Tomokazu Yoshino et al.: TNF-α induces Osteo/Odontoclastogenesis

Original

TNF-α Aggravates the Progression of Orthodontically-induced Inflammatory Root Resorption in the Presence of RANKL

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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 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. Nine days after the tooth movement in the mouse model, an increase in TNF-α and receptor activator of nuclear factor-κB ligand (RANKL) positive cells was observed.

In vitro, their levels increased in the cultures of hPDL cells exposed to the 4 g/cm² 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 synergistic 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 unwanted side effect of orthodontic treatment. It has been reported that 2-5% of orthodontic patients experience more than 5 mm of resorption 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 force1); lengthy treatment and abnormal roots all contribute to the resorption2).

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 compared to without those4).

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. Ustün et al.5) have reported that TNF-α is involved in inflammatory bone destruction in periodontal diseases and rheumatism. TNF-α is produced intravitally at an early stage of resorption due to various stimuli, and is involved in inflammatory bone resorption6) as well as apoptosis7) and immune reactions8). 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)-β9). In contrast, it has been shown that TNF-α induces osteoclastogenesis in the presence of basal levels of RANKL10). The functions of osteoclast and odontoclast are closely related to physiological and pathological character11). There are a lot of similarities in resorption pass ways these cells12). Moreover, it has been reported that excessive expression of RANKL is correlated with bone resorption, and that TNF-α increases the frequency of the expression of RANKL13). Recent study reported that TNF-α and sRANKL expressions in the gingival crevicular fluid obtained from the severe root resorption patients were significantly higher than the non-resorption patients14). 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 examined the relationship between TNF-α and OIIRR by utilizing both an animal model and in vitro studies using hPDL cells and human osteoclast precursor cell (hOCPs) instead of odontoclast.

Materials and Methods

Animal 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 fifteen male (control = 7, orthodontic force (OF) = 8) eight-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.15) 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 left maxillary molar in each mouse to study the experimental tooth movement. The upper first molar was mesially moved by the closed-coil spring with a force of 25 g, which was selected based on the report by Al-Qawasmi et al.4) (Fig. 1-A, B). 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 decalcified and embedded in paraffin using the usual methods. 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 a mouse to study the experimental tooth movement. The force (OF) = 8) eight-week-old wild-type BALB/c mice (body weight 20 ± 5 g; Sankyo Labo Service, Inc., Tokyo, Japan) were used for the experiments.

Immunohistochemistry

Immunohistochemical staining was performed as follows: The tissue sections were deparaffinized, and then the endogenous peroxidase activities were quenched by incubation in 3 % H2O2 in methanol for 30 minutes (min) at room temperature. After being washed in tris-buffered saline (TBS), the sections were incubated with a monoclonal 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 Sommerman et al.10). Briefly, hPDL tissues were collected from the roots of premolars extracted from six healthy young volunteers (three males, three 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 (Forma CO2 incubator MIP-3326; Sanyo Electric Medica System Co., Tokyo, Japan) in 5 % CO2 atmosphere.

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. 1C). 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 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 cell (hOCP) culture

HOCPs were purchased from Lonza Walkersville, Inc. The cells were seeded onto 16-well Lab-Tek chamber slides (Nunc,
Tomokazu Yoshino et al.: TNF-α induces Osteo/Odontoclastogenesis

**Table 1.** Details of the conditioned medium for hOCPs culture.

<table>
<thead>
<tr>
<th>With RANKL (66 ng/ml) group</th>
<th>Without RANKL group</th>
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<tbody>
<tr>
<td>CFM (100 μl)</td>
<td>CFM (100 μl)</td>
</tr>
<tr>
<td>+ rhTNF-α (10 μg/ml)</td>
<td>+ rhTNF-α (10 μg/ml)</td>
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<tr>
<td>CFM (100 μl)</td>
<td>CFM (100 μl)</td>
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<tr>
<td>+ Anti-TNF-α (10 μg/ml)</td>
<td>+ Anti-TNF-α (10 μg/ml)</td>
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<tr>
<td>CFM (100 μl)</td>
<td>CFM (100 μl)</td>
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<tr>
<td>+ OPG (100 μg/ml)</td>
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<td>rhTNF-α (10 ng/ml)</td>
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**Figure 1.** 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) (A, B). 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 (C). Dentine slices stained using Lucifer Yellow CH dilithium salt were observed by laser microscope and steric structure was analyzed (Bar: 30 μm) (D).

