDL cells were exposed to a CF for up to 48 h, the release of TNF-α and RANKL were significantly increased, and peaked at 24 h. In addition, the level of TNF-α and RANKL were also increased in a time-dependent manner compared with the control (Fig. 4-A, B).

The effects of CF on the mRNA expression of TNF-α and sRANKL by hPDL cells

The mRNA expression of TNF-α and RANKL markedly increased after the application of a CF. TNF-α increased immediately at 1h then increased again and peaked at 9h. RANKL increased in a time-dependent manner and peaked at 9 h (Fig. 4-C, D). When hPDL cells were cultured with or without a CF for up to 24 h, the mRNA expression levels of TNF-α and RANKL were significantly increased compared with the control.

The effects of hPDL cell-conditioned medium on TRAP staining of hOCPs

TRAP positive cells of RANKL containing hOCP medium culture groups (groups A-D) were significantly increased compared to the groups without RANKL (group E-H). Furthermore, presence of TNF-α significantly increased TRAP positive cells in RANKL

containing hOCP medium groups (groups B, D). In the group B, 1.9 times increase compared with the control. In the group D, 2.1 times increase compared with control. Whereas TRAP positive cells cultured under presence of anti-TNF-α antibody (group C) were significantly reduced compared to the control cells. (Fig. 5-A)

Pit formation assay

Pit formation assay revealed a similar trend of the TRAP assay. The absorption cavity volume of both TNF-α and RANKL containing hOCP culture medium group (groups B, D) was significantly larger than the control group (group A). In contrast, dentin absorption was not observed in the groups without RANKL (groups E-H). (Fig. 5-B)

Discussion

Dudic *et al.* [19] reported that the GCF composition changes during orthodontic tooth movement. The levels of inflammatory cytokines, such as IL-1 beta, IL-6 and RANKL are elevated in the gingival crevicular fluid during human orthodontic tooth movement [20-22]. Furthermore, Yamaguchi et al. [23] reported that concentrations of IL-6 and IL-17 in GCF were significantly higher in subjects with severe root resorption than in

the non-resorption. Therefore, GCF may be a useful tool for studying OIIRR in a noninvasive manner.

Figure 2A, B showed that the expressions of TNF-α and sRANKL in the GCF were significantly higher in the subjects with severe root resorption than in the subjects without resorption. A recent study demonstrated that the concentrations of RANKL in the GCF were significantly higher in the subjects with mild and severe root resorption than in the controls [24]. The RANK/RANKL system has been suggested to play an integral role in osteoclast activation during orthodontic tooth movement [25]. Brooks *et al.* [26] demonstrated that the expression of RANKL during the application of orthodontic forces is involved in osteoclast precursor signaling. The RANK/RANKL system may also regulate the natural process of root resorption in exfoliated deciduous teeth [27]. Therefore, the RANK/RANKL system may be involved in the process of root resorption resulting from the application of orthodontic forces.

Ren *et al.* [28] reported that the level of TNF-α in the GCF increases during orthodontic tooth movement. Kook *et al.* [29] reported that compression forces induce the mRNA expression of TNF-α and osteoclastogenesis in human periodontal ligament

(hPDL) cells *in vitro*. TNF-α-induced osteoclast recruitment is probably central to the pathogenesis of disorders involving inflammation [30]. Therefore, TNF-α may stimulate bone resorption during orthodontic tooth movement.

Next, to investigate whether TNF-α and RANKL involved in root resorption during orthodontic treatment or not, we induced root resorption by applying excessive orthodontic force in animal models. This is consistent with reports from Yoshimatsu et al.[16] and from Taddei et al.[31]. In the agreement with these results, previous reports described elevated levels of TNF-α and RANKL in compression side following mechanical loading [22,26,31,32]. The TNF-α and RANKL participate in osteoclastogenesis by upregulating osteoclast activity. TNF-α may be a signal for osteoclast recruitment and bone resorption [33]. Furthermore, we demonstrated that a markedly increase in these molecules was found at the resorption lacunae (Fig. 3). Taken together, these findings and our present results suggest that TNF-α and RANKL induced by excessive orthodontic force may activate osteo/odontoclastogenesis.

Finally, the results of the investigation carried out using a PDL pressure model *in vitro* showed a significant increase in the TNF-α gene and protein expression levels in the

group exposed to the CF, compared with the control group; the same result was also obtained for RANKL (Fig. 4). These results are consistent with the report by Mitsuhashi et al. [3]. This led us to hypothesize that exposure to a CF increases the levels of TNF-α and RANKL in the PDL cells. In addition, Yongchaitrakul et al. [13] reported that TNF-α promotes RANKL expression in PDL cells. Therefore, the TNF-α induced by CF may increase the RANKL expression. Furthermore, to determine the relationship between TNF-α and the OIIRR, the pit formation assay was carried out.

Odontoclasts, which are responsible for 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 [34-36]. Therefore, we used osteoclasts instead of odontoclasts to study the mechanism of root resorption in the present study. Our results demonstrated that TNF-α increased the osteoclast differentiation. It is interesting to note that TNF-α alone, in the absence of RANKL, could also slightly increase compared with negative control [RANKL (-), CFM (-)] (data not shown). However, the effect was very weak and was only 20% of that induced by RANKL alone. On the other hand, it was demonstrated that

the osteoclast differentiation is dependent on, and is additively increased by, the coexpression of TNF-α and RANKL. The rate of increase was about 2.1 times that of the control. This clarified that TNF-α alone has a weak differentiation potential, but that it has a very strong effect on promoting osteoclast differentiation in the presence of RANKL. In addition, the pit formation assay was performed in order to investigate the resorption capacity of osteoclasts. The results, which were in agreement with the findings of TRAP staining, indicated that TNF-α significantly promoted the activation of osteoclasts that show RANKL dependency. However, no resorbing activity could be observed by exposure to TNF-α alone (Fig. 5). In this study, the TNF-α induced by a CF was 1.2 pg/ml (at the peak), while Kukita et al. [37] revealed that TNF-α promoted osteoclastogenesis at a concentration of 10 ng/ml. Moreover, Gokul [38] reported that periodontitis patients have 90.22 pg/ml of TNF-α in the GCF, and this increase was dramatic compared with that caused by orthodontic treatment. Therefore, the dose of TNF-α alone produced in response to a CF may not be sufficient to stimulate osteoclastogenesis. Additionally, OPG, a RANKL decoy receptor, is known to suppress osteoclast activity. Kook et al. [29] reported an increase in the expression of OPG in the

presence of CF. On the other hand, the expression of OPG in the presence of CF was not evaluated in the study reported by Diercke et al. [39]. Furthermore, such an increase was also not found in the study by Mitsuhashi et al. [3]. From a comparison of these three studies, it was found that the amount of RANKL expressed was far more based on the RANKL/OPG ratio, and that RANKL was more predominant. In addition, this research used osteoclast precursors for the experiments, and showed that osteoclast differentiation and activity were increased more by exposure to CFM than to control. From such results, the same conclusion was also drawn.

