Expression of Heat Shock Protein 70 in the Periodontal Ligament During Orthodontic Tooth Movement

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Abstract
Heat shock proteins (HSPs) play an important role in maintaining protein homeostasis within the cell, and they are also related to inflammation. However, it is not clear how HSPs regulate inflammatory cytokines in periodontal ligament (PDL) cells during mechanical stress. The present study focuses on the expression of HSP70 in the PDL during experimental tooth movement in rats. A total of 35 6-week-old male Wistar rats were subjected to orthodontic force of 10 g to induce mesial tipping movement of the upper first molars. Experimental tooth movement was accomplished for 14 days. We determined the expression of HSP70 proteins in the PDL during orthodontic tooth movement by immunohistochemistry. HSP70 immunoreactivity was detected in PDL fibroblasts on the compressive side by an orthodontic force of 10 g. On day 1 after tooth movement, the immunoreactivity of HSP70 was weak. On days 2, 3 and 4, we identified a greater positive reaction for HSP70 in the nucleus of fibroblasts than that recognized on the first day. The HSP70–positive reaction further increased on days 7 and 14. From our quantitative evaluation, the rate of HSP70–positive PDL fibroblasts significantly increased compared with control fibroblasts, and this occurred in a time-dependent manner. Therefore, HSP70 may function as a homeostatic factor to compensate for PDL–cell changes that occur during orthodontic force.

Keywords:
orthodontic tooth movement, periodontal ligament, HSP70

Introduction
Heat shock proteins (HSPs), also called stress proteins, are induced in various cells by specific types of stress. HSPs are molecular chaperones that maintain the dynamic stability of protein folding, protein–protein interactions and homeostasis within the cell, and inhibit protein aggregation (1). Cell exposure to stress causes an imbalance in the metabolism of proteins, which challenges the cell to respond rapidly, yet precisely, to minimize the deleterious effects of environmental and physiological stresses (2). In general, the stress response represents a cellular defense mechanism that leads to the decreased production of some polypeptides, while up-regulating the HSPs (3). Recent results show that HSPs play crucial roles in a wide variety of normal and pathological cellular processes, making them an area of interest to specialists in various fields of medicine, including infectious diseases, immunology, oncology, and autoimmunity (4).

Recent studies have reported the presence of HSPs in the periodontal ligament (PDL) and epithelial cells. Both the proliferating epithelial cell rests and the radicular cysts display an over-expression of HSP27 immunostaining intensity coinciding with the presence of local infiltrating immune cells (5). In addition, the expression of HSP25 in the PDL reflects the regeneration process of nerve fibers (6). Furthermore, ultrasound accelerates periodontal wound healing and bone repair via the expression of HSP70 (7). Amemiya et al. (8) reported that PDL cells maintain their osteogenic ability in hypoxia and in re-oxygen-
ation conditions via the expression of HSP70 mRNA in vitro. Yoshimatsu et al. (9) reported increased HSP47 expression on the tension side after application of orthodontic forces. However, few studies have attempted to identify the concomitant expression of HSP70 during orthodontic tooth movement on the compression side.

In the present study, we used immunohistochemistry to investigate the localization of HSP70 in the PDL of the compressive side during experimental tooth movement in rats.

**Materials and Methods**

**Animals**

The Ethics Committee for Animal Experiments at Nihon University School of Dentistry, Matsudo, approved the animal experimental protocol of this study (approval No. ECA-05-0025). A total of 35 6-week-old male Wistar strain rats (Sankyo Labo Service Co., Tokyo, Japan) weighing 180 ± 10 g were used for the experiments. All of the animals were housed in the animal center of the Nihon University School of Dentistry, Matsudo. They were kept in separate cages, on a 12-h light/dark cycle, at a constant room temperature of 23°C, and were provided food and water ad libitum. Health status of the rats was evaluated by daily monitoring of their body weight for 1 week before the start of each experiment.

**Application of orthodontic devices and tissue harvesting**

Thirty-five 6-week-old male Wistar rats with an average body weight of 180 ± 10 g were used. Animals were anesthetized with pentobarbital sodium (40 mg/kg body weight) for the application of orthodontic devices. Experimental tooth movement was performed using the method of Fujita et al. (10), 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 ligation 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. The experimental time period was 14 days.