Naperville, IL, USA) at a density of 1×10⁴ cells/100 μl with hPDL culture medium. We prepared 2 cell culture groups in which cells were cultured with hOCP culture medium with RANKL, 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
3) rhTNF-α

In addition, we added 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). The total RNA was converted to cDNA using a PrimeScript RT reagent kit (TaKaRa Co., Shiga, Japan) in a thermal cycler (TP-800 Thermal Cycler Dice; TaKaRa Co.) following the manufacturer’s protocol. Amplification was performed using SYBR Premix Ex Taq! (TaKaRa Co.). The primers used were as follows:

- **GAPDH**
  - Fw: 5'-GCACCGTCAAGGCTGAGAAC-3’
  - Rv: 5'-TGGTGAAGACGCCAGTGGGA-3’
- **TNF-α**
  - Fw: 5'-GACAAGCCTGTAGCCCATGTTGTA-3’
  - Rv: 5'-CAGCCTTGGCCCTTGAAGA-3’
- **RANKL**
  - Fw: 5'-GACAAGCCTGTAGCCCATGTTGTA-3’
  - Rv: 5'-CAGCCTTGGCCCTTGAAGA-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 NH4OH 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-D).

**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**

**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. 2-A). In the 25 g group, there was a coarse arrangement of fibers and compressed blood capillaries. On day 9 after the application of the orthodontic devices, many root resorption lacunae with multinucleated odontoclasts were recognized on the surface of the root (Fig. 2-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 25 g group, many TNF-α and RANKL-positive cells were observed in the PDL tissues. The multinucleated cells that were considered odontoclasts were observed in the resorption lacunae (Fig. 2-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...
Figure 3. The effects of CF (4.0 g/cm²) 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/cm²) at the respective time point.

Figure 4. 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: 100 μm. 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. 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).

release of TNF-α was significantly increased, and peaked at 24 h. In addition, the level of RANKL was also increased in a time-dependent manner compared with the control (Fig. 3-A, B).

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 and both peaked at 9 h (Fig. 3-C, D). When hPDLCs 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), whereas TRAP positive cells cultured under presence of anti-TNF-α antibody (group C) were significantly reduced compared to the control cells. (Fig. 4-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. 4-B).

**Discussion**

This study showed that there was an increase in TNF-α and RANKL-positive cells around the tooth root on the pressure side in an experimental tooth movement model using mice. This is consistent with reports from Yoshimatsu et al. 15) and from In the agreement with these results, previous reports described elevated levels of TNF-α and RANKL in compression side following mechanical loading 18-21). Given that the TNF-α and RANKL participate in osteoclastogenesis by upregulating osteoclast activity, TNF-α may be a signal for osteoclast recruitment and bone resorption 22). Furthermore, we demonstrated that a markedly increase in these molecules was found at the resorption lacunae consistent with reports from Yoshimatsu et al. 15) in an experimental tooth movement model using mice. This is RANKL-positive cells around the tooth root on the pressure side (groups E-H) (Fig. 4-B).

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 synergistically 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. 4). In this study, the TNF-α induced by a CF was 1.2 pg/ml (at the peak), while Kukita et al. 26) revealed 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. Moreover, Gokul 27) reported that periodontitis patients have 90.22 pg/ml of TNF-α in the gingival crevicular fluid (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, osteoprotegerin (OPG), a RANKL decoy receptor, is known to suppress osteoclast activity. Kook et al. 28) 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. 29). Furthermore, such an increase was also not found in the study by Mitsuhashi et al. 30). 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 moreover, osteoclast differentiation were inhibited in the group added OPG to in CFM (Fig. 4, group G). From such results, the same conclusion was also drawn.

This study has newly demonstrated that OIIRR could be more induced by the coexistence of RANKL than TNF-α or RANKL alone. Furthermore, the results of our present study also suggest that the combined effects of TNF-α and RANKL on OIIRR are
Tomokazu Yoshino et al.: TNF-α induces Osteo/Odontoclastogenesis

more significant than those of RANKL alone. Further studies about the relationship between TNF-α and RANKL in OIIRR in response to orthodontic force are needed. In conclusion, the TNF-α produced by PDL cells in response to heavy orthodontic force may aggregate OIIRR via RANKL.

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References


