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Original Article

**Morphohistological Change and Expression of HSP70, Osteopontin and Osteocalcin mRNAs in Rat Dental Pulp Cells with Orthodontic Tooth Movement**

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**Abstract**

Morphological change and expression of osteopontin, osteocalcin, and HSP70 mRNAs in rat dental pulp cells with experimental orthodontic tooth movement were investigated. Elastic rubber blocks, 0.65 mm in thickness, were inserted between the maxillary first and second molars in rats. In addition to morphological observations of HE staining and TUNEL staining at days 3, 7, 14 and 28 after insertion of elastic rubber blocks, expression of HSP70, osteopontin and osteocalcin mRNAs was also analyzed using quantitative RT-PCR with a LightCycler™. Morphologically, proliferation and vasodilation of capillaries was evident in the pulp at days 3 and 7, and a sparse odontoblast layer and apoptosis in the pulp were observed at days 7 and 14 after rubber block insertion. Expression of HSP70, osteopontin and osteocalcin mRNAs in the experimental groups was higher than that in the control group at all time points. This suggests that orthodontic tooth movement causes degenerative changes and apoptosis in pulp cells, while pulp homeostasis is maintained at the genetic level.

Key words: Tooth movement—HSP70—Osteopontin—Osteocalcin—mRNA

**Introduction**

Orthodontic tooth movement involves dynamic changes in the periodontal ligament and alveolar bone. Tissue change during orthodontic loading is not only evoked in periodontal ligament, but also in pulp tissue. Hamersky demonstrated a significant (27.4%) depression in pulpal respiratory rate when a tooth undergoes orthodontic movement. Kucukkeles reported that vascular degeneration was the main change in pulp during orthodontic tooth movement. Although vascular blood flow affects pulp tissue during degenerative change, homeostasis of the pulp also occurs during...
orthodontic tooth movement. Orthodontic tooth movement also affects dental pulp in a way similar to surgical or chemical stimulation. It has been demonstrated that dental pulp tissue has the ability to form dentin or osteodentin during wound healing induced either by surgical or chemical stimulation\(^2\,25\,27\). The formation of dentin or osteodentin in the dental pulp is known to be one of the reactions of homeostasis. Bone-related protein is related to the calcification of pulp\(^21\), and hard tissue formation is regulated by the activity of pulp cells\(^1\,28\). Heat shock protein 70 (HSP70), which serves as a molecular chaperone to maintain homeostasis, is expressed physiologically in response to stimulation\(^24\). Chen et al. reported that HSP70 expression in pulp increased during reparative dentinogenesis\(^4\). The induction of this stress protein has also been investigated using rat nerve cells or gerbil brain under hypoxic conditions\(^19\,31\,32\). Furthermore, apoptosis plays an important role in homeostasis\(^6\,12\). However, little is known about the homeostatic mechanism of dental pulp cells during orthodontic tooth movement.

Therefore, the aim of this study was to evaluate morphological changes such as programmed cell death by using TdT-mediated dUTP-biotin nick end labeling (TUNEL) method and expression of osteopontin, osteocalcin and HSP70 mRNAs in rat dental pulp cells during experimental orthodontic tooth movement.

Materials and Methods

1. Animals

Thirty-four male Sprague-Dawley rats weighing 200–250 g each (7–8 weeks old), were used in this study. The animals given water and food kneaded with water, and were housed in a room with a 12-hr light/dark cycle; they were acclimatized for 1 week before commencement of the experiments. The study protocol was designed in accordance with the “Principles of Laboratory Animal Care” (NIH publication No. 86-23, revised 1985) and relevant national laws.

2. Experimental design

After administration of general anesthesia using sodium thiopental, elastic rubber blocks, 0.65 × 0.65 × 1.5 mm, were inserted between the maxillary first and second molars bilaterally in each rat, according to the method of Waldo and Rothblatt (Fig. 1)\(^34\). As a control group, we also used rats with no rubber block insertion.

For histological observations, 10 of the rats were divided into 4 experimental groups and a control group consisting of 2 animals each. On days 3, 7, 14 and 28 after insertion of the rubber block (and in the control group), the animals were anesthetized with sodium thiopental and perfused intracardially with 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.3). Each animal was checked to ensure that the rubber blocks remained in place during the examination period. Maxillae were removed and stored in the same fixative at 4°C for 2 days. Each specimen was demineralized in 10% EDTA for 2 weeks, and then rinsed in 0.2 M phosphate buffered saline (PBS), dehydrated with graded ethanols, and embedded in paraffin. Sections in the mesio-distal sagittal plane at a thickness of approximately 5 μm were collected on glass slides, stained with hematoxylin and eosin, and then observed by microscopy. For TUNEL, the sections were incubated with 16.2 μg/ml proteinase K solution (in 10 mM Tris-HCl buffer, pH 7.4) for 20 min following depar-
affinization with xylene. After washing for 5 min, the sections were incubated in TdT solution (TdT buffer, TdT, Biotin-16-dUTP) for 90 min at 37°C, and the reaction was terminated by incubating with termination buffer for 30 min. After the sections were washed with water and PBS, they were stained with 3,3′-diaminobenzidine for 5 min, washed with distilled water, counterstained in hematoxylin, and coverslipped. Finally, the pulp of the distal root was observed histologically.

