Localization of TNF-α and Macrophages in the Periodontal Ligament during Orthodontic Tooth Movement

Mari Funakoshi¹, Masaru Yamaguchi², Shoji Fujita², and Kazutaka Kasai²

¹Nihon University Graduate School of Dentistry at Matsudo, Orthodontics, Matsudo, Chiba 271-8587, Japan
²Department of Orthodontics, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

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Abstract
Remodelling of the periodontium after application of mechanical forces constitutes the basis of clinical orthodontics, and various immunoregulatory molecules are involved in this process. The present study focused on the localizations of the tumor necrosis factor-α (TNF-α) and macrophages in periodontal ligament (PDL) during experimental tooth movement in rats. A total of 15 male 6-weeks-old Wistar rats were subjected to an orthodontic force of 10g to induce a mesially tipping movement of the upper first molars. Experimental tooth movement was accomplished for seven days. We determined the localization of TNF-α and RM-4 (an antibody specific for identification of macrophages) in the PDL during orthodontic tooth movement using immunohistochemistry. Immunoreactivity for TNF-α and RM-4 was detected in PDL fibroblasts in the compressive side by the orthodontic force of 10g. On the first day after tooth movement, the immunoreactivity of TNF-α and RM-4 was weak. On the third and fifth days, more TNF-α and RM-4 positive reactions in some nucleuses of fibroblasts were recognized than on the first day. Furthermore, these positive reactions were decreased after seven days. Therefore, RM-4 (+) cells involved in the expression of TNF-α may play an important role in the initial reaction of the PDL and in the induction of the osteoclastic bone resorption during orthodontic tooth movement.

Introduction
Orthodontic tooth movement is the result of an organized remodeling of the periodontal tissues after application of mechanical forces. At the cellular level, remodeling of the periodontium consists of bone resorption adjacent to the periodontal ligament (PDL) in the compression zone, bone apposition in the tension zone, and degeneration and reestablishment of the PDL (1). A number of factors have been recognized as participants in the rather complex orchestration of tissue remodeling during orthodontic tooth movement. It has been proposed that chemical mediators, such as cytokines, play an important role (2). Cytokines are small protein molecules that regulate cell communication and function and are actively secreted by different cell types in response to external stimuli. It has been proposed that during orthodontic tooth movement, these signaling molecules are produced by inflammatory cells that migrated from dilated PDL capillaries after orthodontic force application (3).

Tumor necrosis factor-alpha (TNF-α) are key mediators in acute-phase inflammatory reactions with overlapping activities. These pro-inflammatory cytokines are members of the formerly known osteoclast-activating factor and have been implicated in the bone remodeling process (4, 5).

At sites of inflammation, TNF-α is expressed in large quantities by macrophages (6), as well as by many other cell types, including fibroblasts (7), osteoblasts (8), and osteoclasts (9).

Increased levels of TNF-α have been detected in the gingival crevicular fluid of orthodontic patients (10–14), speculating that the elevated cytokines observed in gingival
crevicular fluid (GCF) reflect the biological responses induced by mechanical stress. The TNF-α synthesis in GCF during tooth mobilization appeared to level off 24 h after force application (13, 14), suggesting a central role of these cytokines in the early phase of orthodontic tooth movement.

Macrophages are widely distributed throughout the body to play a crucial role in the defense mechanism (15). In different organs, there exists specifically differentiated organ-specific macrophages, such as tangible body macrophages in the lymphatic follicles, sinus macrophages in the lymphnodes, red pulp, marginal metallophilic and marginal zone macrophages in the spleen and Kupffer cells in the liver (16). Nakamura et al. (17) demonstrated that ED1 (anti-mono-ocyte/macrophage-lineage cells and dendritic cells) in the PDL of the compression side were significantly increased during the orthodontic tooth movement. Furthermore, a marked accumulation of ED1-reactive cells was frequently observed in the area of the hyalinized tissue at 5-7 days after the start of tooth movement. These results suggest that after the start of tooth movement ED1-positive cells, which are mostly exudative macrophages, may be actively engaged in bone resorption and the remodeling of tissues on the compression side.

In the present study, we investigated the localization of TNF-α and RM-4 (an antibody specific for identification of macrophages) in PDL of the compressive side during the experimental tooth movement in rats using immunohistochemical analysis.

Materials and Methods

Animals

The animal experimental protocol in this study was approved by the Ethics Committee for Animal Experiments at Niho University School of Dentistry at Matsudo (approval No. ECA-08-0039). A total of 15 male 6-weeks-old Wistar strain rats (Sankyo Labo Service Co., Tokyo, Japan) weighting 180 ± 10 g were used for the experiment. They were kept in the animal center of Niho University School of Dentistry at Matsudo in separate cages, in a 12-hour light/dark environment at a constant temperature of 23°C and provided with food and water ad libitum. The health status of each rat was evaluated by daily body weight monitoring for 1 week before the start of the experiments.

