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ORIGINAL ARTICLE

IL-8 and MCP-1 induced by excessive orthodontic force mediates odontoclastogenesis in periodontal tissues

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OBJECTIVE: The aim of this study was to investigate how interleukin (IL)-8 (cytokine-induced neutrophil chemoattractant; CINC-1) and monocyte chemotactic protein (MCP)-1/CCL2 contribute to root resorption during orthodontic tooth movement.

MATERIALS AND METHODS: Forty 6-week-old male Wistar rats were subjected to orthodontic force of 10 or 50 g to induce a mesially tipping movement of the upper first molars for 7 days. We determined the expressions of CINC-1, CXCR2, and MCP-1 proteins in root resorption area using immunohistochemistry. Furthermore, we investigated the effects of compression forces (CF) on IL-8 and MCP-1 production by human periodontal ligament (hPDL) cells. We observed an effect of chemokine treatment on rat odonto/osteoclasts in dentin slices that recapitulated root resorption.

RESULTS: The immunoreactivity for CINC-1/CXCR2 and MCP-1 was detected in odontoclasts and PDL fibroblasts by the orthodontic force of 50 g on day 7. CF increased the secretion and the expression of mRNA of IL-8 and MCP-1 from PDL cells in a magnitude-dependent manner. Moreover, CINC-1 and MCP-1 stimulated osteoclastogenesis from rat osteoclast precursor cells.

CONCLUSION: IL-8 (CINC-1) and MCP-1 may therefore facilitate the process of root resorption because of excessive orthodontic force.

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Keywords: orthodontic tooth movement; root resorption; IL-8; CXCR2; MCP-1

Introduction

Orthodontically induced inflammatory root resorption (OIIRR) is an unavoidable pathologic consequence of orthodontic tooth movement. It can be defined as an iatrogenic disorder that unpredictably occurs after orthodontic treatment, whereby the resorbed apical root portion is replaced with normal bone. OIIRR is a sterile inflammatory process that is extremely complex and involves various disparate components, including mechanical forces, tooth roots, bone, cells, surrounding matrix, and certain known biologic messengers (Krishnan and Davidovitch, 2006; Meikle, 2006). In the relationship between OIIRR and inflammatory cytokines, Zhang *et al* (2003) indicated that IL-1 and TNF-α are important for the induction and the further processing of mechanically induced root resorption in the rat. Nakano *et al* (2010) demonstrated that the receptor activator of nuclear factor κB ligand (RANKL)/RANK and macrophage colony stimulating factor (M-CSF)/*c-fms* systems may be involved in root resorption as a result of heavy orthodontic force. Therefore, these inflammatory cytokines contribute to alveolar bone remodeling and to resorption during orthodontic tooth movement and OIIRR.

Chemokines form a family of structurally related chemoattractant proteins. Chemokines have been classified into four subfamilies, depending on the number and spacing of the first conserved cysteine residues in the NH_2-terminal region: CXC, CC, XC, and CX_3C. In general, CC chemokines (including monocyto chemotactic protein/MCP, macrophage inflammatory protein/MIP and regulated upon activation normal T expressed and presumably secreted/RANTES) and CXC chemokines (including interleukin/IL-8 and cytokine-induced neutrophil chemoattractant/CINC) are potent chemoattractants for monocytes and neutrophils, respectively. Recent studies have recognized these chemokines as essential signals for the trafficking of osteoblast and osteoclast precursors, and also for the development, activity and survival of bone cells (Bendre *et al*, 2003; Wright and Friedland, 2004). Garlet *et al* (2008) demonstrated the expressions of MCP-1/CCL2, MIP-1alpha/CCL3 and RANKL in compressed and stretched PDLs during orthodontic tooth movement. Both recombinant human MCP-1 and IL-8 directly stimulated human bone marrow mononuclear cell (HBMC) differentiation into osteoclast-like cells (Lu *et al*, 2007). Therefore, these chemokines may

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contribute to alveolar bone remodeling during orthodontic tooth movement.

However, little is known about the relationship between OIIRR and chemokine. In this study, we investigated the expressions of CINC-1, CXCR2 (CINC-1 receptor), and MCP-1 in rat root resorption during experimental tooth movement because of the application of heavy force (50 g) using an immunohistochemical analysis. Furthermore, to reproduce the conditions of pressure during orthodontic tooth movement, we investigated the effects of compression forces (0–4.0 g) on both IL-8 and MCP-1 production by human PDL cells *in vitro*, and also the effects of these chemokines on the formation of osteoclasts from rat osteoclast precursor cells.

Materials and methods

Animal studies

Animals. The animal experimental protocol in this study was approved by the Ethics Committee for Animal Experiments at the Nihon University School of Dentistry at Matsudo (approval No. ECA-08-0039). A total of 40 6-week-old male Wistar strain rats (Sankyo Labo Service, Inc., Tokyo, Japan. body weight 180 ± 10 g) were used for the experiments. Animals were maintained in the animal center of Nihon University School of Dentistry at Matsudo in separate cages on a 12-h light/dark environment at a constant temperature of 23°C, and were 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. Forty 6-week-old male Wistar rats with an average body weight of 180 g were used. Animals were anesthetized with pentobarbital sodium (40 mg kg^{-1} body weight) for the application of orthodontic devices. Experimental tooth movement was induced using the method of Fujita *et al* (2008), with a closed-coil spring (wire size: 0.005 inch, diameter: 1/12 inch, Accurate, Inc., Tokyo, Japan) ligated to the maxillary first molar cleat 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 mesially moved by the closed coil spring with a force of 10 or 50 g. The period of the experiment was performed for 7 days.

Measurement of tooth movement. Tooth movement measurements were performed according to the method of Fujita *et al* (2008). To determine the magnitude of tooth movements, plaster models of the maxillae were made using a silicone impression material (Dent Silicone-V; Shofu, Inc. Kyoto, Japan) before (day 0) and after initiating the tooth movement (days 1, 3, 5 and 7). The plaster models were scanned using a contact-type three-dimensional measurement apparatus (3D-picza; Roland DG Co., Shizuoka, Japan) 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 a three-dimensional morphological analysis software program (3D-Rugle; Medic Engineering Inc., Kyoto, Japan), we measured the distance between the first molar central fossa and second molar mesial surface to determine the total tooth movement.