This study has newly demonstrated that OIIRR could be more induced by the coexistence with RANKL than TNF-α or RANKL alone. In conclusion, the TNF-α produced by PDL cells in response to heavy orthodontic force may aggregate OIIRR via RANKL.

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Figure Legends

Figure 1.

The GCF was sampled at the mesial and distal sides of the upper central and lateral incisors (A). Experimental tooth movement was induced by a Nickel-Titanium closed-coil spring (Tomy International, Inc., Fukushima) inserted between the upper incisor and the upper-left first molar, which was fixed with a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo) around both teeth by means of a dental adhesive agent (Transbond XT; 3M Unitek) (B, C). The upper first molar was moved mesially by the closed-coil spring with a force of 25 g. The force was applied for nine days. The method used to apply the CF. Pre-cultured hPDL cells were continuously compressed using a glass cylinder with different weights. The glass cylinder was placed over confluent cell layers in each well. The number of lead granules placed in the cylinder determined the amount of CF applied (D). Dentine slices stained using Lucifer Yellow CH dilithium salt were observed by laser microscope and steric structure was analyzed Bar: 30 μm (E).

Figure 2.

Western blot analysis for the immunodetection of TNF-α (A) and sRANKL (B) in the gingival crevicular fluid (GCF). Lanes 1 to 5—control group; lanes 6 to 10—severe root resorption group.

Figure 3.

H.E. staining and Immunohistochemistry

H.E. staining (A, B) and the immunorectivity of TNF-α (C, D) / RANKL (E, F). Light microscopic images of the effects of orthodontic forces (OF) (25g) group (B, D, F) and control (0g) group (A, C, E) for nine days.

□D, dentin; C, cementum; PDL, periodontal ligament. Bars: 50 μm

Figure 4.

The effects of CF (4.0 g/cm^2) on the expression of the TNF-α and RANKL proteins. The hPDL cells were cultured with or without a CF for up to 48 h (A, B). The TNF-α and

RANKL levels in the culture medium were determined by an ELISA after 1, 3, 6, 9, 12, 24 and 48 h. The levels of TNF-α and RANKL were significantly increased in the CF group. (C, D) The effects of the CF on the mRNA expression of TNF-α and RANKL. The mRNA levels were determined using real-time PCR after 1, 3, 6, 9, 12 and 24 h. The mRNA expression of TNF-α and RANKL significantly increased, and peaked at 9 h after the application of the CF. The data shown are representative of four separate experiments. Each bar indicates the mean ± SD of four independent experiments. *$P < 0.05$, **$P < 0.01$ from the Mann–Whitney U-test, indicating a significant difference from the corresponding control (0 g/cm^2) at the respective time point.

Figure 5.

The effects of osteoclast formation as determined by TRAP staining. hOCPs were cultured in the commercial medium. The culture medium obtained from hPDL cells loaded with the CF was incubated with or without rhTNF-α (10 ng/ml) and an anti-TNF-α antibody (10 ng/ml) for seven days. In addition, the same experiment was performed without RANKL. Therefore, the RANKL induced from CF in the culture

medium was inactivated by OPG (100 ng/ml). Original magnification 100×, bars: 100μm. The number of TRAP-positive multinucleated cells was significantly increased in the CF and rhTNF-α-treated group compared with the control [RANKL (+), CF (-)] (*P < 0.05, **P < 0.01 Mann–Whitney U-test). In the group treated with the conditioned medium containing a TNF-α antibody and without RANKL, the number of TRAP-positive multinucleated cells was significantly decreased compared with the control [RANKL (+), CF (-)].The data are expressed as the means ± s.d. of four independent experiments. The TRAP-positive multinucleated cells containing three or more nuclei were counted (A).

The effects of osteoclast activation determined by the pit formation assay. hOCPs were cultured in commercial medium for 14 days on dentin slices. The commercial medium was treated with or without TNF-α and an anti-TNF-α antibody (10 ng/ml), then resorption pits were observed by scanning electron microscopy. Original magnification 350×, Bars: 50 μm. Significant differences were observed between the CF and rhTNF-α treated group and the control group [RANKL (+), CF (-)]. In the without RANKL group, no resorption pits were detected (*P < 0.05, **P < 0.01 Mann–Whitney U-test). The

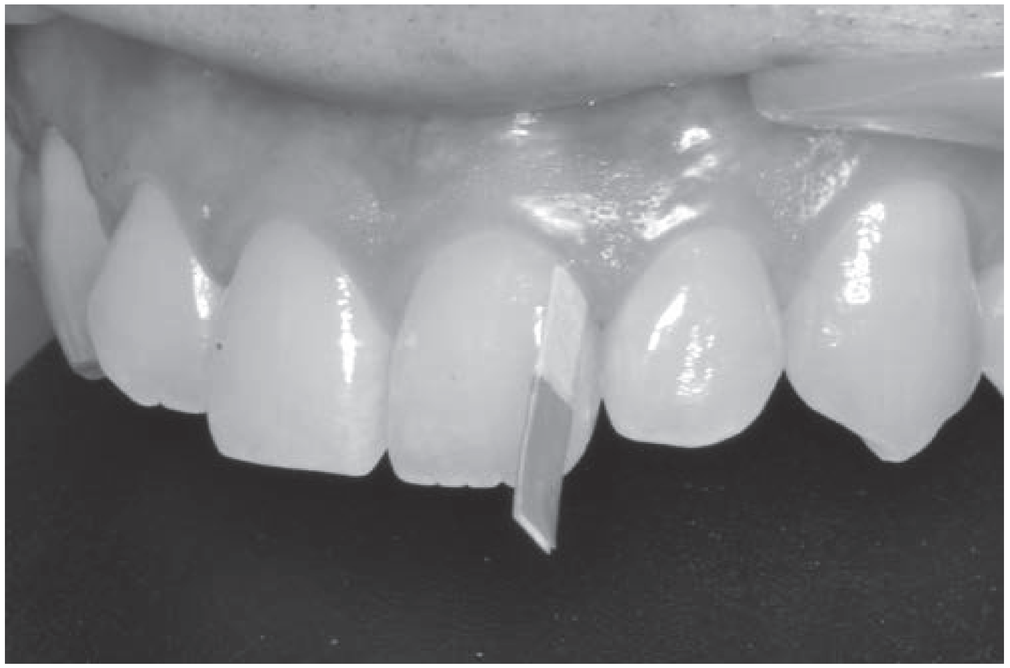
data are expressed as the means ± s.d. of four independent experiments. N.D. = not detectable (B).

