Original

Effect of Compression Force on Apoptosis in Human Periodontal Ligament Cells

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Abstract: The effect of compression force on periodontal ligament (PDL) plays a critical role in orthodontic tooth movement. However, little is known about this effect on apoptosis and the underlying reactive mechanisms in PDL cells. This study focuses on the application of compression force associated with reactive oxygen species (ROS)-induced apoptosis in PDL cells. Following the application of orthodontic force to maxillary first molars, the molars were investigated using immunohistochemical analysis. The cell cycle and apoptosis were assessed in compression force-treated PDL cells using flow cytometry. Furthermore, we assayed the effect on ROS generation by DCFDA fluorescence. *In vivo* study revealed an increased number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and caspase 8-positive cells in the orthodontic force group. In our *in vitro* study model, the compression force increased the G1 phase and apoptotic cells. A large increase in the intracellular ROS levels in human (h) PDL cells was observed in the compression force group. Taken together, these results provide new information on compression force-induced G1 arrest and apoptosis in hPDL cells.

Key words: Apoptosis, ROS, Compression force, Periodontal ligament cell

Introduction

Orthodontic tooth movement is a multistep biological process characterized by sequential reactions of periodontal tissue against biomechanical forces¹⁾. The recruitment of osteoclast- and osteoblast-progenitor cells, and the balanced activation of these cells around and within the periodontal ligament (PDL) are essential for alveolar bone remodeling^{2, 3)}. The application of light orthodontic force causes direct resorption of alveolar bone, while the application of excessive orthodontic force results in excessive compression force, which induces local ischemia, tissue hyalinization, and cell death in the periodontal ligament⁴⁾.

It has been reported in experimental studies that stress produced by orthodontic forces causes a marked increase in the staining intensity of interleukin (IL)-1 α and IL-1 β in all cell types of PDL^{5, 6)}. Tumor necrosis factor (TNF)- α ^{5, 6)}, prostaglandin E2⁷⁾, cyclooxygenase⁸⁾, and interferon-gamma⁹⁾ were observed in PDL in experimental tooth movement. Like IL-1 β , TNF- α plays an important role in mediating immune and inflammatory responses. Moreover, it plays a significant role in the control of proliferation, differentiation and apoptosis¹⁰⁾. Regarding the cells

found in periodontal tissues, TNF- α has been shown to trigger apoptosis in osteoblasts and PDL cells¹¹⁾.

Apoptosis is a programmed cell death, which includes many components that are necessary for its execution. Two main pathways are known: the extrinsic and intrinsic pathway. The extrinsic pathway involves the participation of Fas-FasL, FADD and caspase 8 whereas the intrinsic pathway involves the participation of Bcl-2 proteins, cytochrome c and caspase 9. The activated pathway will depend on the initial stimulus. Reactive oxygen species (ROS) stimulus are considered inducers of both pathways¹²⁾. The extrinsic pathway is triggered by the ROS-induced JNK, which promotes Fas self-activation of the extrinsic pathway to promote cleavage of caspase 8 and subsequent events¹³⁾.

Recent studies have suggested that osteocytes in the alveolar bone adjacent to the hyalinized PDL undergo cell death via apoptosis during rat experimental tooth movement¹⁴⁾. The maximum apoptosis occurs 3 days after the application of orthodontic force¹⁵⁾ and if compounds affect the cyclin and/or CDKs, then it may lead to cell cycle arrest. If a cell continues to cycle with its damaged DNA intact, then the apoptotic mechanism is triggered and the cell will undergo apoptosis¹⁶⁾.

However, little is known about its effect on apoptosis and the underlying mechanisms reactive in PDL cells. This study focuses on the application of compression force associated with ROS-

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induced apoptosis in PDL cells. The maxillary first molar after the application of the orthodontic force were investigated by immunohistochemical analysis. Compression force treated the cell cycle analysis and apoptosis by flow cytometry. Furthermore, we assayed the effect on ROS generation by DCFDA fluorescence.

Materials and Methods

In vivo study

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. AP11MD010). A total of fifteen male 6-week-old Wistar rats of body weight 180 ± 10 g (Sankyo Labo Service, Inc., Tokyo, Japan;) were used for the experiments. The animals were maintained in the animal center of Nihon University School of Dentistry at Matsudo in separate cages in 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 the monitoring of daily body weight for 1 week before the start of the experiments.