Tissue preparation. The experimental periods were set at 1, 3, 5, and 7 days after tooth movement was initiated. Animals were deeply anesthetized by pentobarbital sodium and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the maxilla was immediately dissected and immersed in the same fixative for 18 h at 4°C. The specimens were decalcified in 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for 4 weeks, and the decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin. Each sample was sliced into 6-μm sections continuously in the horizontal direction and was prepared for hematoxylin and eosin staining (H.E.), and also for immunohistochemical staining using tartrate-resistant acid phosphatase (TRAP), CINC-1, CXCR2, and MCP-1. The periodontal tissues in the mesial part of the distal buccal root of a first upper molar were observed. The one that was not moved was defined as the control group.

Immunohistochemistry. Immunohistochemical staining was performed as follows. The sections were deparaffinized and the endogenous peroxidase activities were quenched by incubation in 3% H_2O_2 in methanol for 30 min at room temperature (RT). After washing in Tris Bufferd Saline (TBS), the sections were incubated with polyclonal anti-rabbit TRAP (Santa Cruz Biotechnology, Inc., CA, USA; working dilution, 1:400), polyclonal anti-rabbit MCP-1 (Santa Cruz Biotechnology, working dilution, 1:100), monoclonal anti-rat CINC-1 (American Research Products, Inc., MA, USA; working dilution, 1:50), and polyclonal anti-rabbit CXCR2 (Santa Cruz Biotechnology, working dilution: 1:100) for 18 h at 4°C. CINC-1, CXCR2, and MCP-1 were stained using the Histofine Simple Stain MAX-Po kit (Nichirei, Co., Tokyo, Japan) according to the manufacturer's protocol. The sections were rinsed with TBS and the final color reactions were performed using the 3, 3'-diaminobenzidine tetrahydrochloride substrate reagent and aminoethyl carbazole, and the sections were then counter-stained with hematoxylin. For the immunohistochemical controls, several sections were incubated with either non-immune rabbit IgG or 0.01 M PBS (phosphate bufferd saline) instead of the primary antibody. Negative reactivity was observed. TRAP-positive cell was counted as multinucleate odontoclasts, which were observed on the surface of cementium (n = 5 rats per group).

In vitro study

Human periodontal ligament fibroblasts cell culture. Human periodontal ligament were prepared according to a modification of the method of Somerman *et al* (1988). Briefly, periodontal ligament tissues were taken from the roots of premolars extracted from six healthy young volunteers (three males, three females; 14–16 years of age), during the course of orthodontic treatment, after obtaining informed consent from the donors, and were used according to a protocol reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (#04–021). The periodontal ligament tissues were placed in 35-mm tissue culture dishes and covered with a sterilized glass coverslip. The medium was minimal essential medium (Gibco, NY, USA), which was supplemented with 100 μg ml^{-1} of penicillin-G (Sigma Chemical Co., MO, USA), 50 μg ml^{-1} of gentamicin sulfate (Sigma Chemical Co.), 0.3 μg ml^{-1} of amphotericin B (Flow Laboratories, VA, USA), and 10% fetal calf serum (Cell Culture Laboratories, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326; Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2. When the cells growing from each explant reached confluence, they were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline for 10 min and subcultured in culture flasks. The cells still adhered to the bottom of the flask were discarded to avoid contamination by epithelial cells.

Rat osteoclast cells culture. Rat osteoclast precursor cells purified from rat bone marrow cells were purchased from Hokudo Co. (Hokkaido, Japan). The cells were seeded onto 16-well Lab-Tek chamber slides (Nunc, IL, USA) at a density of 4×10^4 cells per 100 μl and were maintained in essential medium with M-CSF and RANKL (both at 50 ng ml^{-1}) for 10 days. CINC or MCP-1 treatment was used at 20 ng ml^{-1} (Peprotech EC Ltd., London, UK) (Bendre *et al*, 2003).

Application of compression force. To reproduce the conditions of pressure during orthodontic tooth movement, we performed the following *in vitro* experiments as described by Nakajima *et al* (2008). hPDL cells were continuously compressed using a uniform compression method as a model of pressure at the site of orthodontic movement. Static compression force is thought to mimic that found *in vivo* during orthodontic treatments. In the present experiments, the cells were stimulated once. Briefly, a cell disk, 90 mm in diameter, was placed over nearly confluent cell layers in the dishes, on top of which was placed a glass cylinder. Compression force was then controlled by placing lead granules in the cylinder. Before the application of compression force, the cells were pre-incubated for 1 h in culture medium containing 1% fetal calf serum, after which they were subjected to 1.0, 2.0, 3.0 or 4.0 g cm^{-2} of compression force for 24 h. Previous studies have shown that compressive mechanical stress can be applied by the system utilized in the present experiment (Yamaguchi *et al*, 2004). Control cells were covered with thin glass plates without lead weights, which produced a compression force of 0.032 g cm^{-2}. The possibility that removal of the glass plates might cause static electricity, which could affect the cells, was controlled by the use of glass plates with both the CF and control groups.

