While many studies reported the structural changes in the periodontal ligament (PDL) under hypofunctional conditions, the associations of cytokine growth factors are still unclear. They are known to take part in inflammation, and may affect the biological properties of hypofunctional tooth. To investigate the hypofunctional PDL and the recovery from this condition, we focused on interleukin-1 beta (IL-1β) and basic fibroblast growth factor (FGF-2). Male Wistar rats were divided into occluded, non-occluded, and recovery groups. An anterior bite plate was used to eliminate the occlusal contact of molars in the non-occluded group, and was then removed for the recovery group. After occlusal stimuli were eliminated for 7 and 14 days, and after 3 and 7 days of recovery from 7 days in the hypofunctional condition, the PDLs of the lower first molars were investigated immunohistochemically. The lack of occlusal stimuli caused atrophic changes in the PDL with the upregulation of IL-1β and decreased expression of FGF-2, while decreased IL-1β and enhanced FGF-2 expression were observed in the recovery process. These results suggest that occlusal stimuli regulate IL-1β and FGF-2 expression, and the nature of this regulation may differ from that in the healing process of an inflammatory reaction.

Key words: Occlusal stimuli, hypofunction, recovery, IL-1β, FGF-2

Introduction

Occlusal stimulus is an essential factor for the maintenance of the structural integrity of the periodontal ligament (PDL)\(^1\). In orthodontic practices, we often experience malocclusions such as open-bite, high canine, or under-occluded teeth. Those teeth without occlusal stimuli are known as hypofunctional teeth, and lots of atrophic changes in their PDL have been reported; for example, the narrow PDL space\(^6\), the disappearance of functional arrangement of Sharpey’s fiber, the decrease in the fibroblastic proliferation activity\(^5\), the decrease in the number and diameter of blood vessel\(^4\), the unusual distribution of proteoglycans and Ruffini’s nerve ending\(^1,5\), and the decrease of the Nitric oxide (NO) synthase expression\(^6\). In addition, those teeth are also reported to have higher risk for undesirable apical root resorption than the normal occluded teeth during experimental tooth movement\(^7,8\).

By the way, interleukin-1β (IL-1β) is a major inflammatory cytokine that correlates with the root resorption
and bony resorption, and in vitro study has shown that PDL cells respond to mechanical stretching with a marked elevation of IL-1β production. A mechanical stress sensitive procedure of root resorption could be suggested by those reports; however, it is still unclear why hypofunctional teeth were prone to have root resorption. On the other hand, FGF-2 is well known to promote the proliferation of almost all cells associated with wound healing, and known to be a protein that plays an important role in differentiation of mesenchymal cells into fibroblasts and osteoblasts, angiogenesis, and formation of an extracellular matrix. Although tooth with hypofunctional condition tends to cause undesirable changes during tooth movement, this condition can also be recovered by occlusal stimuli.

In this study, we aimed to investigate the dynamic alteration of PDL condition through the influence of occlusal stimuli, and also to clarify the participation of IL-1β and FGF-2.

Materials and Methods

Twenty-five 5 weeks old male Wistar rats were randomly divided into three groups; occluded control (n = 5), non-occluded (n = 10) and recovery group (n = 10). In the non-occluded and recovery groups, rats were divided into 2 subgroups: 7 days non-occluded (n = 5), 14 days non-occluded (n = 5), 3 days recovery (n = 5) and 7 days recovery (n = 5) group, respectively. In the non-occluded and recovery group, an anterior bite plate and a metal cap made of band material (0.180 x 0.005 inch; Rocky Mountain, Colorado, USA) were attached to the maxillary and mandibular incisors, respectively, to eliminate occlusal force at the molar region (Figure 1). In the non-occluded group, rats were sacrificed at 7 and 14 days. In the recovery group, anterior bite plates were removed at 7th day to recover occlusal force at the molar region, and the rats were sacrificed after 3 and 7 days. Untreated rats (5-7 weeks old) were used as normal occluded control group. All animals were fed ad libitum with powder diet (CE-2, Clea Japan INC., Shizuoka, Japan), and had free access to drinking water. All procedures were performed under the guidelines of the Tokyo Medical and Dental University for Animal Research.

Tissue preparation

Animals were deeply anesthetized in diethyl ether, and followed by intraperitoneal injection of chloral hydrate (400 mg/kg), and then all rats were subjected to soft X-ray radiography to confirm occlusal conditions before sacrifice. Sacrifice was carried out by means of transcardiac perfusion with 4% parafomaldehyde in 0.1 M phosphate buffer (pH 7.4). The mandible was immediately immersed in the same fixative solution overnight at 4°C. Subsequently, tissue blocks were decalcified in 10% (W/V) ethylene diamine tetra-acetic acid (EDTA) at 4°C for 4-6 weeks and prepared for frozen and paraffin embedded method.