Figures and Table

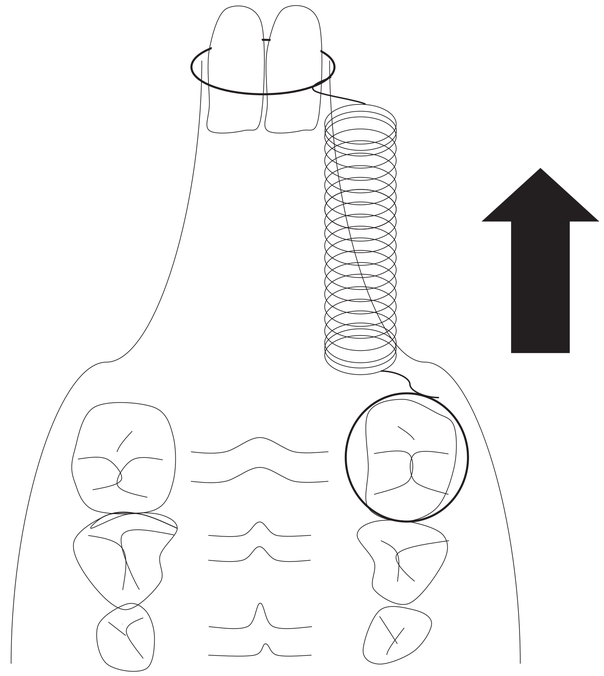
TNF-α induces orthodontic root resorption via the expression of RANKL

(TNF-αはRANKL存在下で歯科矯正治療中の歯根吸収を増悪させる)

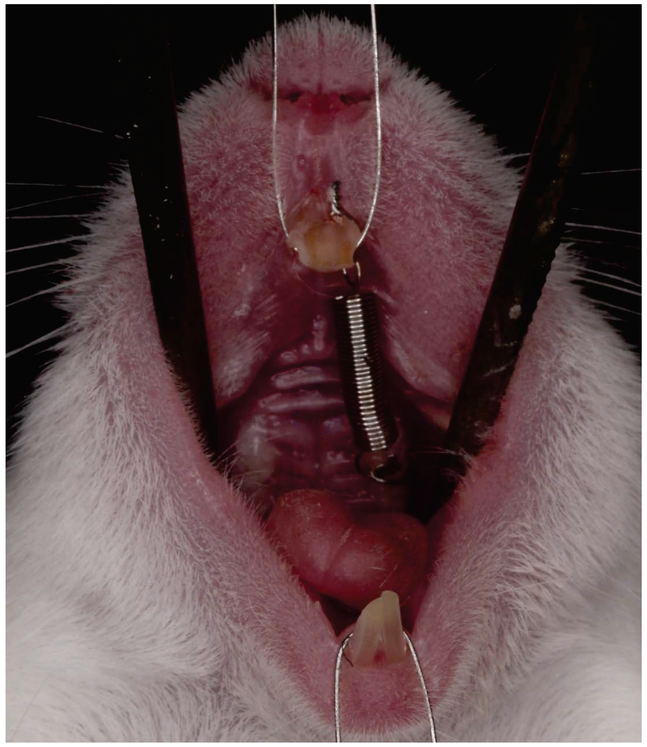
Tomokazu Yoshino



(A)

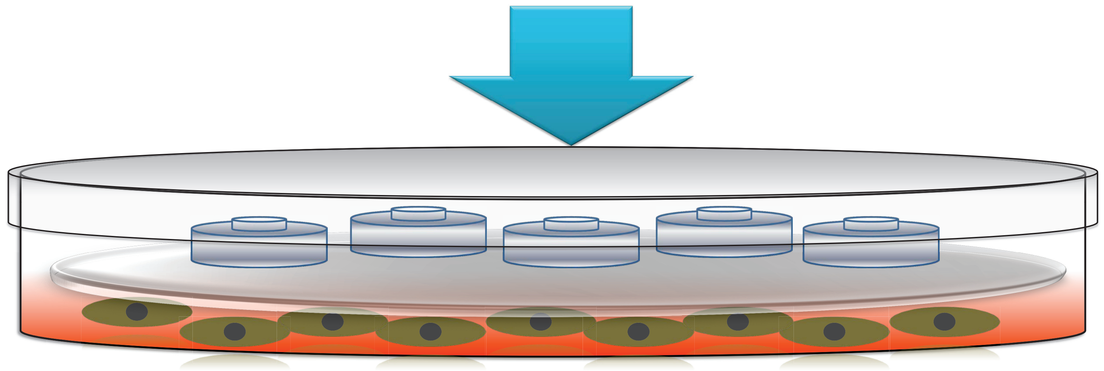


(B)