Real-time polymerase chain reaction. We extracted RNA from hPDL cells treated with compression force an RNeasy Mini-kit (Qiagen Co., Tokyo, Japan), according to the manufacturer's protocol. RNA was amplified with an RT-PCR kit and 40 μl of purified total RNA was obtained, then total RNA was converted to cDNA using a Prime Script RT Reagent Kit (Takara Co., Shiga, Japan). Real-time polymerase chain reaction (PCR) amplification was performed using SYBR Premix Ex Taq (Takara Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; Takara Co.). After a hot start, the samples were denatured at 95°C for 5 s, and the primer was annealed at 60°C for 30 s at 40 cycles. PCR primers for IL-8/CXCL8, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, and GAPDH were purchased from Takara Co., and were designed with reference to the respective cDNA sequences. The primers were designed as follows:

IL-8, 5′-ACACTGCGCCAACACAGAAATTA-3′ and 5′-TTTGCTTGAAGTTTCACTGGCATC-3′; MCP-1, 5′-GCTCATAGCAGCCACCTTCATTC-3′ and 5′- GG ACACTTGCTGCTGGTGATTC-3′; MIP-1α, 5′-CCT GCTCAGAATCATGCAGGTC-3′ and 5′-AGCACTG GCTGCTCGTCTCA-3′; RANTES, 5′-ACCAGTGGC AAGTGCTCCAAC-3′ and 5′-CTCCCAAGCTAGGA CAAGAGCAAG-3′, and GAPDH, 5′-GCACCGTCAA GGCTGAGAAC-3′ and 5′-TGGTGAAGACGCCAG TGA-3′ The levels of the real-time PCR products corresponding to GAPDH were the same in the experimental groups. Therefore, the amount of real-time PCR products reflected the level of each mRNA. We used the real-time PCR products treated without compression force as the control.

Enzyme-linked immunosorbent assay. IL-8 and MCP-1 released into the culture supernatants were measured using a Human IL-8/CXCL8 Enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Co., MN, USA) and a Human MCP-1/CCL2 ELISA kit (R&D Systems Co.) according to the manufacturer's protocol.

Tartrate-resistant acid phosphatase staining. When the incubation schedule was completed, the osteoclasts were washed twice with PBS and stained with TRAP using a commercially available staining kit (Hokudo Co.).

Pit formation assay. The resorptive activity of mature rat 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). After incubation for 10 days, 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 for scanning electron microscopy and then were sputter-coated with platinum or onto glass slides for light microscopy. The entire surface of each dentin slice was examined using a scanning electron microscope (JSM-6340F; JEOL, Tokyo, Japan). The resorption pit area in a single well was determined by means of an image processing system (Win Roof; Mitani Co., Tokyo, Japan).

Statistical analysis

The values in each figure represent the mean ± standard deviation (SD) for each group. Intergroup comparisons of average values were evaluated using a one-way analysis of variance (ANOVA), followed by Tukey's test, and *P*-values of less than 0.05 were considered to be statistically significant.

Results

Animal studies

Body weights and tooth movement during experimental period. The body weights of the rats in the application of orthodontic devices (10 and 50 g groups) decreased transiently on day 1 and then recovered. No significant differences were found among the three groups (10, 50 g, and control groups) (data not shown). The amount of tooth movement was also not significantly different between the 10 and 50 g groups during the experimental period (7 days) (Figure 1).

Histologic changes in periodontal tissues during tooth movement (H.E. staining). In the control group, the rat PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that were regularly arranged in a horizontal direction from the root cementum toward the alveolar bone. Blood capillaries were mainly recognized near the alveolar bone in the PDL. The alveolar bone and root surfaces were relatively smooth, but only a few mononuclear and multinucleate osteoclasts and resorption lacunae were rarely observed on the alveolar bone surface (Figure 2a). In 10 g group, the arrangement of the fibers and fibroblasts become coarse and irregular, and the blood capillaries were pressured on 1 and 3 days. Resorption lacunae with few multinucleate osteoclasts were observed on the surface of the alveolar bone surface (Figure 2b, d). At 5 and 7 days 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 on the first day (Figure 2f, h). In the 50 g group, the PDL was composed of a coarse arrangement of fibers and expanded blood capillaries. Many resorption lacunae with multinucleate osteoclasts appeared on the alveolar bone on day 1 (Figure 2c). At 3 and 5 days, the number of resorption lacunae with multinucleate osteoclasts was found to have increased on the alveolar bone (Figure 2e, g). On day 7, on the surface of the root many root resorption lacunae with multinucleate odontoclasts were found (Figure 2i).

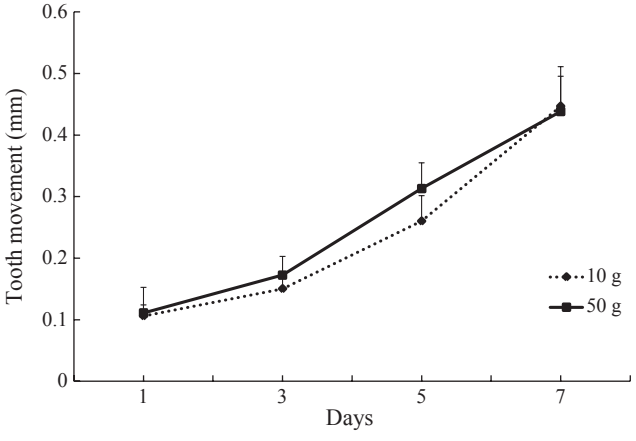

Figure 1 Effect of 10 and 50 g force on tooth movement. Values are shown as the mean ± s.d.. of five rats

Immunohistochemical findings of TRAP. In the control group, the resorption lacunae with TRAP-positive multinucleate osteoclasts were not observed on the surfaces of the alveolar bone or root (Figure 3Aa). In the 10 g group, several resorption lacunae with TRAP-positive multinucleated osteoclasts appeared on the alveolar bone surface of pressure side on day 1 and 3 (Figure 3Ab, d). On day 5 and 7 on the surface of the alveolar bone, bone resorption lacunae with multinucleate TRAP-positive osteoclasts were recognized. The multinucleate TRAP-positive osteoclasts on the alveolar bone were increased in comparison with these on day 1 (Figure 3Af, h). In the 50 g group, on the surface of the alveolar bone many bone resorption lacunae with multinucleated TRAP-positive osteoclasts were identified on day 1 (Figure 3Ac). At 3 and 5 days, TRAP-positive osteoclasts increased on the alveolar bone (Figure 3Ae, g). On day 7, many root resorption lacunae with TRAP-positive odontoclasts were observed (Figure 3Ai). In our quantitative evaluations, the number of TRAP-positive odontoclasts was found to be significantly increased in the 50 g group on day 7 (6.0-fold) in comparison with the 10 g groups ($P < 0.01$) (Figure 3B).