**Tissue preparation**

The experimental time periods were set on days 1, 2, 3, 4, 7 and 14 after tooth movement. Animals were deeply anesthetized by 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 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 then dehydrated through an ethanol series and embedded in paraffin. Each sample was sliced into continuous 6-μm sections in the horizontal direction, and prepared for both hematoxylin and eosin (HE) staining and for immunohistochemistry with HSP70 antibodies. Periodontal tissues in the mesial part of the distal buccal root of the first upper molar (M1) were observed. Specimens in which no movement was observed were defined as the control group.

**Measurement of tooth movement**

Measurement of tooth movement was performed according to the method of Fujita et al. (10). To determine the degree 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, 2, 3, 4, 7 and 14). The plaster models were scanned using a contact-type three-dimensional measurement apparatus (3D-pizza; Roland DG Co., Shizuoka, Japan) by setting the plane to pass through 3 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), the
distance between the first molar central fossa and second molar mesial surface were measured to determine the total tooth movement.

**Immunohistochemistry**

Immunohistochemical staining was performed as follows. The sections were deparaffinized and endogenous peroxidase activity was quenched by incubation with 3% H$_2$O$_2$ in methanol for 30 min at room temperature (RT). After washing in Tris-buffered saline (TBS), the sections were incubated with anti-HSP70 mouse monoclonal antibody C92F3A-5 (StressMarq Biosciences INC, Victoria, Canada; working dilution, 1:100) overnight at 4°C.

The sections were rinsed with TBS and the final color reactions were performed using the substrate reagent 3, 3’-diaminobenzidine tetra-hydrochloride and aminoethyl carbazole, and counter-stained with hematoxylin. For the immunohistochemical controls, sections were incubated with either non-immune rabbit IgG or 0.01 M phosphate-buffered saline (PBS) alone, instead of the primary antibody.

The percentage of positive cells was determined using the following formula:

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\text{Percentage of positive cells (\%) = \frac{\text{number of positive cells}}{\text{number of whole cells}}} \times 100.
\]

**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 the Tukey test, with values of \( p < 0.01 \) considered to be statistically significant (Figs. 1 and 3B).

**Results**

**Body weights and tooth movement during the experimental period**

Body weights of the rats during the application of orthodontic devices (10 g) decreased transiently on day 1 and recovered thereafter (data not shown). The amount of tooth movement increased from 0 to 14 days during the experimental period; however, the increasing became less pronounced after 7 days (\( p < 0.01 \), Tukey test; \( p < 0.01 \), by one-way ANOVA) (Fig. 1).

**Histological changes in periodontal tissues during tooth movement (HE staining)**

Fig. 2 shows the histological changes in the periodontal tissues during tooth movement. In the 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 days 1 and 2 after tooth movement, blood capillaries were mainly recognized near the alveolar bone in the PDL. Only a few mononuclear and multinucleate osteoclasts were observed on the alveolar bone surface. The arrangement of the fibers and fibroblasts become coarse and irregular, and blood capillaries were pressured on days 3 and 4. 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 day 2. On days 7 and 14, the PDL was recomposed of a coarse arrangement of fibers and expanded blood capillaries. The resorption lacunae with multinucleate osteoclasts decreased on the alveolar bone compared with that on day 3.
Protein expression of HSP70

Immunoreactivity of HSP70 was performed on day 14 after tooth movement (Fig. 3A). An HSP70-positive reaction was recognized in the nucleus of fibroblasts and collagen fibers. On day 1 after tooth movement, the immunoreactivity of HSP70 was weakly localized in some nuclei of fibroblasts and pericytes near the alveolar bone surface. On days 2, 3 and 4, more HSP70-positive nuclei of fibroblasts were recognized than on day 1 in the compressed PDL. HSP70-positive reactions in some nuclei of fibroblasts were further increased on days 7 and 14. Most of the collagen fibers showed an HSP70-positive reaction on all of the days. In our quantitative evaluation, the rate of HSP70-positive PDL fibroblasts was found to be significantly increased as compared with the control (p<0.01, Tukey test), which occurred in a time-dependent manner (p<0.01, by one-way ANOVA) (Fig. 3B).

