Biomechanical and biochemical factors are involved in bone remodeling. Occlusal loading is a well-known mechanical modulator of alveolar bone remodeling. Neuropeptides, such as vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), have been described within the biochemical bone regulators. In this study, the influences of occlusal loading on the alveolar bone remodeling and the distribution of VIP- and CGRP-immunoreactive (IR) fibers were investigated 1, 3 and 5 days after tooth replantation in rats. At day 5, occlusal loading induced a significant increase (p<0.05) in osteoclast number and osteoblast surface compared to those in the non-occluded group. VIP-IR fibers were observed beside osteoblastic layers and their distribution was significantly enhanced (p<0.05) at day 5 in the occluded group, compared to the non-occluded group. Although there was immunoreactivity for CGRP in the periodontal ligament and alveolar bone apically, CGRP-IR fibers were not detected above the furcation. These results suggest that, after tooth replantation in rats, occlusal loading induced an increase in osteoclast and osteoblast formation, and that VIP might play a functional role in osteoblasts.

Key words: occlusal loading, neuropeptides, VIP, CGRP, alveolar bone.

Introduction

Bone tissue is continuously remodeled through the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. It is well known that mechanical loading plays a role in bone remodeling, however, the mechanism(s) by which loading stimuli on bone are translated into biomechanical stimuli that regulate bone remodeling are not fully understood.

Bone cells are considered to respond directly or indirectly to local strains in their vicinity due to loading in normal function. These strains are the product of the bones’ external loads and their structural properties and thus include the information necessary to be the controlling input for adaptive bone modeling and remodeling.

The regulation of bone remodeling is a complex process that involves the activities of and interactions between different bone cells that are regulated by a variety of systemic hormones, cytokines, growth factors and inflammatory mediators. Another proposed regulatory element is the nervous system, which, through
Vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) are the two neuropeptides which have been most extensively studied in this field. These neuropeptides affect the activities of osteoblasts as well as osteoclasts. Moreover, immunoreactive (IR) fibers for VIP and CGRP have been demonstrated on the periodontal ligament (PDL) and alveolar bone (AB). Changes in the distribution of these fibers have been described after experimental tooth movement.

Compared to other bone tissues in the body, the AB is subjected to continual and rapid remodeling associated with the functional demands of mastication. It has been suggested that the PDL plays a role in the mechanotransduction of signals required for bone homeostasis and participates in the regulation of alveolar bone volume.

Tooth transplantation is a valuable treatment option for replacing missing or extracted teeth and has been largely used in conjunction with orthodontic treatment. While previous studies have focused on the effects of occlusal force on the healing of the PDL after tooth replantation, little is known about the effects of occlusal force on AB remodeling after this procedure. Since tooth replantation causes rupture of the PDL and impairs its mechanical properties, changes in AB remodeling are expected. Therefore, the purposes of this study were to investigate, the effects of occlusal loading on AB remodeling and changes in the disposition and distribution of VIP- and CGRP-IR fibers on the AB after molar replantation in the rat.

Materials and Methods

Animals and experimental model
Twenty-four male 5-week-old Sprague-Dawley rats (Sankyo Lab Service Corporation, Inc., Tokyo, Japan), body weight 144 ± 7g (mean ± SD), were used in this study. The animals were selected due to the pulpal and periodontal characteristics of rat at this age. To address the influence of occlusal loading on bone remodeling after tooth replantation, the experimental group was divided into occluded (n = 12) and non-occluded groups (n = 12).

All the procedures were performed under anesthesia. After the administration of diethyl ether for anesthesia, the animals were deeply anesthetized by the intraperitoneal injection of chloral hydrate (400 mg/Kg). The right maxillary first molars of the animals were replanted according to the method described by Kvinnsland. The molars were loosened, the roots were rotated anteriorly so that they came out of the socket while leaving part of the attached mesial gingiva intact, and then immediately repositioned. No postoperative splinting was used. Postoperative antibiotic treatment consisted of amoxicillin (32.4 mg/Kg). The contralateral first molars of the occluded group animals served as control; these specimens were denominated as non-replantation group.