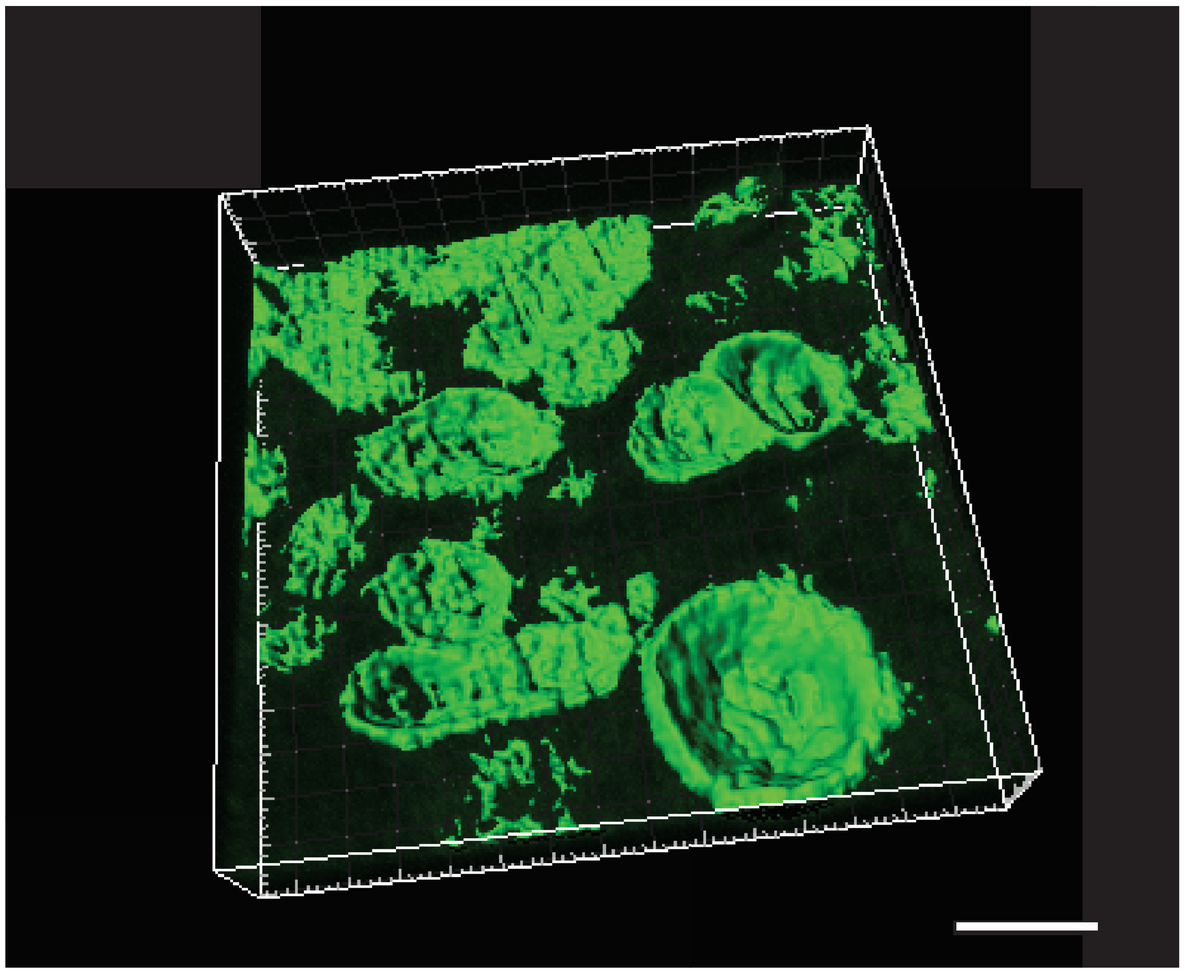


(C)

Figure 1



(D)



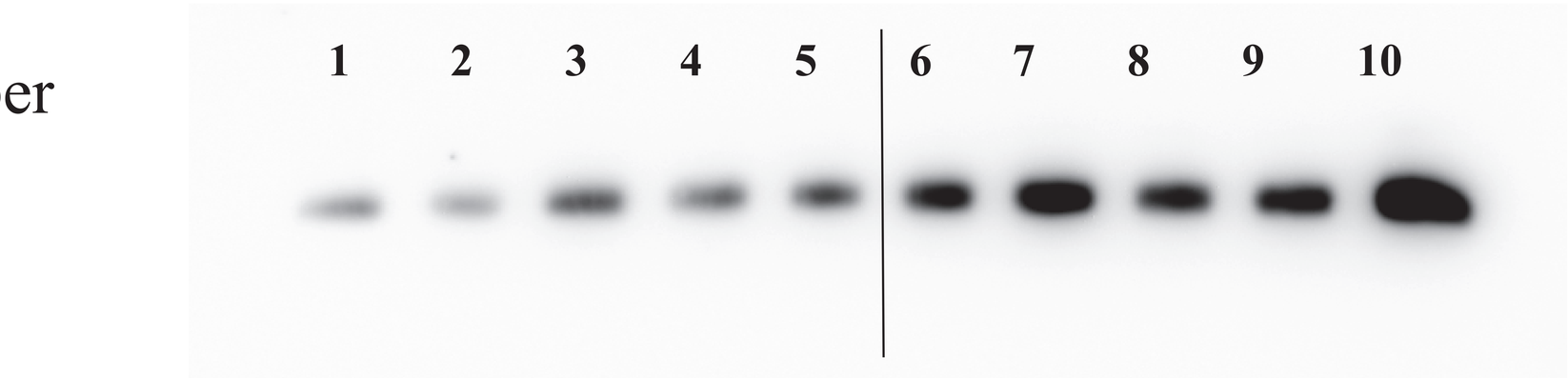
(E)

Figure 1

	With RANKL(66 ng/ml) group				Without RANKL group			
		CFM (100 μl)	CFM (100 μl) + Anti-TNF-α (10 μg/ml)	rhTNF-α (10 ng/ml)	CFM (100 μl)	CFM (100 μl) + Anti-TNF-α (10 μg/ml)	CFM (100 μl) + OPG (100 ng/ml)	rhTNF-α (10 ng/ml)
group	A	B	C	D	E	F	G	H

Table 1
Details of the conditioned medium for hOCPs culture.
CFM, Culture supernatant obtained from CF treated hPDL cell culture.