Discussion

Considering the method of tooth movement, 10 g of a light force application resulted in tooth movement without root resorption over a period of 14 days in rats. The resorption lacunae with multinucleate osteoclasts appeared on the alveolar bone on days 3, 4, 7 and 14 after tooth movement (Figs. 2 and 3A). Gonzales et al. (11) showed that 10 g of a 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 the rats. The optimum force for the movement of the rat upper molar may be less than 10 g as previously suggested (12). Therefore, the model in this study was considered to be an efficient method of tooth movement.

Fig. 3A shows that most of the collagen fibers were HSP70-positive on all days. HSPs are constitutively expressed in unstressed cells at low levels in all
D. Dentin : C. Cements : PDL. Periodontal ligament : AB. Alveolar bone

Bar : 50 µm

Fig. 3. (A): Effect of orthodontic forces on HSP70-positive PDL fibroblasts by immunohistochemistry. The immunoreactivity of HSP70-positive cells was observed in the PDL fibroblasts on the alveolar bone surface. HSP70-positive fibroblasts increased on days 7 and 14. AB : alveolar bone, PDL : periodontal ligament, C : cementum, D : dentin. Bar = 50 µm.

Fig. 3. (B): The rate of HSP70-positive PDL fibroblasts during orthodontic force of 10 g for 14 days. The rate increased from 0 to 14 days in a time-dependent manner (p<0.01, Tukey test; p<0.01, by one-way ANOVA).

HSP70 in collagen fibers in the PDL was reflective of the unstressed condition.

The rate of HSP70-positive reactions recognized in the nucleus of fibroblasts increased on days 7 and 14 compared with that on days 1, 2, 3 and 4 (Figs. 3A and 3B). HSP70 participates in the maintenance of cell homeostasis and is induced reversibly during either disrupted energy metabolism, protein synthesis or calcium homeostasis in cells (13). In a previous report, a high level of expression of HSP70 was shown to correlate well with the healing process (14). Ikai et al. (7) demonstrated immunohistochemically that HSP70-positive cells were localized only in the low-intensity pulsed ultrasound-exposed gingival epithelium around the basal and spinous layers. The authors concluded that elevated expression of HSP70
might have contributed to the proliferation of epithelial cells, preventing their downgrowth. Chen et al. (15) suggested that HSP70 plays an important role as a molecular chaperone during reparative dentin formation. They reported a statistically significant difference in HSP70 staining between traumatized and non-traumatized teeth only in the group observed at 24 h after the trauma. The expression of HSP70 forms part of the early pulpal response to trauma (16). HSP70 mRNA expression in dental pulp during orthodontic tooth movement was found to be higher on days 3, 7, 14 and 28 after insertion of elastic rubber blocks (17). This suggests that orthodontic tooth movement causes degenerative changes and apoptosis in pulp cells, while pulp homeostasis is maintained at the genetic level.

Hayama et al. (18) demonstrated that osteocytes in alveolar bone adjacent to the hyalinized PDL underwent cell death via apoptosis during rat experimental tooth movement. Rana et al. (19) demonstrated that maximum apoptosis occurred on days 3 after the application of orthodontic force. In addition, Noxon et al. (20) demonstrated that the highest percentages of apoptosis appeared to be on the bone surface and in the PDL on days 5 and 7 after the application of force, and suggested that recruitment of osteoclasts during orthodontic tooth movement are, at least in part, cleared by apoptosis. Furthermore, Kroemer (21) reported that HSP70 apparently exerts its death-inhibitory function by binding and neutralizing apoptosis-inducing factor. Yenaru et al. (22) reported that HSP70 may directly interfere with cell death pathways, such as apoptosis and inflammation.

A recent study reported that expression of HSPA1A, which is a member of the HSP70 family, was significantly increased in the pressure zone of the PDL at 6 h after tooth movement and was seldom observed in the PDL days 5 after tooth movement (23). That these findings conflict with the present results might be related to the different force mechanics used between the studies. As it is known, magnitude and duration of force are key factors that produce second messengers needed to stimulate cellular differentiation (24). Continuous force in our study was applied, while interrupted force was used in the study of Arai et al. (23). Karacay et al. (25) reported that the periodontal tissue response to continuous force begins later than it does to interrupted force and declines at 1 week because of tissue adaptation to the orthodontic force. Therefore, the expression of HSP70 in our study might be expressed in the late phase after tooth movement. Taken together, these findings and our present results suggest that the expression of HSP70 may function in a homeostatic mechanism to compensate for PDL cell changes occurring during orthodontic force.

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