We then evaluated osteocalcin, osteopontin and HSP70 mRNAs in 3 further experimental groups and a control group consisting of 6 animals each. On days 3, 7 and 14 after insertion of the rubber block, the animals were anesthetized with an overdose of sodium thiopental and checked to ensure that the rubber blocks remained in place during the examination period. The first molars were extracted, and the periodontal ligament was removed mechanically (using a knife observed through a loupe). The periodontal ligament was then immersed in a solution of sodium hypochlorite. Following washing with PBS, the teeth were broken using a dental chisel and a mallet. Each pulp was then carefully collected into an Eppendorf tube with a dental explorer and a dental scaler using a microscope. Six animals without rubber block insertions were used as controls. For quantitative analysis of osteopontin, osteocalcin and HSP70 mRNA expression, total RNA was extracted using Isogen reagent (Nippon Gene, Japan) according to the manufacturer’s instructions. Briefly, cells were homogenized and solubilized in the Isogen/chloroform solution at 4°C. Supernatants were obtained following centrifugation at 12,000×g for 20 min at 4°C. The precipitates were obtained by decantation and washed with 75% ethanol. The RNA pellets were then dissolved in RNase-free water, and preserved at −20°C until used. Using the extracted RNA as a template, reverse transcription reactions were conducted with an RT-PCR kit (RNA-PCR kit ver. 2.1, Takara Biomedicals, Japan) to synthesize cDNA. Quantitative PCR was then conducted using primers for osteopontin, osteocalcin, and HSP70 with a LightCycler® using double-stranded DNA dye SYBR Green I (Roche Diagnostics, Germany). The PCR conditions and primer sequences used in the LightCycler™ are shown in Table 1. Quantification was performed by comparing the levels obtained with standard samples. In the present study, the concentrated solutions of cDNA in the unstimulated samples were 0.2, 0.5, 1.0 and 2.0 µl. PCR with the LightCycler™ was carried out over 40 cycles. After PCR amplification, melting curve analyses were also performed to confirm the absence of the primer dimer in the PCR products. The ratios of osteopontin mRNA expression were adjusted according to the value of the housekeeping gene, GAPDH. Four independent experiments were performed for all assays of cell behavior; two specimens of each sample

<table>
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<tr>
<th>Target cDNA</th>
<th>Primer sequence (5’ to 3’)</th>
<th>PCR condition</th>
<th>Product size</th>
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<tr>
<td>Osteopontin</td>
<td>Forward; CTC GGA GGA GAA GGC GCA TTA</td>
<td>40 cycles (95°C 10sec, 60°C 5sec, 72°C 12sec)</td>
<td>207bp</td>
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<tr>
<td>Reverse; CCA TCG TCA TCG TCG TCA</td>
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<tr>
<td>Osteocalcin</td>
<td>Forward; GGT GCA AAG CCC AGC GAC TCT</td>
<td>40 cycles (95°C 10sec, 60°C 5sec, 72°C 12sec)</td>
<td>199bp</td>
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<tr>
<td>Reverse; GGA AGC CAA TGT GGT CCG CTA</td>
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<tr>
<td>HSP70</td>
<td>Forward; GTG TGC AAC CCG ATC ATC AGA</td>
<td>40 cycles (95°C 10sec, 60°C 10sec, 72°C 7sec)</td>
<td>180bp</td>
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<tr>
<td>Reverse; CAC CAG CAG CCA TCA AGA GT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward; TGC CCA GAA CAT CAT CCC TG</td>
<td>40 cycles (95°C 10sec, 60°C 10sec, 72°C 12sec)</td>
<td>307bp</td>
</tr>
<tr>
<td>Reverse; TCA GAT CCA CGA CGG ACA CA</td>
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type were used in each experiment.

3. Statistical analysis
The results obtained at each experimental time point were compared with those of the control group using the Mann-Whitney U-test.

Results

1. Histological observations (Fig. 2a–f)
In the control group, a layer of densely packed odontoblasts was observed close to the dentin, under which there was a cell-rich layer. A few undilated vessels were observed in the center of the pulp (Fig. 2a). At days 3 and 7 after rubber block insertion, multiplication and vasodilation of capillary vessels were distinguished (Fig. 2b, c). Furthermore, a sparse odontoblast layer was recognized at days 7 and 14 after insertion, and cells in both the odontoblast layer and underneath it were degenerated and vacuolated (Fig. 2c, d). On the other hand, at day 28 after insertion, morphologically, no vasodilation was observed, and the odontoblasts lay in a densely-packed arrangement (data not shown).