Application of orthodontic devices and tissue harvesting

The animals were anaesthetized with pentobarbital sodium (40 mg/kg body weight) for the application of orthodontic devices. Experimental tooth movement was induced using the method of Asano et al.¹⁷⁾, with a closed-coil spring (wire size: 0.005 inch, diameter: 1/12 inch, Accurate, Inc., Tokyo, Japan) ligated to the maxillary first molar 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 number 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 g. The duration of the experiment was day 5.

Tissue preparation

The experimental periods were set at 1, 3 and 5 days after tooth movement was initiated. The animals were deeply anesthetized by pentobarbital sodium and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and hereafter 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 decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin by the usual methods for preparation. Each sample was sliced into 4- μ m sections continuously in the horizontal direction, and were prepared for hematoxylin and eosin staining (H-E), for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

(TUNEL) assay and also for immunohistochemical staining. The periodontal tissues in the mesial part of the distal buccal root of a first upper molar were observed.

TUNEL assay

Apoptosis was detected by the TUNEL assay using the TACS 2TdT-DAB In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA). The paraffin sections (4 μ l) were incubated with proteinase K (Trevigen), diluted 1: 200 at 37 °C for 15 min, washed with deionized water, incubated with 3 % H_2O_2 for 5 min, and then washed with deionized water again. For the positive control, sections were incubated with DNase I at 37°C for 30 min. All sections were incubated with terminal deoxynucleotide transferase (TdT) and Biotin-dUTP at 37°C for 1 h (the negative control sections were not incubated with the TdT enzyme). After washing, the sections were incubated in Strep-HRP solution at 37°C for 10 min. After washing, the diaminobenzidine (DAB) was added to demarcate the apoptotic cells. The sections were counterstained with 1% methyl green, and were finally dehydrated and mounted.

Immunohistochemistry

The immunohistochemical staining was performed as described in this section. 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-buffered saline (TBS), the sections were incubated with polyclonal anti-rabbit caspase 8 (Novus, working dilution, 1:1000), antibodies for 18 h at 4°C. Caspase 8 was stained using the Histofine Simple Stain MAX-PO (multi) (Nichirei Biosciences, Tokyo, Japan) according to the manufacturer's protocol. The sections were rinsed with TBS and the final color reactions were performed using DAB. Thereafter, the sections were counter-stained with hematoxylin.

In vitro study

Human periodontal ligament (hPDL) cell culture

The hPDL cells were prepared according to a modification of the method of Somerman et al.¹⁸⁾ Periodontal ligament tissues were taken from the roots of premolars that were extracted from donors between 15 to 18 years of age for orthodontic reasons with informed consent, and these specimens were used according to a protocol reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019). The periodontal ligament tissues were placed in 35-mm tissue culture dishes and were covered with a sterilized glass coverslip. The cells were maintained in α -MEM medium (Gibco, Grand Island, NY, USA), which was supplemented with 100, μ g/ml of penicillin-G (Sigma Chemical Co., St.Louis, MO, USA), 50 μ g/ml of gentamicin sulphate (Sigma Chemical Co.), 0.3 μ g/ ml of amphotericin B

(Flow Laboratories, McLean, VA, USA), and 10% fetal calf serum (Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator in the presence of 95% air and 5% CO₂. When the growing cells from each explant reached confluence, they were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline for 2 min and were subcultured in culture flasks. Any cells which adhered to the bottom of the flask were discarded to avoid contamination by epithelial cells.

Application of compression force

In order to reproduce the conditions of pressure during orthodontic tooth movement, we performed the following *in vitro* experiments as described by Asano et al.¹⁷⁾. hPDL cells were continuously compressed using a uniform compression method which serves as a model of the pressure at the site of orthodontic movement. The static compression force is thought to mimic that found *in vivo* during orthodontic treatments. In the present experiments, the cells were stimulated once. A cell disk 35-mm in diameter was placed over subconfluent cell layers in the dishes, on top of which a slide glass was placed. The compression force was then controlled by placing lead granules in the slide glass. Before the application of the compression force, the cells were pre-incubated for 18 h in culture medium containing 1% fetal calf serum, and thereafter were subjected to 1.0 g/cm² of CF for 24 h.