Protein expressions of CINC-1, CXCR2, and MCP-1. The immunoreactivity of CINC-1, CXCR2, and MCP-1 was performed on days 1, 3, 5, and 7 after tooth movement. CINC-1-, CXCR2- and MCP-1-positive cells were rarely observed from the control group (Figures 4a, 5a and 6a). In the 10 g group, few CINC-1- and CXCR2-positive fibroblasts in the PDL and osteoclasts at the root surface were increased. CINC-1- and CXCR2-positive odontoclasts were rarely observed from 1 to 7 days (Figures 4b, d, f, h and 5b, d, f, h). MCP-1-positive PDL fibroblasts were rarely observed from 1 to 7 days (Figure 6b, d, f, h). In the 50 g group, many CINC-1- and CXCR2-positive odontoclasts and fibroblasts were observed. Many odontoclasts showed strong CINC-1 and CXCR2 immunoreactivity (Figures 4c, e, g and 5c, e, g). The number of MCP-1-positive PDL fibroblasts were also

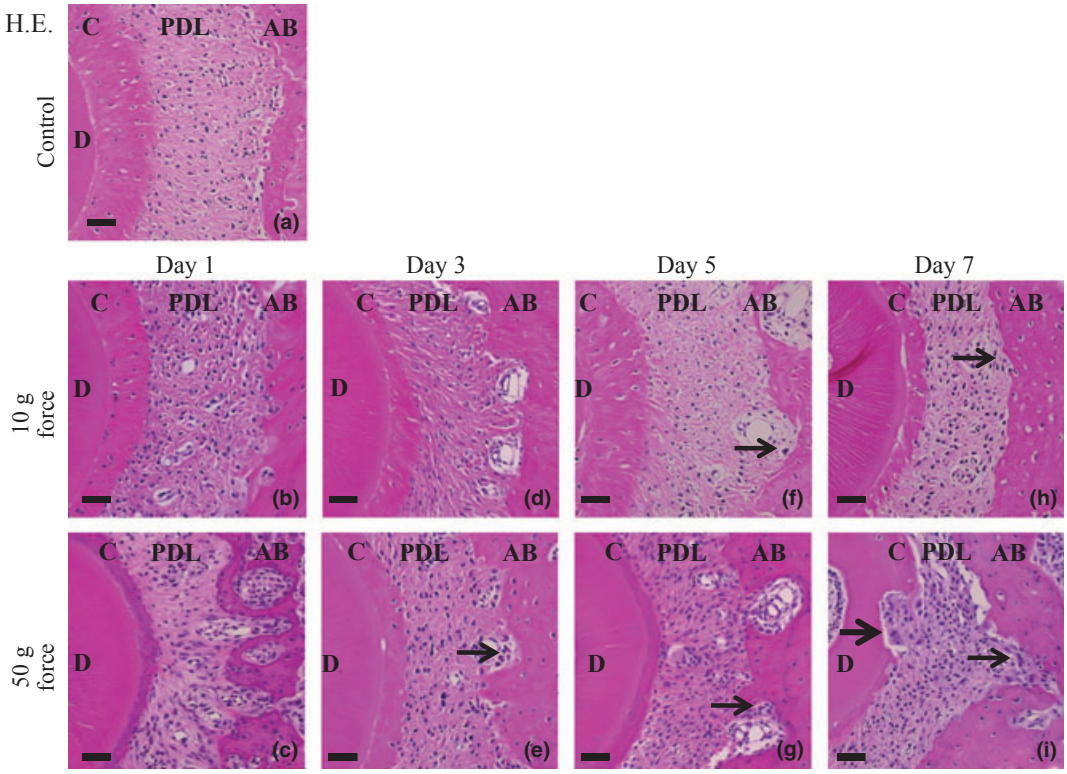

Figure 2 Effects of different orthodontic forces on the multinucleate osteoclasts by light microscopic images (haematoxylin and eosin (H.E.); original magnification 400 ×). The osteoclasts (thin arrow) appeared on the alveolar bone surface in both groups on days 5 and 7. The odontoclasts (thick arrow) on the cementum in the 50 g group were greater than that in the 10 g group on day 7. AB: alveolar bone, PDL: periodontal ligament, C: cements, D: dentin. Bar: 50 μm

found to have increased in comparison with that of the corresponding 10 g group (Figure 6c, e, g). Many CINC-1- and CXCR2-positive odontoclasts and fibroblasts were observed. Many odontoclasts showed strong CINC-1 and CXCR2 immunoreactivity (Figures 4i and 5i). PDL fibroblasts were strongly immunohistochemically positive for MCP -1 (Figure 6i).

In vitro study

Effect of CF on the release of IL-8 and MCP-1. The release of IL-8 and MCP-1 in PDL cells was determined using ELISA. When hPDL cells were subjected to a CF for 24 h, the release of IL-8 and MCP-1 was increased compared with the control cells ($P < 0.05$, Tukey's test), in a time- and magnitude-dependent manner ($P < 0.01$, one-way ANOVA) (Figure 7A, B).

Effect of CF on the gene expression of IL-8 and MCP-1 by real-time PCR. Human periodontal ligament cells were cultured with or without CF for up to 24 h, and the expression of mRNA for IL-8 and MCP-1 in the PDL cells was determined using a real-time PCR analysis after 3, 6, 9, 12, and 24 h. The mRNA expression of IL-8 and MCP-1 was significantly increased in a magnitude-dependent manner with increasing CF, and peaked at 6 h thereafter ($P < 0.01$, Tukey's test) (Figure 7C, D).

Effects of CINC and MCP-1 on osteoclast formation. We performed TRAP staining following 10 days of CINC and MCP-1 treatment. The number of TRAP-positive cells was significantly increased by CINC and MCP-1 treatment for 10 day when compared with the controls (without CINC and MCP-1) ($P < 0.01$, Tukey test) (Figure 8A).

Pit formation assay. The resorption of mineralized tissue was investigated by measuring the capacity of osteoclasts formed *in vitro* to resorb dentin. When osteoclast precursor cells were cultured for 10 days on dentin slices, resorption lacunae appeared on the surfaces of the slices. Resorption pits were more abundant in the CINC and MCP-1 treatment groups than in the control group ($P < 0.05$, Tukey's test). Significant differences were observed between the CINC and MCP-1 treatment groups and the control group (Figure 8B).