TNF-α



1-5 : Control group 6-10 : Severe root resorption group

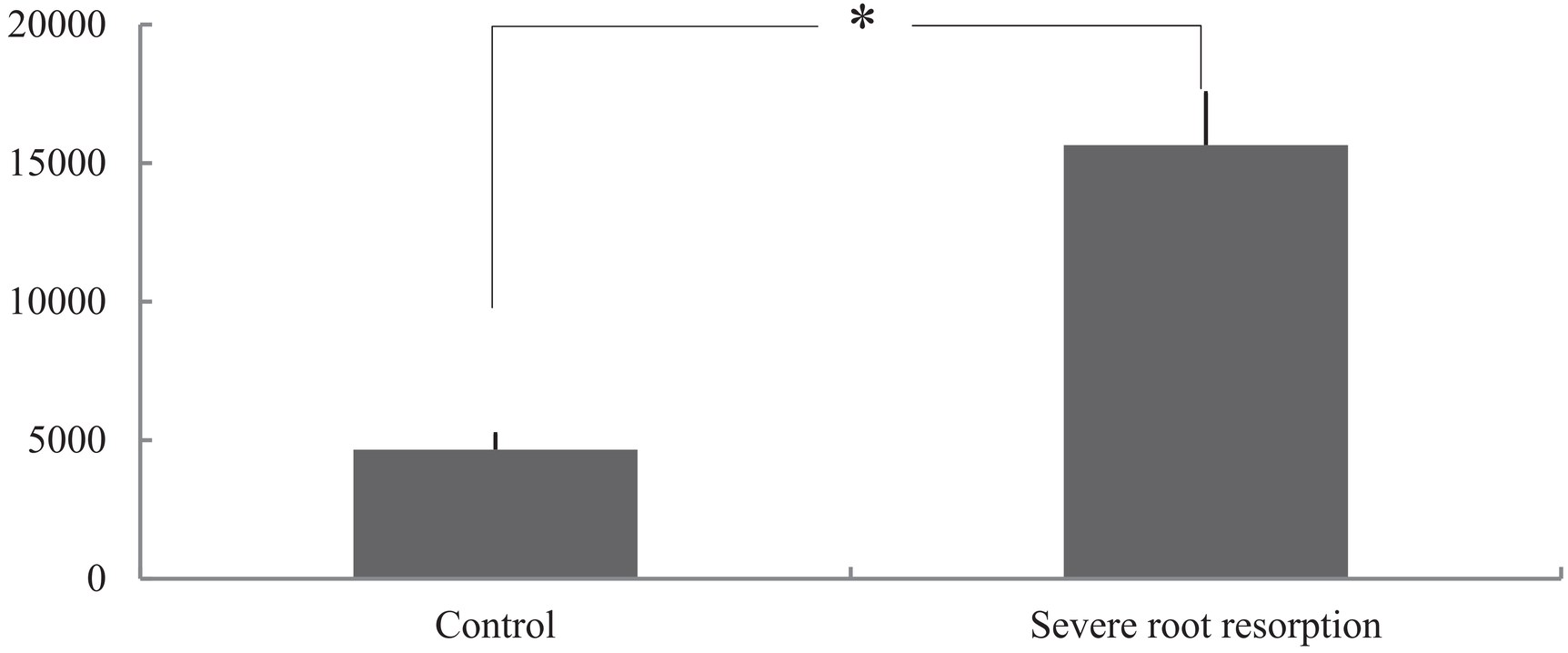
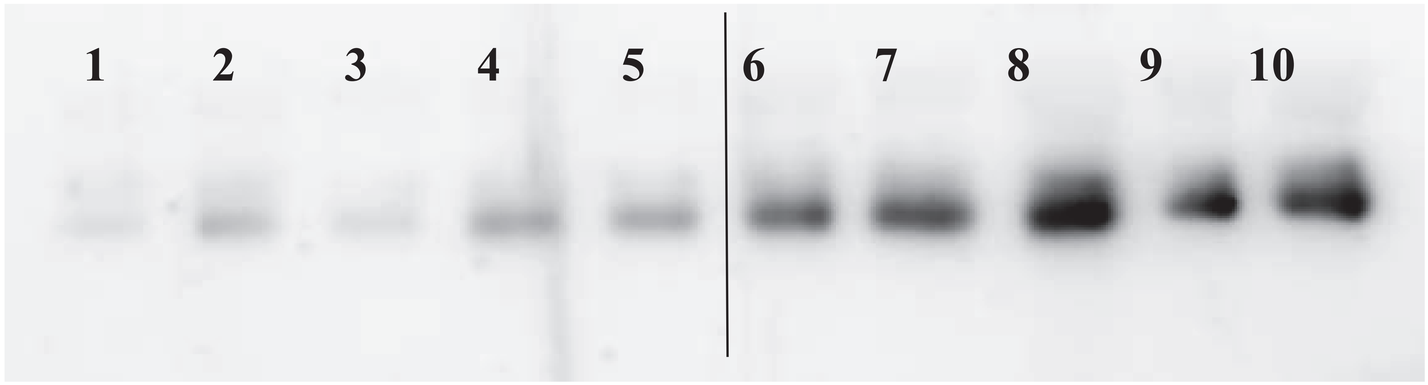


Figure 2A

sRANKL



1-5 : Control group 6-10 : Severe root resorption group