Frozen tissue preparation:

Decalcified specimens were immersed in 10%, 15% and 30% sucrose in 0.01 M phosphate buffer saline (PBS) for 6 hours each before embedding in O.C.T. Compound (Tissue -Tek®, Sakura, Tokyo, Japan).

The 20 μm thick longitudinal frozen sections were performed with a cryostat (CM3000, Leica Instrument GmbH, Nussloch, Germany). The sections were prepared for immunohistochemical staining.

Paraffin embedded tissue preparation:

Decalcified specimens were embedded in paraffin, and then 6 μm thick longitudinal sections were performed, and prepared for HE and immunohistochemical staining.

Immunohistochemical staining

IL-1β staining procedure:

Frozen sections for immunohistochemical examination were prepared for immunoreacted avidin-biotin complex (ABC) procedure using ABC-PO kit (Vector Laboratories, CA, USA), according to the manufacturer’s instruction. In brief, air drying and incubation in PBS with 0.3% Triton X-100 (PBST) for 20 minutes was...
performed. Then, endoperoxidase enzyme was inhibited with 3% hydrogen peroxide in methanol, and the sections were pre-incubated with 2% normal goat or rabbit serum (NSS) (Vector Laboratories) in PBST for 30 minutes depending on the specification of each primary antibody, and followed by incubation with anti-rat IL-1β (1:300) rabbit polyclonal antibodies (Endogen, MA, USA) in 2% NSS for 20 hours at 4°C. Subsequently, incubation with biotinylated anti-goat or rabbit serum and ABC solution for 30 minutes each was performed, followed by immunoprecipitating visualization with 0.02% 3, 3-diaminobenzidinetetrahydrochloride (DAB kit, Vector Laboratories). Finally, all sections were counterstained with methyl green and mounted in Entellan (Merck, Darmstadt, Germany). For negative control in immunohistochemical procedure, the sections were incubated in the same way with the omission of primary antibodies and replacement with normal rabbit/goat fraction (Vector Laboratories).

**FGF-2 staining procedure:**
Paraffin embedded tissue sections were prepared for HRP method (DAKO LSAB®-2 kit, DakoCytomation, Inc., CA, USA), according to the manufacturer’s instruction. In brief, sections were deparaffinized, then endoperoxidase enzyme was inhibited with 3% hydrogen peroxide in methanol, rinsed with distilled water and placed in fresh PBS, and followed by incubation with anti-rat FGF-2 (1:100) rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., CA, USA) in 2% NSS for 30 minutes at 37°C then placed in PBS. Subsequently, sections were incubated with biotinylated anti-rabbit and anti-mouse immunoglobulins in PBS for 30 minutes, and then washed with fresh PBS, followed by incubation with streptavidin peroxidase for 30 minutes, and then washed with fresh PBS, followed by immunoprecipitating visualization with 3, 3-diaminobenzidinetetrahydrochloride (DAB kit). Finally, all sections were counterstained with methyl green and mounted in Entellan. For negative control, the same procedure was followed with IL-1β staining.

**Quantitative Analysis**
The middle one third mesial aspect of distal root PDL (Figure 2) was photographed by a digital camera (DXm1200, Nikon, Tokyo, Japan) connected with microscope magnification 200X. Quantitative images were measured using image analysis software (Scion Image Beta 4.02, Scion Corporation, Frederick, Maryland, USA). One of the positive cells in control group was chosen to quantify the density, and those cells with the same density were counted as positive cells in all groups. The number of IL-1β and FGF-2 positive PDL cells and positive alveolar bone lining cells in this region was used for the measurement.

**Mesial root**
**Distal root**
*Fig. 2. The middle 1/3 of the mesial aspect of distal root was chosen for observation. A rectangular area (100 X 300 μm) including PDL cells and alveolar bone lining cells in this region was used for the measurement.*

**Statistical Analysis**
The number of immunopositive PDL cells and alveolar bone lining cells per area (mm²) in rat PDL was represented as the mean ±1 SD, (n = 5 in each group). Multiple comparisons of these groups were carried out using Kruskal-Wallis test, followed by Post hoc (Bonferroni) test. Statistical analysis software (SPSS for window, version 13.0, SPSS Inc., Chicago, Illinois, USA) was used and the level of significance was set at P = 0.05 for all statistic analyses.

**Results**
The body weight of each animal was monitored during the experiments. All animals exhibited normal growth and no significant difference was seen between all experimental groups.

**The occlusal condition**
In order to confirm the condition of the occlusal contact in the molar region, the soft X-ray images were taken before sacrifice for each group (Figure 3).