Application of orthodontic devices and tissue harvesting

Fifteen male 6-weeks-old Wistar rats with an average body weight of 180 ± 10 g were used. Animals were anaesthetized with pentobarbital sodium (40 mg/kg body weight) for application of orthodontic devices. Experimental tooth movement was performed using the method of Fujita et al. (18), with a closed-coil spring (wire size: 0.005 inch, diameter: 1/12 inch. Accurate Sales Co., Chiba, Japan) ligated to the maxillary first molar cleft by a 0.008 inch stainless steel ligature wire (Tomy International Inc., Tokyo, Japan). The other side of the coil spring was also ligated, with the holes in the maxillary incisors drilled laterally just above the gingival papilla with a #1/4 round bur, using the same ligature wire. The upper first molar was moved mesially by the closed coil spring with a force of 10 g (Fig. 1). The period of experiment was performed for 7 days.

Tissue preparation

The experimental periods were set at 1, 3, 5 and 7 days after tooth movement. Animals were deeply anesthetized with pentobarbital sodium, and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) in a trans-cardial manner, after which the maxilla was immediately dissected and immersed in the same fixative overnight at 4°C. The specimens were decalcified in 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for 4 weeks, and then decalcified specimens were dehydrated through an ethanol series and embedded in paraffin. Each sample was sliced into 6 μm continuous sections in the horizontal direction, and prepared for Hematoxylin and Eosin (H.E.) and also for immunohistochemistry staining for TNF-α and RM-4. The periodontal tissues in the distal part of the distal buccal root of a first upper molar (M1) was observed. The one that was not moved was defined as the control group.

Measurement of tooth movement

Measurement of tooth movement was performed according to the method of Fujita et al. (18). To determine the amount of tooth movement, plaster models of the maxillae were made using silicone impression material (Dent Silicone-V, Shofu Inc. Kyoto, Japan) before (day 0) and after initiating tooth movement (days 1, 3, 5, and 7). The plaster models were scanned using a contact-type three-dimensional measurement apparatus (3D-picza, Roland Co.) by setting the plane to pass through three points, which were the bilateral interpapillary crests between the first and
second molars, and the interpapillary crest between the second and third molars. Using three-dimensional morphological analysis software (3D-RUGLE, Medic Engineering Inc.), we measured the distance between the first molar central fossa and second molar mesial surface to determine tooth movement.

Immunohistochemistry

Immunohistochemical staining was performed as follows: The sections were deparaffinized and the endogenous peroxidase activities were quenched by incubation in a Peroxidase-blocking solution (DAKO, Japan) for 10 minutes at room temperature (RT). After washing in PBS, the sections were incubated with polyclonal anti-rabbit TNF-α (IHCWORLD, ready to use), and polyclonal anti-rabbit RM-4 (Trans Genic, working dilution, 1:200) antibodies for 18 hours at 4°C. TNF-α and RM-4 were stained using the histofine simple stain MAX-Po (multi) kit (Nichirei, Co., Tokyo, Japan) according to the manufacturer’s protocol.

The sections were rinsed with Tris-buffered saline (TBS) and final color reactions were performed using the substrate reagent 3, 3′-diaminobenzidine tetra-hydrochloride and aminoethylcarbazole, then they were counter-stained with hematoxylin. As the immunohistochemical controls, some sections were incubated in the same way, and then incubated with either non-immune rabbit IgG or 0.01 M phosphate buffered saline (PBS) alone, instead of the primary antibody. Negative reactivity was observed for the controls.

Statistical analysis

The values in each figure represent the mean ± standard deviation (SD) for each group. Intergroup comparisons of average values were evaluated by one-way analysis of variance (ANOVA), followed by a Tukey test, with values of $p < 0.01$ considered to indicate a significant difference (Fig. 2).

Results

The body weights and tooth movement during experimental period

The body weights of the rats in the application of orthodontic devices (10 g) decreased transiently on day one and then recovered (data not shown). The amount of tooth movement increased from zero to seven days during experimental period ($p < 0.01$, Tukey test; $p < 0.01$, by one-way ANOVA) (Fig. 2).

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

Fig. 3a showed histological changes in periodontal tissues during tooth movement. In control (day 0), rat PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that regularly ran in a horizontal direction from the root cementum toward the alveolar bone. On the first day after tooth movement, blood capillaries were mainly recognized near the alveolar bone in the PDL. Only a few mononuclear and multinucleate osteoclasts were rarely observed on the alveolar bone surface (Fig. 3b). The arrangement of the fibers and fibroblasts become coarse and irregular, and blood capillaries were pressured on the third and fifth days (Figs. 3c, d). On the surface of the alveolar bone, bone resorption lacunae with multinucleate osteoclasts were recognized. Osteoclasts on the alveolar bone were increased in comparison with these after 3 days. On the seventh day, the PDL was recomposed of the coarse arrangement of fibers and expanded blood capillaries. The resorption lacunae with multinucleate osteoclasts decreased on the alveolar bone compared to the third day (Fig. 3e).
Fig. 2  Tooth movement by the orthodontic force of 10 g for seven days. The amount of tooth movement increased from 0 to 7 days during the experimental period ($p < 0.01$, Tukey test; $p < 0.01$, by one-way ANOVA).