Discussion

Considering the methods used to induce root resorption, the amount of tooth movement was not significantly different between the 10 and 50 g groups during the experimental period (days 7). King *et al* (1991) demonstrated that the effective tooth movement of rat molars for 14 days ranged from 20 to 40 g, and the velocity did not increase over 40 g. Gonzales *et al* (2008) showed that the amount of tooth movement was not significantly different among the 10, 25, or 50 g groups over a period of 14 days. These reports were consistent with

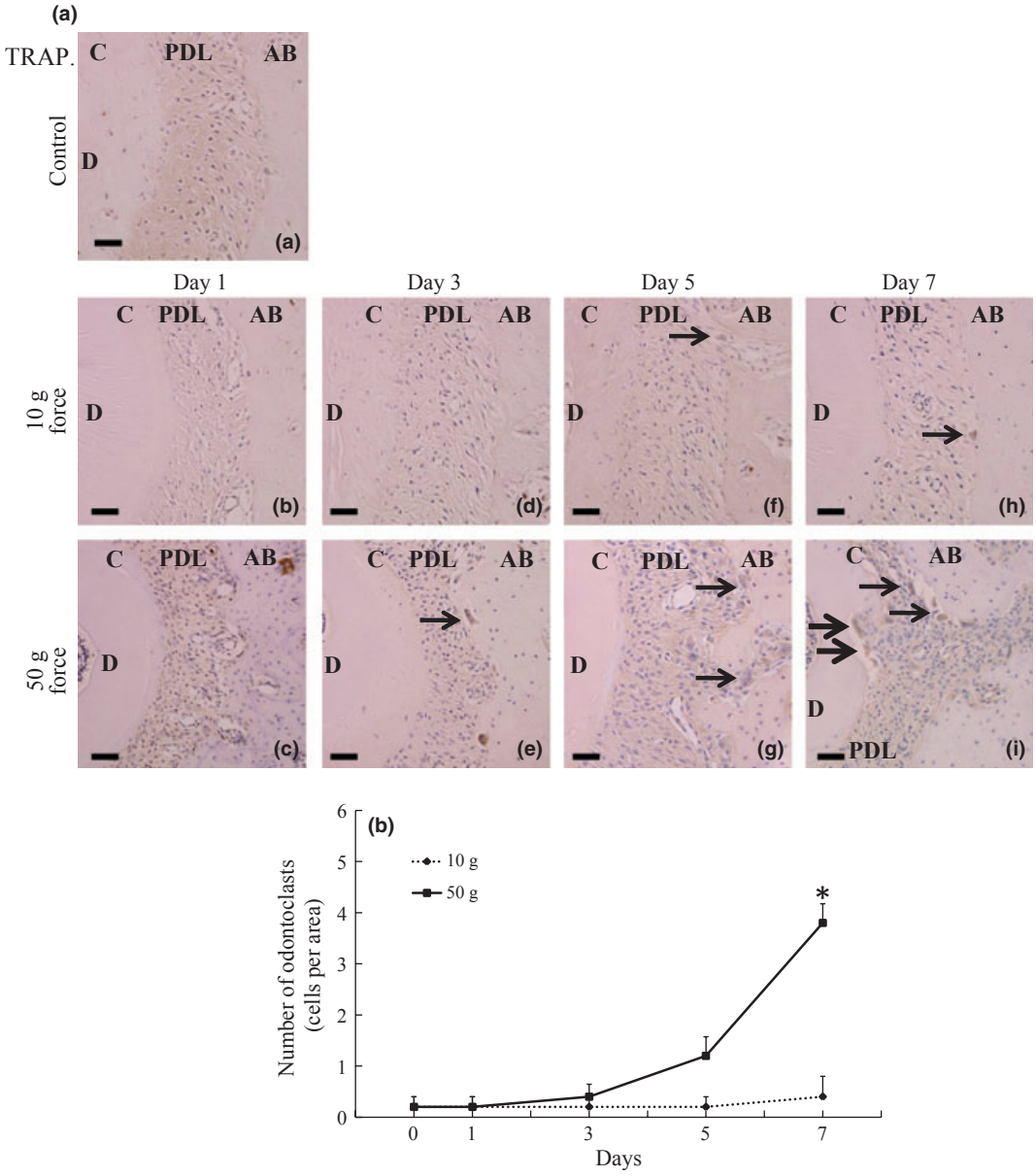

Figure 3 (**a**) Effects of different orthodontic forces on TRAP-positive odontoclasts by immunohistochemistry (original magnification 400×). The osteoclasts (thin arrow) appeared on the alveolar bone surface in both groups on days 5 and 7. The immunoreactivity of TRAP-positive odontoclasts was observed in the odontoclasts (thick arrow) on the cementum in the 50 g group, but not in the 10 g group, on day 7. AB: alveolar bone, PDL: periodontal ligament, C: cements, D: dentin. Bar: 50 μm. (**b**) Statistical diagram depicting the quantitative assessment of the cellular changes. The number of TRAP-positive odontoclasts in the 50 g group was greater than that in the 10 g group on day 7. Significantly different from the corresponding 10 g group. ($*P < 0.01$)

the present results. Many resorption lacunae with multinucleate osteoclasts and odontoclasts appeared on the alveolar bone and root on days 5 and 7 after tooth movement. Many investigators have reported that root resorption is aggravated by increasing force magnitudes (Reitan, 1985; Chan and Darendeliler, 2005). Gameiro *et al* (2008) demonstrated osteoclastic resorption of roots on the mesial surfaces of teeth subjected to heavy orthodontic force (50 g). Therefore, the model in this study was an effective method to induce root resorption.