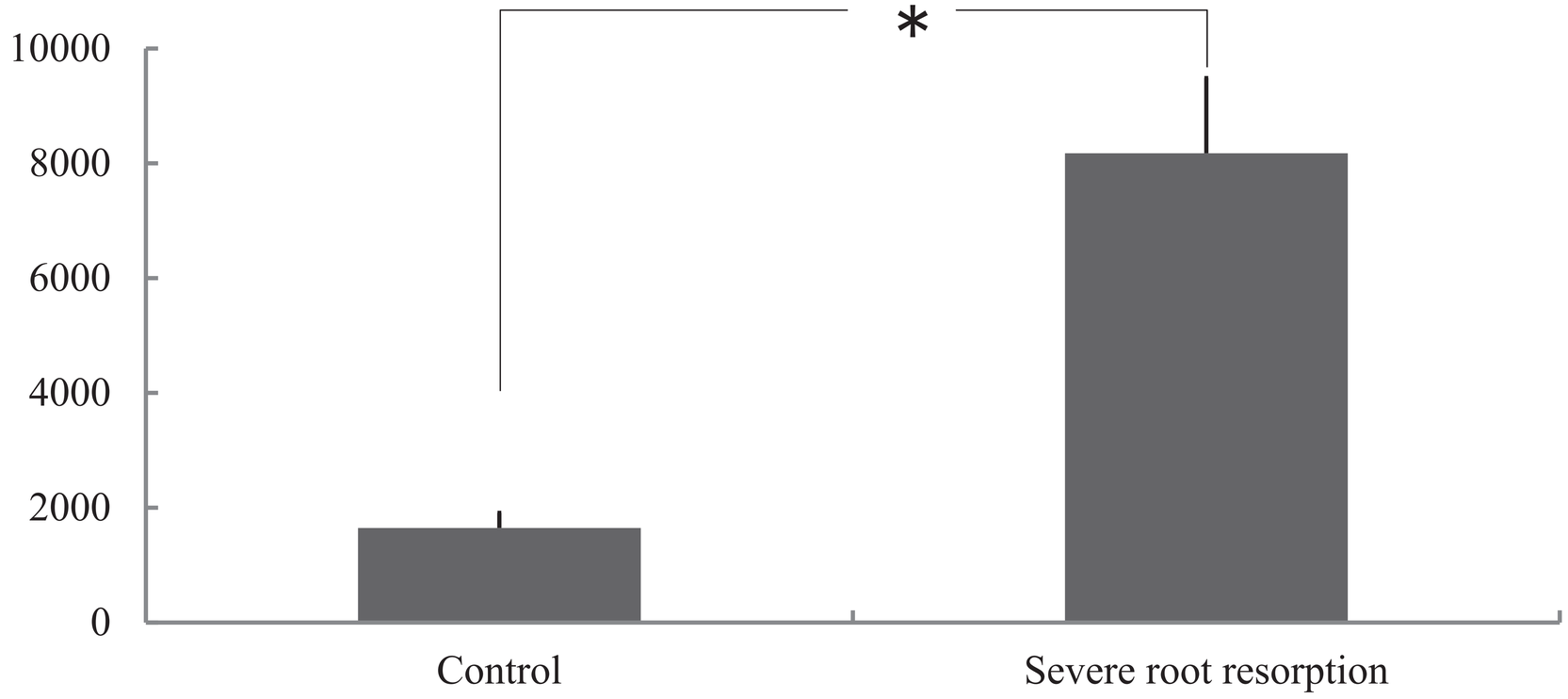
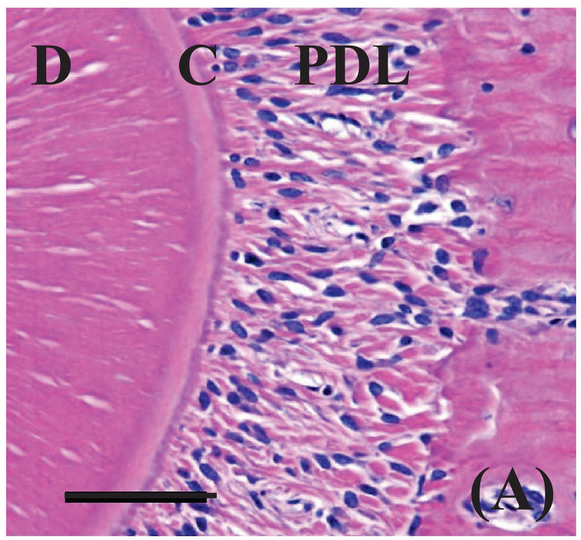
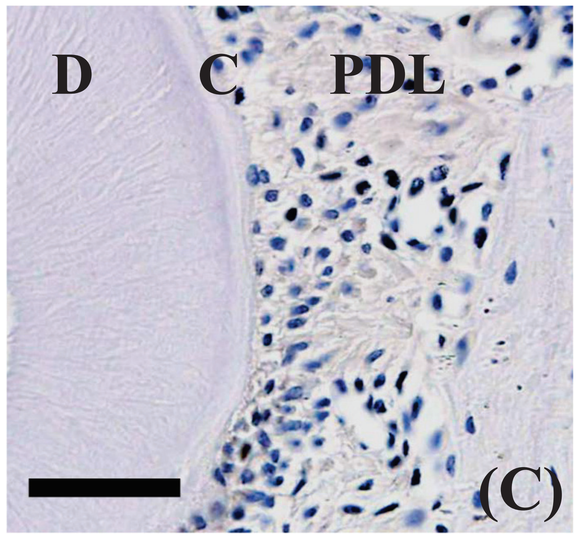
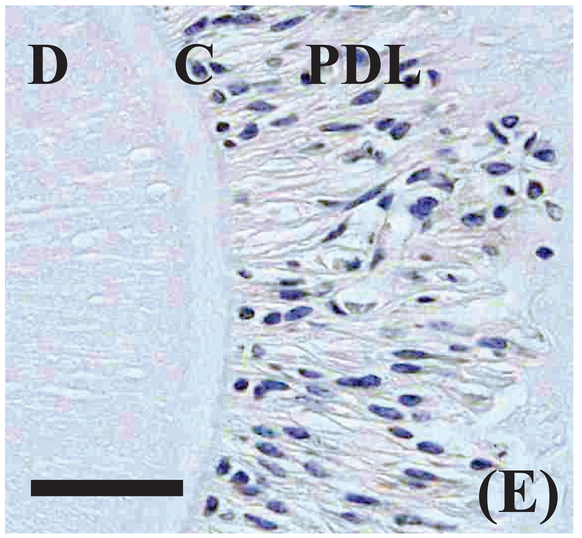
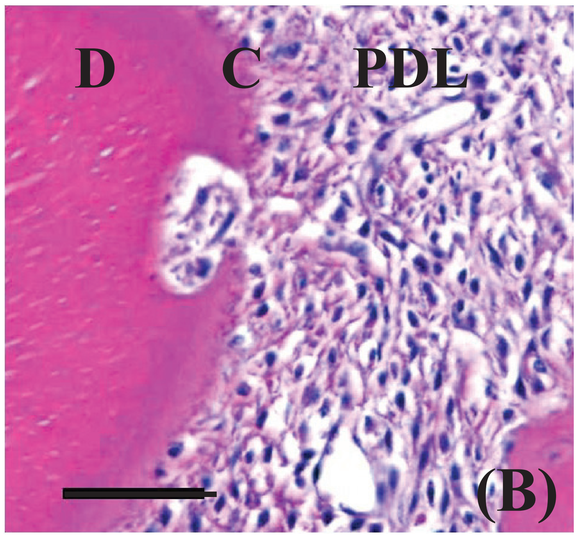