**Histological finding**
Compared to the normal PDL, the non-occluded PDL
showed disorder of collagen fiber arrangement in 7 days after the elimination of occlusal force. And after 14 days, the irregularity of collagen fiber arrangement became more evident (Figure 4a, b). On the other hand, when occlusal stimuli were reloaded for 7 days, the arrangement of collagen fiber seemed to be recovered (Figure 4c, d, e).

Immunohistochemical finding of IL-1β and FGF-2 expression

In the non-occluded group, the number of IL-1β immunopositive cells was significantly increased compared to the normally occluded control group ($P<0.05$). And those IL-1β immunopositive cells were found most abundantly in the bone lining cells, and secondary in fibroblasts and endothelial cells (Figure 5a, b,
When occlusal stimuli were reloaded with the removal of the bite plate, the number of IL-1β immunopositive cells was decreased significantly after 3 days compared to both 7 and 14 days in non-occluded groups (P<0.05), and the level was decreased almost similar to that of the control group. On the other hand, the elimination of occlusal stimuli significantly decreased the number of FGF-2 immunopositive cells in both PDL cells and alveolar bone lining cells after 3 and 7 days compared to the 7 and 14 days non-occluded groups (P<0.05; Figure 7).

**Discussion**

The periodontal ligaments (PDL) are filled with vessels, collagen fibers, extracellular substances and various cells, and play vital roles not only in tooth stability but also in the homeostasis of periodontal tissue. It is also well known that the physiological occlusal stimulus is an essential factor for the maintenance of those properties, and that the lack of occlusal forces...
may cause atrophic changes in PDL. Although many publications have reported the structural alterations of PDL, the associations of cytokine growth factors have not been studied extensively yet. Cytokine growth factors are often reported to take part in the inflammatory reactions, and may have influences in some undesirable side effect in tooth movement such as root resorption. According to a recent study that was mentioning the higher risk of root resorption in moving hypofunctional tooth, investigating the cytokine growth factors in hypofunctional PDL may assist our knowledge for preventing those side effects.

In this study, we focused on interleukin-1 beta (IL-1β) and basic fibroblast growth factor (FGF-2). IL-1β is one of the pro-inflammatory cytokine, while FGF-2 regulates fibroblast function, differentiation and proliferation, and they are correlated to each other in the healing process of inflammatory reaction. FGF-2 is reported to participate in the suppression of cytokine induced iNOS mRNA expression and in the prevention of inflammatory reaction. On the other hand, with a structure similar to FGF-2, IL-1β was also reported to bind to fibrin and fibrinogen to support wound healing. In addition, some investigation revealed that IL-1β is a major stimulator of FGF-2 expression. These studies have indicated that the upregulation or downregulation of IL-1β and FGF-2 expression were generally observed at the same time in inflammatory reaction; however, our results were differing to those findings. In our study, enhanced IL-1β and diminished FGF-2 expression was observed as the occlusal stimuli being recovered. Our results suggested that a distinctive co-relationship between IL-1β and FGF-2 may exist under occlusal-stimulus-dependent condition, which could be differentiated from ordinary inflammatory reaction.

It has been previously reported that the lack of occlusal stimuli increases osteoblastic deposition and leads to the narrowed width of PDL space, and the enhanced IL-1β expression might have been produced from those bone lining cells. Moreover, an in vitro study has reported that low magnitude equibiaxial tensile strain acts as a potent antagonist of IL-1β actions and suppresses transcriptional regulation of multiple pro-inflammatory genes. Therefore, reduced occlusal stimuli may have increased the osteoblastic deposition and resulted in enhanced expression of IL-1β in our experiment, which suggested a possibility of a higher risk for inflammatory reaction in hypofunctional PDL. On the other hand, FGF-2 is a growth factor that may be induced by mechanical stimuli. FGF-2 has been reported to increase the expression with fluid shear stress in bovine aortic endothelial cells, and the elimination of occlusal stimuli might have reduced the vascular shear stress and led to the decrease of FGF-2 expression in our study. FGF-2 has also been revealed to inhibit osteoblasts lineage differentiation. This indicated that the increase of FGF-2 expression in PDL may have inhibited the IL-1β expression especially in alveolar bone lining PDL cells as the occlusal stimuli were recovered. These findings of our study suggested that the regulation of IL-1β and FGF-2 in PDL was strongly dependent on occlusal stimuli; however,
further studies are still needed to clarify the detail of the interactive influences between IL-1β and FGF-2.

In conclusion, occlusal stimuli play an important role in the maintenance of the PDL stability, with the regulation of IL-1β and FGF-2 in an occlusal-stimulus-dependent manner.

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References