On day 7, CINC-1 and CXCR2 immunoreactivity was detected in both odontoclasts and PDL fibroblasts in the 50 g group. MCP-1 was also strongly detected in PDL fibroblasts. Oshiro *et al* (2001) suggested that osteoblast/stromal cells and PDL fibroblasts are involved in supporting osteoclast differentiation during tooth movement. Odontoclasts, which are responsible for the resorption of teeth, are thought to be derived from mononuclear precursors in the monocyte/macrophage lineage, and demonstrate characteristics similar to osteoclasts, which are responsible for bone resorption (Brezniak and Wasserstein, 2002a,b; Sasaki, 2003). Bendre *et al* (2003) demonstrated that osteoclasts and their progenitors expressed the IL-8-specific receptor (CXCR1) on the cell surface. Lu *et al* (2007)

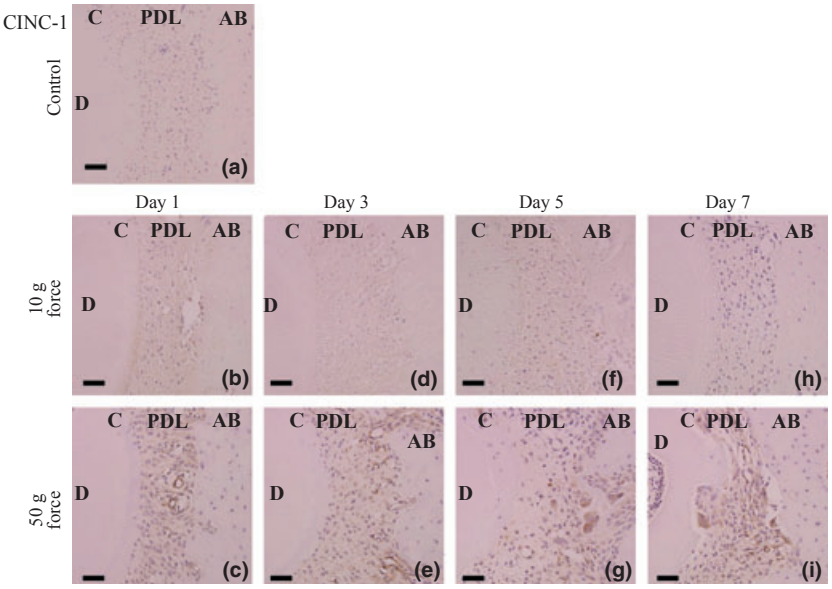

Figure 4 Effect of different orthodontic forces on CINC-1-positive odontoclasts by immunohistochemistry (original magnification 400×). The immunoreactivity of CINC-1 was observed in the odontoclasts on the cementum in the 50 g group, but not in the 10 g group, on day 7. AB: alveolar bone, PDL: periodontal ligament, C: cements, D: dentin. Bar: 50 μm

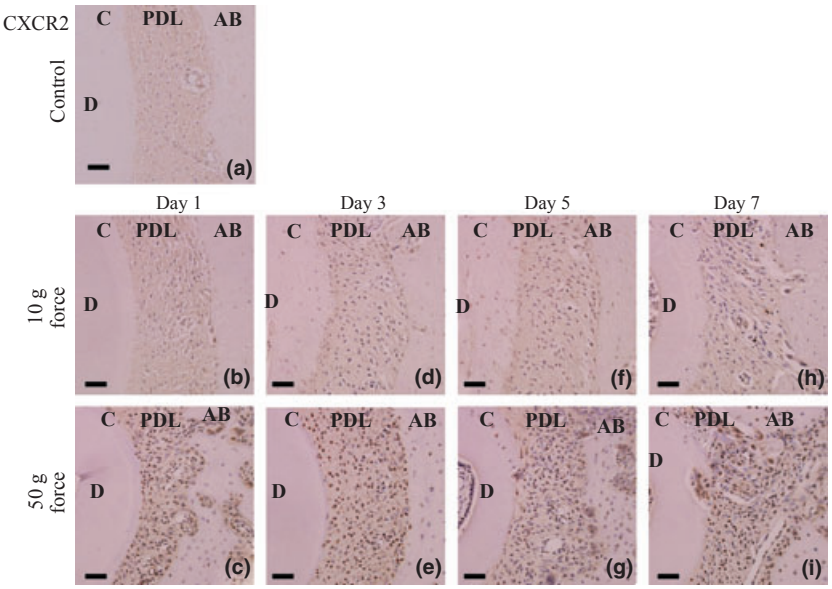

Figure 5 Effects of different orthodontic forces on CXCR2-positive odontoclasts by immunohistochemistry (original magnification 400×). The immunoreactivity of CXCR2 was observed in the odontoclasts on the cementum in the 50 g group, but not in the 10 g group, on day 7. AB: alveolar bone, PDL: periodontal ligament, C: cements, D: dentin. Bar: 50 μm

demonstrated that activation of the MCP-1/CCR2 axis promotes the tumor cell-induced osteoclast activity *in vitro*. These studies suggest that each receptor for IL-8 and MCP-1 exists on the cell surface of osteoclast progenitor cells. Therefore, CINC-1 and MCP-1 may stimulate the differentiation to odontoclasts from osteoclast progenitor cells.

Orthodontically induced inflammatory root resorption is an inflammatory process in the PDL that leads to orthodontic force (Pizzo *et al*, 2007). Gauglitz *et al* (2008) demonstrated that the serum levels of CINC-1 and MCP-1 increased after the inflammatory response during the acute phase after severe burn injury in a rat burn model. In previous studies, Alhashimi *et al* (1999) reported MCP-1 mRNA in PDL subjected to orthodontic forces. Therefore, CINC-1/CXCR2 and MCP-1 are induced in the application of heavy force, and this induction may contribute to the inflammatory response that may contribute to the ensuing OIIRR.

