# Effects of Pulpectomy on the Amount of Root Resorption during Orthodontic Tooth Movement

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## Abstract

**Introduction:** Previous studies have revealed that orthodontic force affects dental pulp via the rupture of blood vessels and vacuolization of pulp tissues. We hypothesized that pulp tissues express inflammatory cytokines and regulators of odontoclast differentiation after excess orthodontic force. The purpose of this study was to investigate the effects of tensile force in human pulp cells and to measure inflammatory root resorption during tooth movement in pulpless rat teeth. **Methods:** After cyclic tensile force application in human pulp cells, gene expression and protein concentration of macrophage colony-stimulating factor, receptor activator of nuclear factor kappa-B ligand, interleukin-1 beta, and tumor necrosis factor alpha were determined by real-time polymerase chain reaction and enzyme-linked immunoassay. Moreover, the role of the stretch-activated channel was evaluated by gadolinium ($Gd^{3+}$) treatment. The upper right first molars of 7-week Wistar rats were subjected to pulpectomy and root canal filling followed by mesial movement for 6 months. **Results:** The expression of cytokine messenger RNAs and proteins in the experimental group peaked with loading at 10-kPa tensile force after 48 hours ($P < .01$). $Gd^{3+}$ reduced the expression of these cytokine messenger RNAs and protein concentrations ($P < .01$). The amount of inflammatory root resorption was significantly larger in the control teeth than the pulpectomized teeth ($P < .05$). **Conclusions:** This study shows that tensile forces in the pulp cells enhance the expression of various cytokines via the S-A channel, which may lead to inflammatory root resorption during tooth movement. It also suggests that root canal treatment is effective for progressive severe inflammatory root resorption during tooth movement. *(J Endod 2014;40:372–378)*

## Key Words

Odontoclast, orthodontic tooth movement, pulp cells, pulpectomy, root resorption, tensile forces

During orthodontic tooth movement, apical root resorption is an undesirable side effect that is difficult to predict and repair. Massler and Malone (1) found that root resorption occurred in 86.4% of orthodontic patients, and root resorption associated with orthodontics is reported to be related to factors such as patient age, sex, and systemic conditions (2). Root resorption is also associated with inflammatory reactions by odontoclasts, which are similar to osteoclasts in morphology, activity, functions, and features (3). Previous studies have shown that macrophage colony-stimulating factor (M-CSF) plays an essential role in osteoclastogenesis (4). In addition, it was clarified that M-CSF supports osteoclast differentiation in cooperation with receptor activator of nuclear factor kappa-B ligand (RANKL) (5). Moreover, cementoblasts are able to express RANKL and can modulate osteoclast cytogenesis (6). Thus, M-CSF and RANKL are indispensable to odontoclast differentiation for root resorption, and it is important to clarify how and from where M-CSF and RANKL are released when root resorption occurs during orthodontic tooth movement.

Because orthodontic force is recognized as a type of trauma, pulp tissue can be injured during orthodontic tooth movement. McDonald and Pitt Ford (7) found that human pulpal blood decreased when continuous tipping forces were applied. Miura (8) suggested that continuous heavy orthodontic forces predispose pulp tissue to pulp necrosis via rupture of blood vessels in the apical root. Furthermore, some researchers have investigated the relationship between pulp tissue and root resorption during orthodontic tooth movement. Remington et al (9) and Spurrier et al (10) showed that root resorption is more often observed in intact teeth when compared with pulpless teeth. Indeed, we have noted severe apical root resorption in intact teeth and mild root resorption in pulpectomized teeth. Based on these findings, we hypothesized that stretched and injured pulp cells express M-CSF and RANKL as well as inflammatory cytokines; as a result, inflammatory apical root resorption occurs because of the derived odontoclasts.