Fig. 3  Effect of orthodontic forces on the multinucleate osteoclasts by light microscopic images (H.E.). After 7 days, the resorption lacunae with multinucleate osteoclasts decreased on the alveolar bone compared with after 3 days. AB: alveolar bone, PDL: periodontal ligament, C: cementum, D: dentin. Bar = 50μm.
Protein localization of TNF-α and RM-4

The immunoreactivity of TNF-α and RM-4 was performed 7 days after tooth movement (Figs. 4 and 5). The positive reaction of TNF-α and RM-4 was recognized in the fibroblastic PDL cells. On the first day after tooth movement, some of TNF-α and RM-4 was localized in fibroblastic PDL cells and pericytes near the alveolar bone surface (Figs. 4c and 5c). On the third day, more TNF-α and RM-4 positive reactions in fibroblastic PDL cells were recognized than on the first day in the compressed PDL (Figs. 4d and 5d). TNF-α and RM-4 positive reactions in fibroblastic PDL cells were increased after five days (Figs. 4e and 5e). Furthermore, these positive reactions were decreased after 7 days (Figs. 4f and 5f).

Discussion

Considering the method of tooth movement, 10 g of light force application produced tooth movement without root resorption over a period of 7 days in rats. The resorption lacunae with multinucleate osteoclasts appeared on the alveolar bone on the third, fifth, and seventh days after tooth movement (Fig. 2 and 3). Gonzales et al. (19) showed that 10 g of light force application produced significantly larger tooth movement with significantly less root resorption over a period of 28 days in relation to a heavier force application in rats. The optimum force for the movement of the rats’ upper molars may be less than 10 g as previously suggested (20). Therefore, the model in this study was supported as the method of efficient tooth movement.

Fig. 4 and 5 demonstrated that after the start of experimental tooth movement, significant changes in TNF-α and RM-4 positive reaction found in the compression side of PDL on the fifth day. TNF-α is a candidate cytokine involved in orthodontic tooth movement (14, 21, 22). Yoshimatsu et al. (23) demonstrated that on days 2, 6, and 10 after application of the orthodontic force, expression of TNF-α was identified in the osteoclasts and mononuclear cells located on the alveolar bone surface as well as in fibroblastic cells in the PDL on the compression side. Mitsuhashi et al. (24) reported that the mRNA expression of TNF-α in compressed PDL
cells was found to be increased in a time and magnitude-dependent manner, detectable after nine hours, and TNF-α expression gradually decreased after 12 hours. In this study, TNF-α were detected during tooth movement by immunohistochemistry.

Osteoclasts are derived from macrophage-lineages precursors. RM-4 is an antibody that can recognize this lineage of cells. Xie et al. (25) reported that the number of ED-1 positive cells, an antibody for macrophage-lineages precursors, increased in the bone marrow on 5 days after application of orthodontic force. Thereafter, these cells decreased. Among the anti-rat macrophage monoclonal antibody (mAB), ED-1 reacts with monocyte/macrophages and dendritic cells. However, ED-1 mAB react with monocytes and with some cell types other than macrophages and dendritic cells (26). Iyonaga et al. (27) reported that RM-4 did not label monocytes, granulocytes or fibroblasts, and concluded that RM-4 is considered to be a useful tool for identifying macrophage/dendritic cells. Therefore, the macrophages were detected specifically during tooth movement in this study.

Considering the relationship between TNF-α and macrophages during orthodontic tooth movement, Baba et al. (28) demonstrated that macrophages expressed TNF-α in the PDL during orthodontic tooth movement by double-immunofluorescence staining. These findings suggested that macrophages involved in the localization of TNF-α may play an important role in the initial reaction of the PDL. Previous studies reported that activated macrophages, monocytes, lymphoid cells, and fibroblasts produce TNF-α (29, 30). Kook et al. (31) suggest that PDL fibroblasts secreted relatively higher levels of TNF-α at the compression side than at the tension side, and this imbalance leads to RANKL expression by activating CD4+ T cells, thereby facilitating bone resorption during orthodontic tooth movement. Previous in vitro studies suggest that macrophages in the PDL may be closely associated with bone resorption during tooth movement by the production of TNF-α (32, 33). Therefore, TNF-α may play an important role in osteoclastic recruitment and activation. However, more research is required to under-

Fig. 5 Effect of orthodontic forces on RM-4 positive PDL fibroblasts by immunohistochemistry. The immunoreactivity of RM-4 positive was observed in the fibroblastic PDL cells on the alveolar bone surface. RM-4 positive fibroblasts increased after 5 days. AB: alveolar bone, PDL: periodontal ligament, C: cementum, D: dentin. Bar = 50 μm.
stand the effects of macrophage-derived cytokines such as interleukin (IL) -1β, IL-6, which are known to be very important stimulators of osteoclastic bone resorption on bone remodeling during tooth movement.

**Conclusion**

Macrophages involved in the localization of TNF-α may play an important role in the initial reaction of the PDL and in the induction of the osteoclastic bone resorption during orthodontic tooth movement.

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