ROS detection

In order to detect the ROS in the cells, an ROS detection kit was used Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Inc, Eugene, OR, USA). The kit was used as described in the manufacturer's protocol. Intracellular, unspecific ROS were marked with carboxy-H₂DCFDA solution and the cell nuclei with Hoechst 33342 solution. After washing with HBSS (Gibco), the cells were fixed. Tert-butyl hydroperoxide (TBHP, at a concentration of 100 µM) which served as a positive control was incubated for the same duration as the samples.

Wound healing assay

hPDL cells were cultured on 35-mm dishes until they reached confluence. A linear wound track was made by a P-1000 pipette tip to scratch on the confluent cell monolayer. The wounds were marked and scored in each dish and the plates were then washed twice with culture medium. The cells migrating into the wound were photographed 24 h after the incubation following wounding.

Flow cytometry for the cellular propidium iodide (PI) assay and cell cycle analysis

After the compression force was applied, the cells were washed with PBS, and detached with 0.05% trypsin. After washing and

fixing with 70% ice-cold alcohol overnight at 20°C, the cells were collected and resuspended in PBS containing 40 µg/ml PI, 100 µg/ml RNase. They were then incubated at 37°C for 30 min and were subsequently analyzed by flow cytometry.

Analysis of apoptosis by flow cytometry

A FITC-Annexin V Apoptosis Detection Kit I (BD Biosciences Pharmingen, San Jose, CA, USA) was used according to the supplier's protocol. After the compression force was applied, the cells were gently trypsinized and collected by centrifugation. Approximately 5×10⁶ cells were washed twice with cold PBS and resuspended in 500 µl of 1×binding buffer. Five microlitres of annexin V and 5 µl of PI were then added to the cell suspensions and the samples were incubated in the dark at room temperature for 15 min. The fluorescence observed following annexin V-FITC (which binds to apoptotic cells) binding and PI (which stains necrotic cells) staining was measured quantitatively using a flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Detection of apoptosis by Hoechst 33258 staining

The nuclear fragmentation was visualized by staining the apoptotic nuclei with Hoechst 33258. The cells were fixed with 4% paraformaldehyde for 30 min at RT and then washed once with 1×PBS/0.1% triton-X for 5 min at RT. Hoechst 33258 (2.5 µg/ml) was added to the fixed cells, followed by incubation for 30 min at RT; the cells were then washed with 1×PBS/0.1% triton-X. The cells were mounted and examined by fluorescence microscopy. The apoptotic cells were identified by the condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to the total cells counted.

Statistical methods

The values in each figure represent the mean ± standard deviation (S.D.) for each group. Values are shown as the means ±S.D. A Mann-Whitney's U-test was used to compare the means of the groups. The values of *p* < 0.05 considered to indicate a significant difference.

Results

In vivo study

Histological changes in the periodontal tissues during tooth movement

In the control group, PDL specimens were composed of relatively dense connective tissue fibres and fibroblasts that regularly ran in a horizontal direction from the root cement towards the alveolar bone. In the compression force group, the arrangement of the fibres and fibroblasts became coarse and irregular, and blood capillaries were compressed on day 3 (Fig. 1).

Figure 1. Light microscopic images of the effects of TUNEL staining and the immunohistochemical staining for caspase 8. The control specimen shows that there are few TUNEL and caspase 8-positive cells in PDL. The increased levels of TUNEL and caspase 8 were shown after the application of the orthodontic force. C: cementum, PDL: periodontal ligament, AB: alveolar bone. Bar =50 μ m

Figure 2. The compression force increases the intracellular ROS production in hPDL cells. The compression force was applied to the hPDL cells for 6 and 24 h in the presence of DCFDA and measured by fluorescence microscopy. Bar =100 μ m

Induction of apoptosis of PDL cells with orthodontic force in rat

To confirm the apoptotic effect of periodontal ligament cells after the application of the orthodontic force, TUNEL stain and caspase 8 immunohistochemistry were used. The PDL cells after the application of the orthodontic force increased TUNEL and caspase 8 -positive cells at day 1, reached to a maximum peak at

day 5. Only a few TUNEL and caspase 8-positive cells were present in the control (Fig. 1). These results showed that cells subjected to a continuous compression force had strong staining in comparison to that of the control cells.