A stretch-activated channel (S-A channel), which is an ionic channel activated by stretching, was detected in tissue-cultured embryonic chick skeletal muscle (11). Vascular endothelial cells have been reported to contain a cation-selective S-A channel permeable to calcium ions (12). It was also shown that intracellular $Ca^{2+}$ increased in response to mechanical stretching via the S-A channel in human umbilical endothelial cells, and this response was blocked by gadolinium ($Gd^{3+}$), an S-A channel blocker (13). Based on these findings, it was assumed that mechanical stimuli such as tensile forces in pulp cells are controlled through the S-A channel, and, consequently, osteoclast-inducing factors and inflammatory cytokines can be produced.

Therefore, in this study, we examined the effects of cyclic tensile forces on the expression of M-CSF, RANKL, interleukin-1 beta (IL-1$\beta$), and tumor necrosis factor alpha (TNF-$\alpha$) in human pulp cells as well as the inhibition of these actions by blockade

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of mechanosensory S-A channels. Furthermore, we investigated differences in root resorption during orthodontic tooth movement between pulpless and intact teeth.

## Materials and Methods

### Cell Culture

Freshly extracted and intact teeth were used. These teeth were indicated for extraction for orthodontic treatment in patients aged 18–30 years. The protocol was reviewed and approved by the Ethics Committee of Hiroshima University, and informed consent was obtained from each tooth donor. Teeth were cut vertically, and pulp tissues were removed aseptically and rinsed with phosphate buffered saline (PBS). The outgrowth method was performed for culture of human dental pulp cells in accordance with a previous study (14). Explants were cut into small fragments with a sharp blade and transferred to 10-cm Petri dishes and cultured in $\alpha$-Modified Eagle's Medium (MEM) (Sigma-Aldrich, St Louis, MO) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel), 32 U/mL penicillin G (Meiji Seika, Tokyo, Japan), 250 $\mu$g/mL amphotericin B (Nacalai Tesque, Kyoto, Japan), and 60 $\mu$g/mL kanamycin (Meiji Seika, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% $CO_2$. The medium was changed twice a week. Cells were subcultured by treatment with 0.25% trypsin/EDTA and plated at $3 \times 10^5$ cells per 100-mm culture dish. For all experiments, cells between the 4th and 6th passages were used.

### Application of Cyclic Tensile Forces

The Flexcell strain unit FX-2000 (Flexcell International Co, Hillsborough, NC) consists of a vacuum unit and a valve controlled by a computer program. Pulp cells ($1 \times 10^5$) cultured on a flexible membrane base were subjected to cyclic tensile forces produced by computer-controlled application of sinusoidal negative pressure. Flexible membranes supporting the cultured cells were deformed by negative pressure. Application of a vacuum results in a maximum cell elongation of 20% at the periphery of wells, with the strain declining toward the center. Cells were placed in a humidified incubator under an atmosphere of 5% $CO_2$ at 37°C. To examine the expression of M-CSF, RANKL, IL-1$\beta$, and TNF-$\alpha$ messenger RNAs (mRNAs) and their concentrations, a tensile force of 10 kPa was loaded at a frequency of 30 cycles per minute for 12, 24, and 48 hours. Next, we investigated the influence of various tensile forces of 1, 3, 5, 10, and 15 kPa on the amounts of M-CSF, RANKL, IL-1$\beta$, and TNF-$\alpha$ for 48 hours.

### Total RNA Extraction and Complementary DNA Synthesis

Total RNA was isolated from the cell cultures using a Quickprep Total RNA extraction kit (Amersham Biosciences, Tokyo, Japan). Single-stranded complementary DNA (cDNA) was synthesized from 1 $\mu$g total RNA using Oligo$(dT)_{20}$ primer (Toyobo, Osaka, Japan) and a Rever Tra Ace-$\alpha$ first-strand cDNA synthesis kit (Toyobo).