No cells positive for TUNEL in the pulp were found in the controls (data not shown). At days 7 and 14 after insertion, TUNEL-positive cells, showing separation, condensation and fragmentation of chromatin, were found in the pulp underlying the odontoblast layer and central root area (Fig. 2e, f, g, h).

2. Expression of osteopontin, osteocalcin and HSP70 mRNA
Expression of osteopontin mRNA in the experimental groups was approximately 1.3 times higher than that in the control group at days 3, 7 and 14 after insertion of the rubber block (Fig. 3). Osteopontin mRNA expression at day 3 after insertion was significantly higher than that in the controls (p<0.01). Osteocalcin mRNA expression at day 3 after insertion was approximately 2 times higher than that in the controls (p<0.01). At day 14 after insertion, expression of osteocalcin mRNA showed a decrease to a level similar to that of the controls (Fig. 4). Furthermore, HSP70 mRNA expression at day 3 after insertion was 2.8 times higher than that of the controls, and at days 7 and 14 was approximately 2 times higher (p<0.01) (Fig. 5).

Discussion
A number of phenomena such as vacuolar degeneration of odontoblasts, bulging of microvessels, and production of denticles have all been reported as effects of orthodontic tooth movement on pulp. In this experiment, we found vacuolar degeneration, a sparse odontoblast layer, and dilatation of capillary vessels, as reported in earlier studies. During tooth movement, pulpal changes may occur primarily due to alterations in blood vessels in the periodontium and those entering the pulp. There have been several reports on circulatory disturbance in dental pulp during orthodontic tooth movement, so it is well known that orthodontic tooth movement affects dental pulp cells when blood circulation is disturbed. Apoptosis is an essential mechanism during development and normal tissue injury. Since the concept of apoptosis was first postulated, many studies on apoptosis in the oral region have investigated periodontal tissues during tooth eruption. The TUNEL method, which is based on the specific binding of TdT to 3'-OH of DNA, has been reported as an in situ labeling method. Rana et al. demonstrated that TUNEL-positive cells were found in pulp. In this study, TUNEL-positive cells were also observed outside the coronal area in the pulp. This study demonstrated that orthodontic tooth movement evoked not only degenerative change, but also apoptosis in pulp cells. Amemiya studied pulp cell responses during hypoxia in vitro, and showed that alkaline phosphatase activity in pulp cells under hypoxic conditions was significantly higher than in the controls. Further, Wong et al. reported that orthodontic tooth movement caused vasodilation in the pulp. Moreover, it is known that local blood circulatory disturbances affect calcification in...
Fig. 2  Histological observations of dental pulp
bar: a–e, g: 500 μm, f, h: 100 μm, a: H&E staining of control group, b: H&E staining at day 3 after rubber block insertion, c: H&E staining at day 7 after rubber block insertion, d: H&E staining at day 14 after rubber block insertion, e: TUNEL staining at day 7 after rubber block insertion (arrow: TUNEL-positive cell), f: high magnification of box area of figure e, g: TUNEL staining at day 14 after rubber block insertion (arrow: TUNEL-positive cell), h: high magnification of box area of figure g.
14, osteocalcin mRNA expression showed a decrease, while osteopontin mRNA expression remained high. Osteocalcin is involved in matrix mineralization, while osteopontin is involved in differentiation of odontoblasts. Morphologically, in this study, we found vacuolar degeneration of odontoblasts, which suggests that pulp cells differentiate into odontoblasts to compensate for this regressive change. HSP70 is a molecular chaperone needed to maintain homeostasis\(^2\). Here, HSP70 mRNA expression during orthodontic tooth movement was higher than that in the controls. This result differs from that of an in vitro experiment where HSP70 mRNA expression showed no change with hypoxia\(^3\). Amemiya demonstrated that HSP70 expression increased after reoxygenation\(^4\). In their in vitro experiment, only fibroblast-like pulp cells were selected, but the reactions of the dental pulp in our in vivo experiment involved a complex of interacting factors. Chen et al.\(^5\) suggested that HSP70 plays an important role as a molecular chaperone during reparative dentin formation\(^5\). The increase of HSP70 mRNA expression found in this study supports Chen’s hypothesis\(^5\).

In conclusion, these results suggest that expression of HSP70, osteopontin, and osteocalcin mRNA functions as a homeostatic mechanism to compensate for pulpal cell changes occurring during orthodontic force as a result of circulatory disturbance.
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