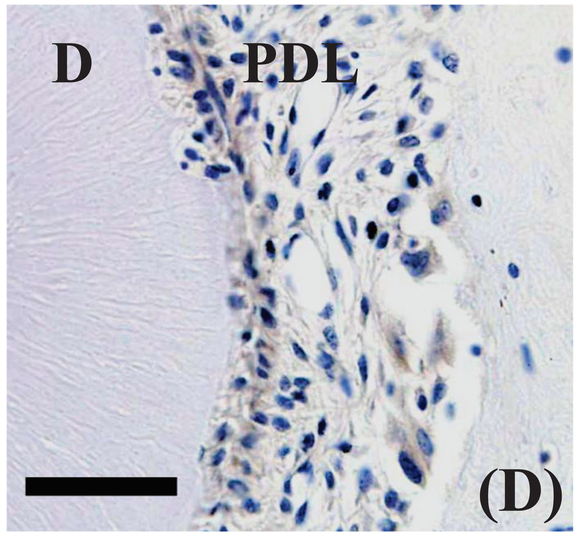
Figure 2B











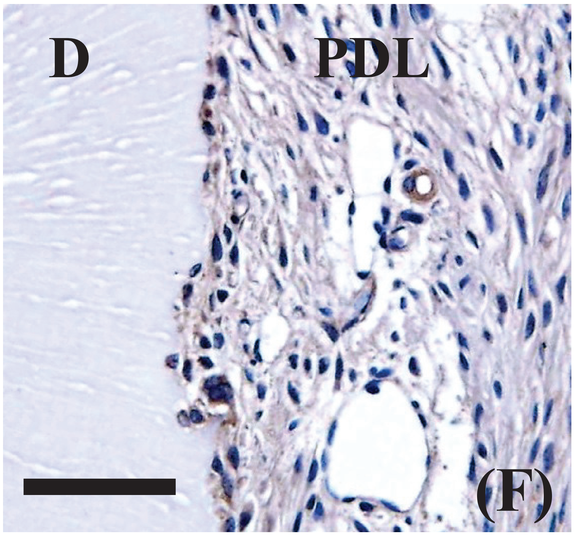
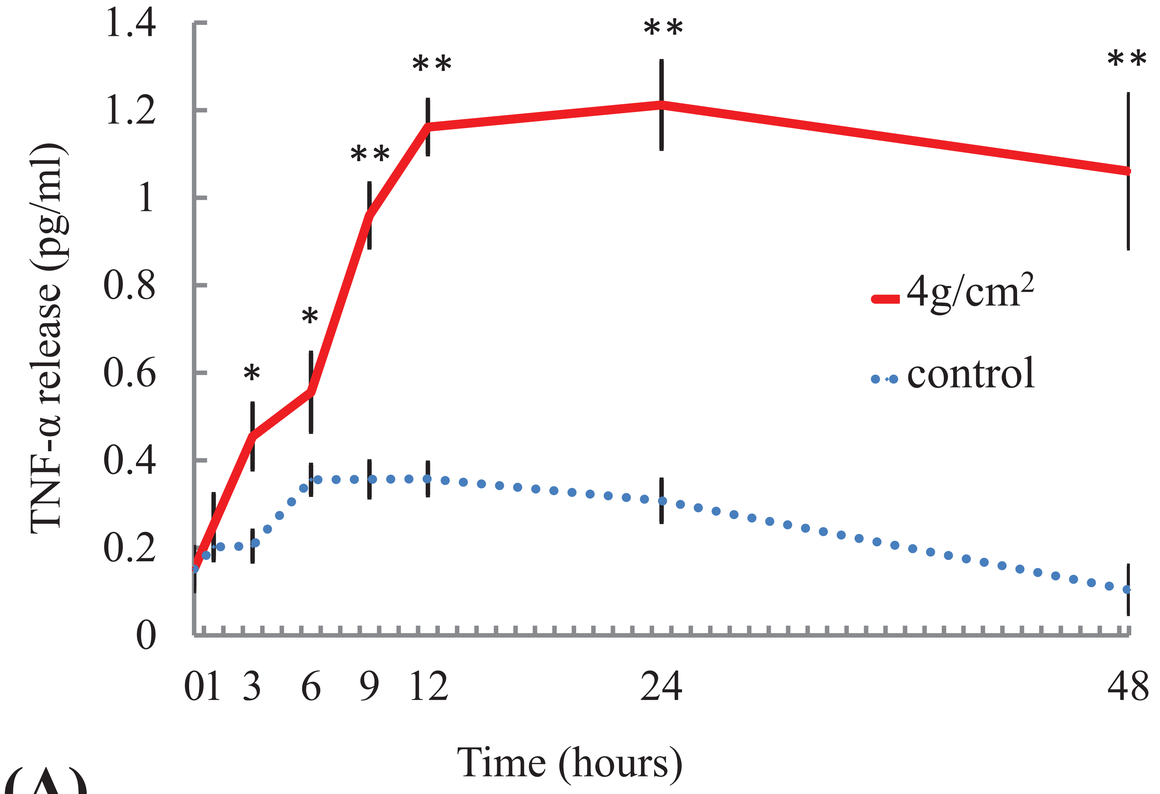
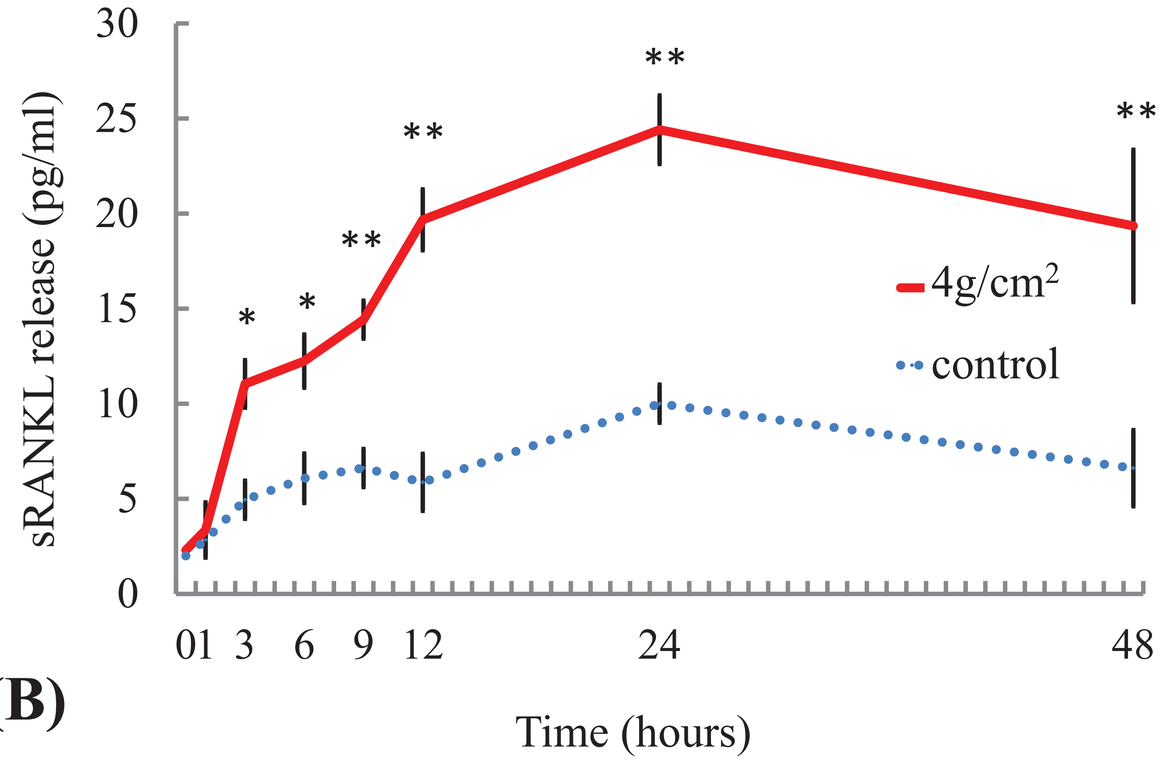
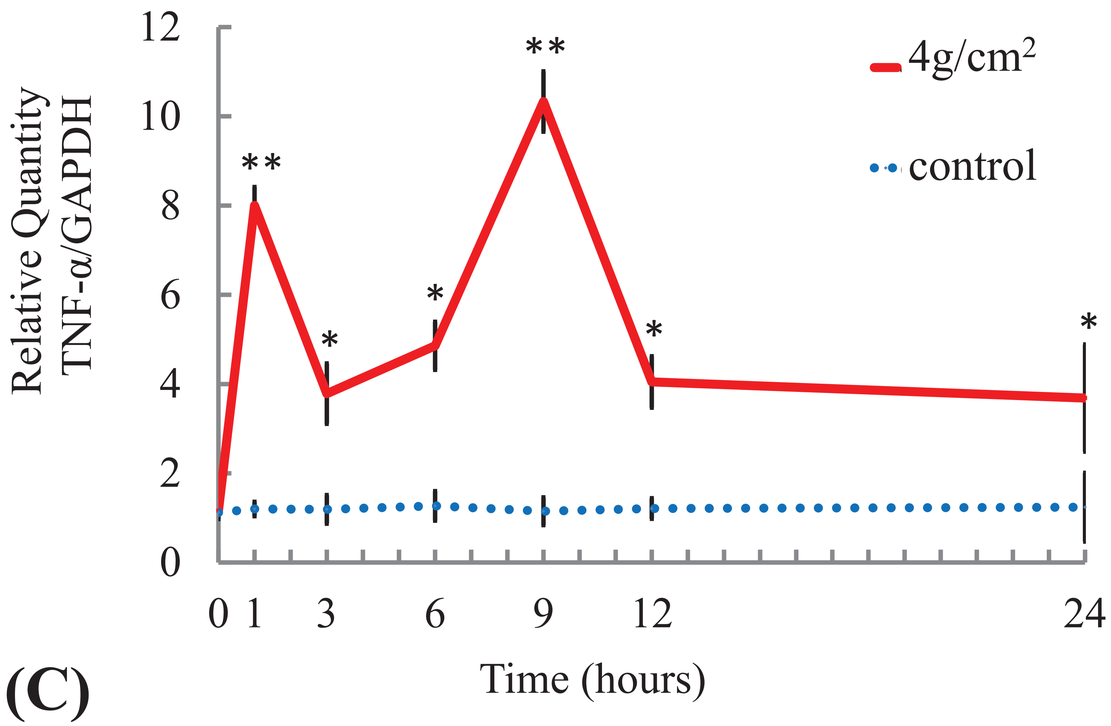