### Primers

The following primers were used: M-CSF (15) 5′-GGCCATGAGAGGCAGTCCGAGGG-3′ (forward), 5′-CACTGGCAGTTCCACCTGTCTGTC-3′ (reverse); RANKL (16): 5′-TCAGAAGATGGCACTCACTG-3′ (forward), 5′-AACATCTCCCACTGGCTGTA-3′ (reverse); IL-1$\beta$ (17): 5′-CTCAGGTGTCCTCGAAGAAATCAA-3′ (forward), 5′-GCTTTTTTGCTGTGAGTCCCG-3′ (reverse); and TNF-$\alpha$ (18): 5′-CCCCAGGGCTCCAGGCGGTGCTTGT-3′ (forward), 5′-GGAGACGGCGATGCGGCTGATGGTG-3′ (reverse). G3PDH primer (Rever Tra Ace-$\alpha$ first-strand cDNA synthesis kit, Toyobo) was used as a control primer; 5′-ACCACAGTCCATGCCATCAC-3′ (sense), 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense).

### Real-time Quantitative Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was performed using SYBR Green I assay and an ABI Prism 7700 sequence detection system (Biosystems, Foster City, CA) from a 1-$\mu$L sample of cDNA under the following conditions: denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and primer extension at 72°C for 22 seconds for 45 cycles. PCR for each sample was repeated 3 times for both the target gene and control (without tensile force).

Quantitative results of real-time fluorescence PCR were assessed based on the cycle threshold value, which identifies a cycle when the fluorescence of a given sample becomes significantly different from the baseline signal. Relative quantification of the M-CSF, RANKL, IL-1$\beta$, and TNF-$\alpha$ signals was normalized and expressed relative to G3PDH signals.

### Measurement of M-CSF, RANKL, IL-1$\beta$, and TNF-$\alpha$ Concentrations

Conditioned medium from cultured pulp cells and controls with and without the application of cyclic tensile were collected and cleared at 2000 rpm for 5 minutes. The amounts of M-CSF (Quantikine Human M-CSF Immunoassay Kit; R&D Systems, Inc, Minneapolis, MN), RANKL (BI-20452 Ampli sRANKL Human Immunoassay Kit; Biomedica-Gruppe, Inc), IL-1$\beta$, and TNF-$\alpha$ (Quantikine Human IL-1$\beta$ Immunoassay Kit, Quantikine Human TNF-$\alpha$ Immunoassay Kit; R&D Systems, Inc, Wien, Austria) were measured using the quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions. Standard curves were obtained as usual, and the experiment was repeated 5 times.

### Blockade of the S-A Channel

The role of the S-A channel in the effects of cyclic tensile forces was examined by gadolinium ($Gd^{3+}$) treatment. Cells were incubated with 10 or 100 $\mu$mol/L $Gd^{3+}$ chloride hexahydrate (Wako, Osaka, Japan) for 30 minutes and were prepared to assess the effects of cyclic tensile forces of 10 kPa on the amounts of M-CSF, RANKL, IL-1$\beta$, and TNF-$\alpha$ for 48 hours. To examine the cytotoxicity of $Gd^{3+}$, cells were stained with 0.5% Trypan Blue (Sigma-Aldrich) after $Gd^{3+}$ treatment. The numbers of surviving and dead cells were then counted.

### Experimental Animals and Treatment

Seven 7-week-old Wistar rats were used in this experiment. All animals were handled in accordance with the ethical regulations for animal experiments defined by the Ethics Committee of the Hiroshima University Faculty of Dentistry. Dental pulp from the upper right first molars was exposed by drilling cavities on the central portion of the occlusal surface with a round bur (1-mm diameter). A #25 endodontic file was then used to remove pulp tissue. Canals were irrigated with 2.5% sodium hypochlorite followed by 0.9% sterile saline solution using long needles. Canals were then dried and filled with a #25 gutta-percha point and sealer. The condition of root canal filling was evaluated by dental radiographic imaging. The left first molars were used as controls. An experimental appliance with a closed-coil spring was bonded onto the upper right and left first molars and incisors. Both molars were subjected to mesial movement for 6 months. Rats were killed under general anesthesia with sodium pentobarbital. Specimens were fixed in 4% paraformaldehyde, decalcified in 14% EDTA (pH = 7.4) for 28 days, and embedded in paraffin. Premaxillary bones, including the upper molars, were cut into 7-$\mu$m thick frontal sections.