According to the method developed by Suhr, in the non-occluded group an anterior bite-plate and a metal cap constructed from band material (4.6 x 0.13 mm; Tomy International, Tokyo, Japan) were attached to the maxillary and mandibular incisors, respectively, using light-curing composite resin (Clearfil Photo SC, Kuraray Inc., Okayama, Japan). The appliance was designed to impede occlusal function in the molar region, whereas in the occluded group, occlusal contact was maintained.

Rats in the non-occluded group were fed liquid diet (Liquid fodder, Clea Japan Inc, Shizuoka, Japan) and the ones in the occluded group were fed standard rat chow (CE-2, Clea Japan Inc, Shizuoka, Japan), both ad libitum. Both groups had free access to drinking water. The animals on both groups were sacrificed at 1, 3 and 5 days after tooth replantation. All procedures followed the guidelines of the Tokyo Medical and Dental University for Animal Research and were approved by the Animal Ethics Committee.

Tissue preparation
After deep anesthesia, the animals were sacrificed by transcardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The upper jaws were excised en bloc and further immersed in the same fixative solution at 4°C for 12 hours. After being rinsed with PBS, the specimens were decalcified in 4.13% EDTA-2Na (ethylene diamine tetra acetic acid disodium) solution at 4°C for 4-5 weeks and embedded in paraffin by conventional methods. Five-µm-thick serial sagittal sections were cut parallel to a line that passed through the disto-buccal and mesial roots of the first maxillary molar (M1) (RM 2155, Leica Co LTDA, Nussloch, Germany); these sections also included the surrounding tissue (Figs. 1A and 1B).

Immunohistochemistry
To evaluate the morphology of the PDL and AB, and the time-related changes throughout the experimental
period, some sections were stained for hematoxylin and eosin (HE) by conventional methods.

To analyze the disposition and distribution of neuropeptidergic fibers and bone cells at the AB, double-staining was carried out. Double-staining was performed to reveal immunoreactivity for neuropeptides and tartrate-resistant acid phosphatase (TRAP) in the same sections. Serial sections were stained for either VIP or CGRP while alternating the first antibody. Immunohistochemical studies were performed on paraffin-embedded sections using polyclonal antibody to VIP diluted 1:8000 (Euro-Diagnostica, Malmo, Sweden) and to CGRP diluted 1:2000 (Yanaihara Institute, Shizuoka, Japan). First-antibody reactivity was revealed by Catalyzed Signal Amplification (CSA) System HRP (Dako, Carpinteria, CA, USA). The protocol followed was essentially that described by Tolcos. After color development, TRAP staining was performed on the same sections. The sections were incubated in de-ionized water containing naphthol AS-MX phosphate (Sigma, St. Louis, MO, USA) as the substrate and Fast Red Violet LB salt (Sigma, St. Louis, MO, USA) for a color reaction at pH 5.4 with 50 mM sodium tartrate. The color reaction was carried out in a humid chamber under 37°C for 15-20 minutes and the reaction was then stopped by washing with distilled water. The sections were counterstained with hematoxylin, mounted with aqueous mounting medium (Gel/ Mount, Biomeda Corp., Foster City, CA, USA) and cover-slipped. Negative controls were prepared by replacing the primary antibody with TRIS buffered saline-Tween (TBST) buffer (0.05 M Tris-HCl [pH 7.6], 0.3 M NaCl, and 0.1% Tween 20). In these negative controls, immunoreactivity was not detected (data not shown). The sections were observed and photographed by a light microscope (Nikon Microphoto-FXA, Nikon, Tokyo, Japan) equipped with a digital camera (DXm1200, Nikon, Tokyo, Japan).

Histomorphometry

Histological examination focused on the interradicular area. Since there were signs of distal drift in all of the groups throughout the experimental period, and the most remarkable changes occurred at the interradicular bone adjacent to the furcation, the histomorphometric parameters were measured in a quadrangular area (400×400) µm² on the AB alongside the disto-buccal root (Fig. 1A).