To investigate the mechanism of alterations in CINC-1/CXCR2 and MCP-1 in OIIRR during orthodontic tooth movement, the levels of chemokine secretion and the expression of chemokine mRNAs from human PDL cells stimulated by various amounts of compression force were measured. The results indicated that the compression force significantly increased the secretion and the expression of mRNA of IL-8 and MCP-1 in a magnitude-dependent manner. We observed that IL-8 and MCP-1 treatments were sufficient to reproduce root resorption in rat odonto/osteoclasts in dentin slices. Rats do not synthesize IL-8 but synthesize CINC/KC, which is widely assumed to be the functional rat homolog of human IL-8 (Yoshimura *et al*, 1999). CINC has many similarities to IL-8 with respect to its

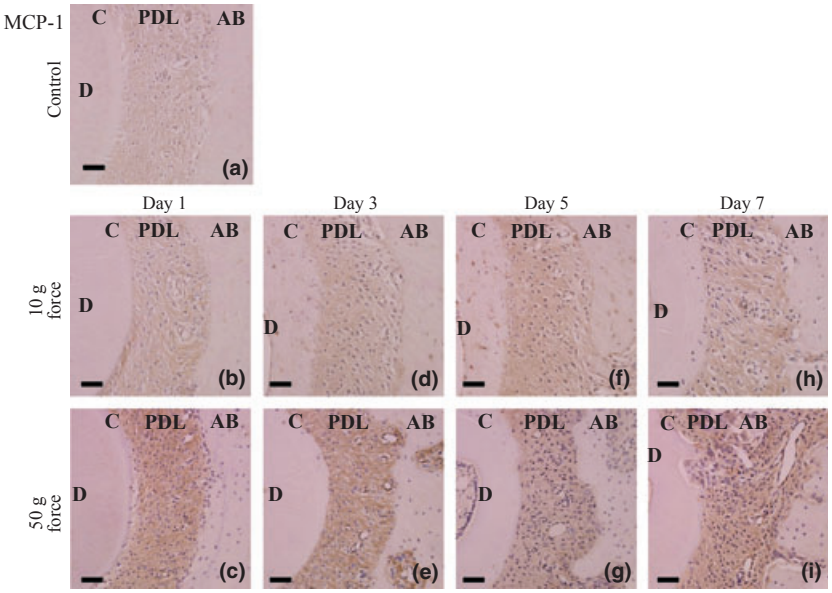

Figure 6 Effects of different orthodontic forces on MCP-1-positive PDL by immunohistochemistry (original magnification 400×). The immunoreactivity of MCP-1 was observed in the PDL in the 50 g group, but not in the 10 g group, on day 7. AB: alveolar bone, PDL: periodontal ligament, C: cements, D: dentin. Bar: 50 μm

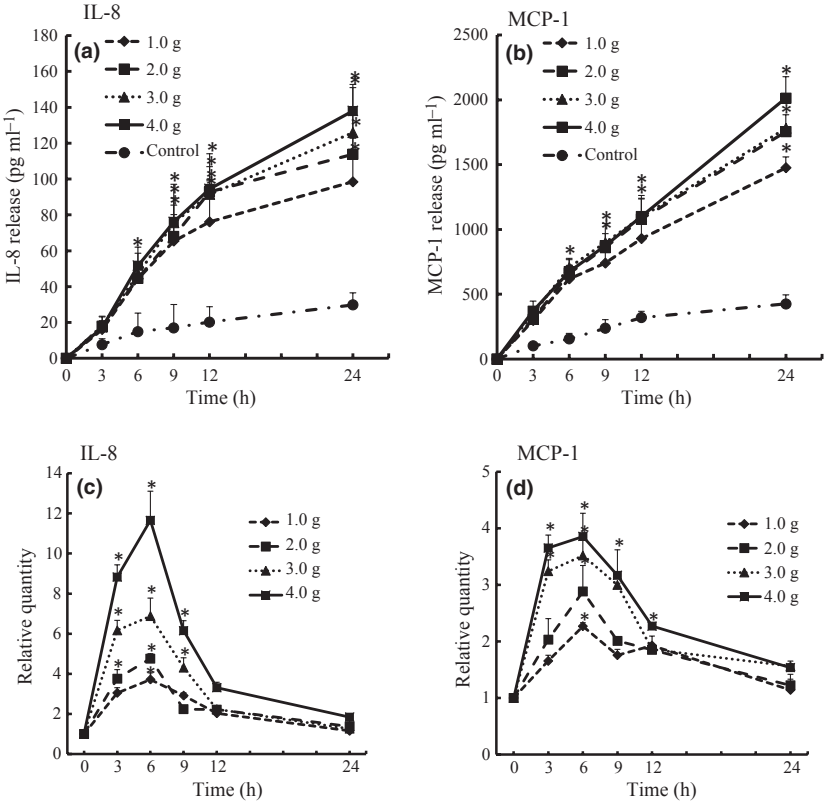

Figure 7 The effect of CF on the protein and mRNA production of IL-8 and MCP-1. hPDL cells were cultured with or without CF (1.0–4.0 g cm^{-2}) for up to 24 h. The protein release of IL-8 (**a**) and MCP-1 (**b**) was determined using ELISA after 3, 6, 9, 12, and 24 h. The mRNA expression of IL-8 (**c**) and MCP-1 (**d**) was determined using real-time PCR after 3, 6, 9, 12, and 24 h. Each bar indicates the mean ± s.d. of six experiments using ANOVA, followed by a Tukey's test, with values of $^*P < 0.01$, CF treatment *vs* control at the respective time point

induction and physicochemical properties. Osteoclast precursor cells were treated with CINC and MCP-1 for 10 days. The mature cell formation ability and the resorption ability of odonto/osteoclasts were increased in the CINC and MCP-1 treatment groups. Maeda *et al* (2007) reported the pressure force to rapidly activate the ERK, JNK, and p38 pathways in PDL cells. Lee *et al* (2008) reported that H_2O_2 induced the secretion of IL-8 via the ERK, JNK, and p38 pathways in PDL cells. Therefore, these chemokines in PDL cells in response to CF may mediate the expression induced via the MAPK (ERK, JNK and p38 MAPK) pathways. Taken together, these findings and our present results suggest that increased IL-8 (CINC-1) and MCP-1 secretion from PDL cells by orthodontic load may directly activate odonto/osteoclastogenesis.