Compression force induced ROS production

Production of ROS was monitored using DCFH-DA. As shown

Figure. 3 Effect of the compression force on the repopulation hPDL cells. A monolayer of hPDL cells was scratched with a P-1000 pipette tip, producing a single straight-wound line and the application of the compression force on the cultures for various time points. Following the application, the cultures were maintained in the appropriate conditions for various periods of time prior to making the observations and taking the photographs Original magnification 40 $\times$ (a). The wound healing, rated in percentage, was determined as the mean $\pm$ standard deviation (b).

Figure. 4 Compression force induced G1 phase arrest in hPDL cells. The histogram plotting and quantification of each cell cycle phase, showing a decrease in the population of cells in the S and an increase in the population of cells in the G1 phases for 6 and 24 h (a). Flow cytometric analysis by annexin-V-FITC/PI dual staining. The cells were labeled with FITC conjugate and were quantified following analysis in a FACS. The upper right quadrant indicates the late stages of apoptosis (VFITC+/PI+) was increased from 49.97% to 69.07% at 24 h (b).

in Figure 2, the intracellular ROS levels were increased after compression force treatment for 6 h, and the levels of ROS production was further increased at 24 h. Results showed that a time-dependent increase in ROS production was observed in the PDL cells after the application of compression force.

Wound healing assay

PDL cells were used to study cell motility and migration using an in vitro wound healing assay. Figure 3 showed the treatment area of wound after the application of the compression force.

Compression force group showed the delayed closure than the control group. At 24 h the compression force group had significantly less motility and migration movement than the control groups.

Analysis of compression force-induced cell cycle arrest and apoptosis

To assess the application of compression force for various periods of time on cell cycle progression, we have performed flow cytometry analyses in PDL cells. The results show that compression

Figure. 5 The nucleus condensation was observed with Hoechst 33258 after the compression force was applied to the cells for 6 and 24 h. The arrows indicate apoptotic cells. Bar =50µm.

Figure. 6 Schematic model for compression force induced apoptosis in hPDL cells. Application of the compression force-induced G1 arrest, caspase 8 protein and ROS in hPDL cells and they induced apoptosis.

sion force contributed to gradual increase and decrease in percentage of cells in G1 phase and S phase, respectively compares to the control (Fig. 4a). By staining the cells with annexin V-FITC and PI, FACS was used to determine the compression force-induced apoptotic cells. The upper right quadrant indicates the late stages of apoptosis (VFITC+/PI+) was increased from 49.97% to 69.07% at 24 h (Fig. 4b). The experiment indicated a time-dependent induction of apoptosis of hPDL cells in response to the compression force for 24 h. Hoechst 33258 staining was used to determine the effects of apoptosis induced by application of compression force. As shown in Figure 5, Hoechst 33258 staining showed the presence of nuclear condensation at 6 h after the application of the compression force, and a gradual increase in

the number of condensed or fragmented nuclei (apoptosis) was observed for 24 h.

Discussion

This study demonstrated the induction of apoptosis and the subsequent decrease in cellular viability of the PDL cells following the application of compression force.

In this study, it was observed that TUNEL and caspase 8-positive cells are highest at day 5 *in vivo* (Fig. 1). During tooth movement *in vivo*, recent studies have reported that the number of TUNEL-positive periodontal ligament cells increases at 12 h and reaches a maximum at 24 h¹⁹. In addition, *in vivo*, the ratio of TUNEL-positive cells on the pressure side of the periodontal ligament increased up to day 28²⁰. The effect of the compression force on PDL cells may occur through an apoptotic mechanism. Hatai et al.¹⁹ reported that they applied mechanical stress during orthodontic tooth movement by using the 50 g nickel titanium and Mabuchi et al.²⁰ reported using the Waldo method. It is believed that it varies in a tooth migration method and a movement power that a day of the expression is different from them.

Apoptosis consists of two main pathways: intrinsic (mitochondrial) and extrinsic (receptors-related). Caspases are common to both pathways. Caspases are cysteine proteases that cleave a set of proteins in order to initiate and promote apoptotic signaling. Caspase 8 participates in the extrinsic pathway and is activated by Fas-FasL signal transduction. Caspase 9 is the mediator of the intrinsic pathway, and an alteration in oxidative phosphorylation, hypoxia and oxidative stress can induce the activation of caspase 9¹². We previously studied the mRNA expression of TNF-α and found that it increased significantly

after the application of compression force²¹⁾. We therefore hypothesize that the mechanism of apoptosis through caspase 8 activation is mediated by TNF- α .