Figure 3


TNF-α release (pg/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
01 3 6 9 12 24 48
Time (hours)
4g/cm2
control
(A)


sRANKL release (pg/ml)
30
25
20
15
10
5
0
01 3 6 9 12 24 48
Time (hours)
4g/cm2
control
(B)


Relative Quantity
TNF-α/GAPDH
12
10
8
6
4
2
0
0 1 3 6 9 12 24
Time (hours)
4g/cm2
control
(C)

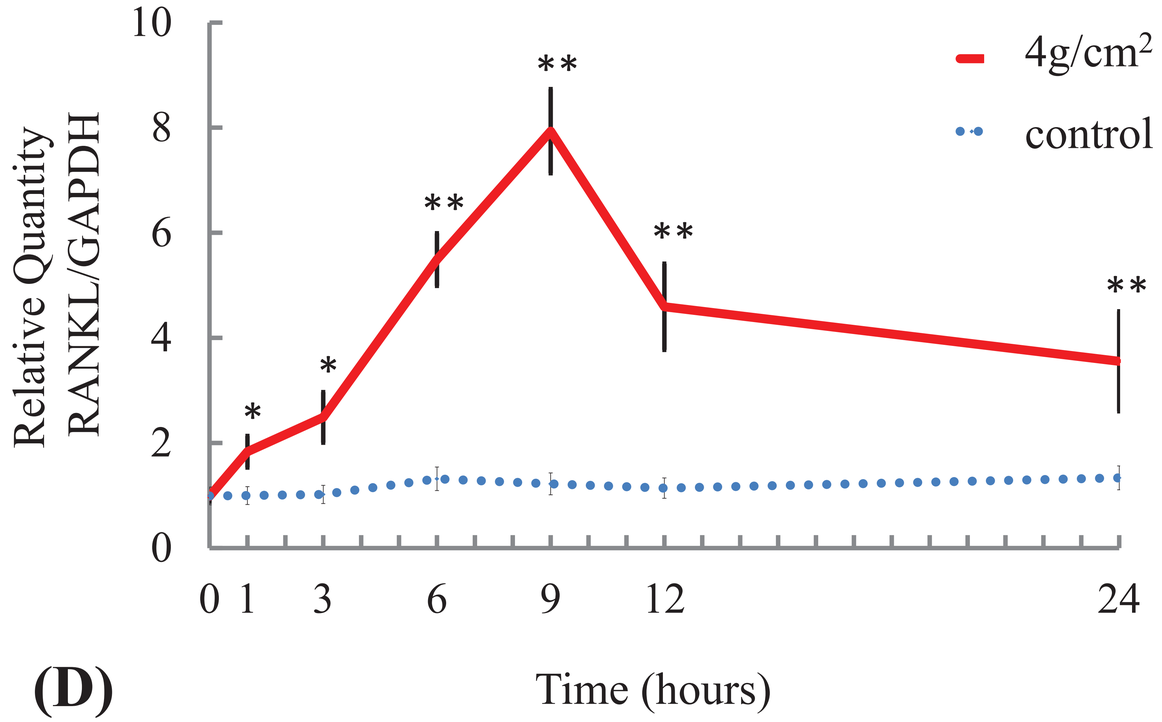
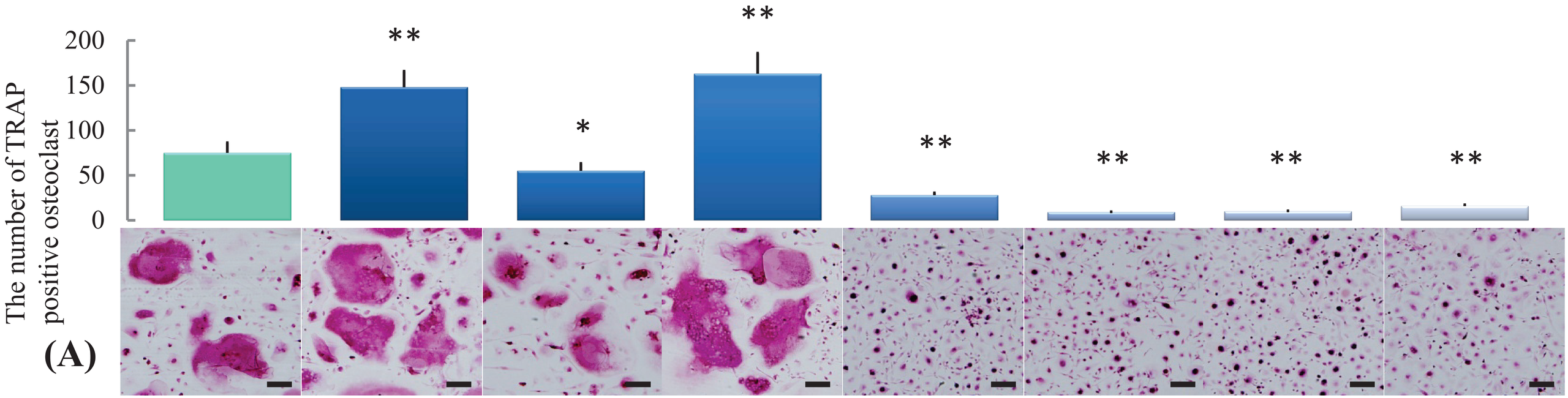

Relative Quantity
RANKL/GAPDH
10
8
6
4
2
0
0 1 3 6 9 12 24
Time (hours)
4g/cm2
control
(D)

Figure 4



	With RANKL group				Without RANKL group			
		CFM	CFM + Anti-TNF-α	rhTNF-α	CFM	CFM + Anti-TNF-α	CFM + OPG	rhTNF-α
group	A	B	C	D	E	F	G	H

Figure 5