Bendre *et al* (2003) reported that recombinant IL-8 induces RANKL expression in stromal osteoblastic cells and promotes the differentiation of human peripheral blood mononuclear cells into bone-resorbing osteoclasts

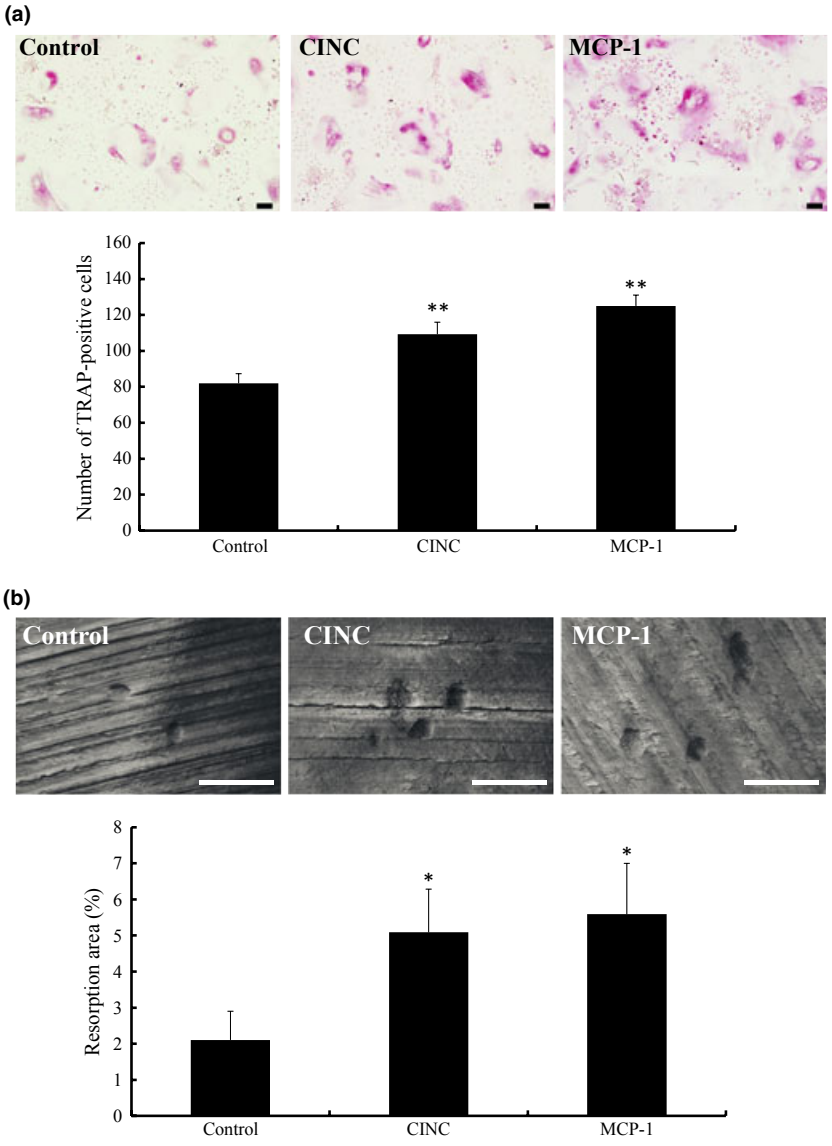

Figure 8 Osteoclast precursor cells (4×10^4 cells per 100 μl) were cultured for 10 days. (**a**) The effects of CINC and MCP-1 treatment on osteoclast formation (TRAP staining). TRAP-positive multinucleated cells containing three or more nuclei were counted. (**b**) The effects of CINC and MCP-1 treatment on resorption pits. Scanning electron micrograph images of resorption pits on the dentin slice surfaces. Osteoclast precursor cells were cultured for 10 days on the dentin slices. Data are expressed as the means ± s.d. of five wells. Significant differences from the corresponding controls are indicated. (*$P < 0.05$, **$P < 0.01$). Bar: 100 μm

in a RANKL-independent manner. Miyamoto *et al* (2009) reported that MCP-1 expressed by osteoclasts plays a role in stimulating osteoclastogenesis induced by RANKL in an autocrine/paracrine manner. Our laboratory reported that RANKL expression was increased by compression force *in vitro* and *in vivo*. Yamaguchi *et al* (2006) demonstrated that compressed PDL cells obtained from tissues with severe external apical root resorption may produce a large amount of RANKL and upregulate osteoclastogenesis *in vitro*. Nakano *et al* (2010) observed RANKL immunoreactivity in rat odontoclasts with an orthodontic force of 50 g on day 7 *in vivo*. These findings suggest that IL-8 (CINC-1) and MCP-1 may partially affect odonto/osteoclast formation via RANKL signaling. However, it remains unclear whether osteoclast precursor cells grow as a result of the influence of RANKL and M-CSF or directly because of the presence of MCP-1 and CINC. Therefore, further studies are required to investigate whether MCP-1 and IL-8 directly causes HBMC to differentiate into osteoclast-like cells, or whether the chemokines indirectly stimulate it via RANKL signaling.

According to Wang and McCauley (2010), the osteoclast and odontoclast functions are closely related to the physiological and pathologic clinical scenarios, including craniofacial abnormalities, tooth eruption and root resorption. Additionally, *in vitro* studies have shown that bone marrow osteoclasts, as well as odontoclasts, are able to resorb dentin and bone (Boyde *et al*, 1984; Jones *et al*, 1984; Sasaki *et al*, 1989). As a result, elucidating the complex mechanisms that control odonto/osteoclast development and activation is therefore expected to provide valuable insight into the early detection and management. Therefore, further studies should be carried out to investigate how the odonto/osteoclast function can be effectively modulated at the molecular level.

In conclusion, the immunoreactivity for CINC-1/CXCR2 and MCP-1 were detected in odontoclasts and PDL fibroblasts by orthodontic force of 50 g on day 7. CF increased the secretion and the expression of mRNA

of IL-8 and MCP-1 from PDL cells in a magnitude-dependent manner. Moreover, CINC-1 and MCP-1 stimulated osteoclastogenesis from rat osteoclast precursor cells. Therefore, the presence of both IL-8 (CINC-1) and MCP-1 may therefore facilitate the process of root resorption because of excessive orthodontic force.

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