We previously reported the increased expressions of cytokines and chemokines during experimental tooth movement because of the application of heavy force (50 g) *in vivo* and the effects of compression forces (0-4.0 g/cm²) *in vitro*^{17,21)}. The highest amount of cell damage was 16.9% in the compression force (4.0 g) group²¹⁾. It is difficult to determine the optimal force during therapeutic orthodontic tooth movement, because periodontal tissue reactions vary among individuals. Based on the aforementioned facts, we hypothesized that an optimal compression force (1.0 g/cm²) applied to periodontal ligament cells can induce *in vitro* apoptosis via ROS generation and cell cycle arrest and that may accelerate their downstream signals. As expected, the apoptotic periodontal ligament cells were increased by the application of 1.0 g/cm² compressive force. We demonstrated that the compression force increases ROS production in hPDL cells (Fig. 2). Compression force-induced G1 arrest and apoptosis in human periodontal ligament cells was at least partly mediated by ROS generation.

When we assayed ROS generation in the human PDL cells, we found that compression force induced a large increase in the fluorescence of the H₂O₂ indicator DCF, indicating that compression force stimulated the intracellular generation of peroxides in PDL cells. Previous studies have shown that the mechanical compression of the spinal nerve increases the recruitment of inflammatory cells to these tissues, resulting in ROS production²²⁾. The elevation of ROS may deplete cellular glutathione, damage DNA, activate check-point kinases and regulate the cell cycle and apoptosis-related genes^{23, 24)}. In promoting PDL cells apoptosis, the compression force may hamper wound healing. In this study, the proliferation decreased in hPDL cells 24 h after the application of the compression force (Fig. 3). Cell cycle progression is achieved through a tightly controlled cascade of cellular events. In the present study, we found that the compression force suppressed the PDL cell proliferation by arresting the cell cycle in the G1 phase (Fig. 4a). The highest accumulation of cells at the G1 phase was observed after 24 h of incubation with compression force, suggesting that ROS generation preceded cell-cycle arrest by compression force. The G1 arrest is always used as the marker for cellular differentiation^{25, 26)}, and an apparent G1 arrest has been observed during the differentiation in previous research^{27, 28)}. Recent research has shown that cyclic tensile stress on PDL cells arrested cells in the G1 phase, and there were gene changes related to apoptosis and the cell cycle. Cell cycle-associated genes developed by the stretch force were directly related to the change of the cell cycle²⁹⁾. It controls G2/M checkpoint and contributes to the switch-like, all-or-nothing behavior of the cell in deciding to commit to mitosis³⁰⁾. Like all cyclins, levels of cyclin B1 oscillate over the course of

the cell cycle. Just prior to mitosis, a large quantity of cyclin B1 is present in the cell, but it is inactive due to the phosphorylation of Cdk1³¹⁾. This suggests that the cell cycle arrest under the compression force may result in the expression of cell cycle-associated genes.

To verify that these events occur, a flow cytometric analysis was performed. The flow cytometric analysis of apoptosis is indicative of the extent of apoptosis induced by the compression force. The fraction of apoptotic hPDL cells was significantly upregulated, in comparison to the control, following exposure to 6 or 24 h. Figure. 4b showed that the rate of apoptosis reached a peak when the cells were compressed for 24 h. These results are supported by those previously reported in other models namely the stretching force model in hPDL cells³²⁾ and the compression force model in osteoblast³³⁾. Furthermore, after staining with Hoechst 33258, the PDL cells which were subjected to a compression force exhibited the typical morphological features of apoptosis (Fig. 5). As the time and dose increased, the number of apoptotic cells increased, as indicated by the presence of fragmented nuclei and apoptotic bodies. The compression force therefore exerted a significant effect on the hPDL cells by increasing the number of apoptotic cells (Fig. 6). These suggest that both caspase 8 and caspase 9 signaling pathways may have contributed to the compression force-induced apoptosis in hPDL cells.

In conclusion, we herein elucidated the mechanisms by which compression force affects hPDL cells. Our findings suggest that the application of compression force to hPDL cells produces ROS which initiates the activation of the apoptosis pathway, thus leading to cell cycle arrest in the G1 phase.

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