The measurements and calculations for bone histomorphometry were referenced to the standard nomenclature described by Partiff. The parameters measured included osteoclast number (N.Oc/BS, #/mm) and osteoblast surface (Ob.S/BS, %). Osteoclasts were identified as multinucleated TRAP-positive (red-staining) cells that were situated at the bone surface. Osteoblasts were identified as cuboidal cells that lined the bone surfaces. The measurements were performed as described by Yamashiro.

Since CGRP-IR fibers were not regularly found on the evaluated area, measurements were only performed for the area of VIP-IR fibers.

Four rats were evaluated in each group. The measurements were made with image analysis software (Image-Pro, Media Cybernetics, Silver Spring, MD, USA). The data were analyzed by one-way analysis of variance (ANOVA) or Kruskal-Wallis test for intergroup comparisons. To detect significant changes over time between each experimental group and the non-replantation group, and between the experimental groups, the data were compared using repeated measures ANOVA with Bonferroni post hoc-test. Statistical analyses described above were done using SPSS version 10.0J (Chicago, IL, USA). Data are expressed as mean ± SD. The level of significance was set at 0.05.

Results

The body weight in the experimental animals regularly increased during the experimental period. There was no significant difference in the mean body weight between the groups (data not shown), which indicates that differences in diet or dietary consistency had no effect on the animals’ general growth weight and development.
**Histological Observation**

At day 1, in the line of PDL rupture, blood cells were observed and a lack of continuity between the alveolar and root portions of the PDL was evident. Also, disruption of the normal blood supply occurred (Figs. 2A and 2D). At day 3, the first signs of re-arrangement of PDL cells were observed with concomitant tissue revascularization. Continuity between the alveolar and root portions of the PDL was re-established in both groups. Numerous osteoclasts were observed on the AB in the occluded group, but were scarce in the non-occluded group (Figs. 2B, 2E, 3B and 3E). At day 5, PDL healing had progressed, although disorientation of the periodontal fibroblasts was still evident. In the occluded group, the bone surface was covered by numerous osteoblasts, and in the non-occluded group a large area of the bone surface was covered by hard tissue (Figs. 2C, 2F, 3C and 3F). At days 3 and 5, whereas the PDL in the occluded group animals was apparently wider than that in the non-replantation group ones, in the non-occluded group the PDL thickness appeared to be similar to that in the non-replantation group animals (Figs. 2B, 2C, 2E, 2F and 2G).

VIP- and CGRP-IR fibers were usually found along blood vessels, however isolated fibers were also identified. VIP-IR fibers were often found bordering areas that were covered by osteoblasts (Fig. 3). At day 1, although there was no reactivity for VIP-IR fibers, such activity was observed in both groups at days 3 and 5 inside the AB (Fig. 3). Although CGRP-IR fibers were observed in the PDL apically and in superior portions of the AB and bone marrow, in the study area these fibers were not detected (Fig. 4).

**Histomorphometry**

The data regarding osteoclast number, osteoblast surface and VIP-IR fiber area are expressed graphically in Fig. 5.

Osteoclast number and osteoblast surface

At days 1, 3 and 5, osteoclast number in the

---

**Fig. 2.** HE sections of the AB-PDL complex adjacent to the furcation. (A), (B), (C), occluded group days 1, 3 and 5, respectively; (D), (E), (F), non-occluded group days 1, 3 and 5, respectively; (G) non-replantation group. (A) and (D) At day 1, disruption of the PDL (asterisks) was caused by the replantation procedure. (B) and (E) At day 3, continuity of the PDL was restored which coincided with tissue revascularization. Numerous osteoclasts were observed on the bone surface in the occluded group (arrows). (C) and (F) Several osteoblasts (ob) covered the bone surface in the occluded group, whereas hard tissue was common in the non-occluded group. AB, alveolar bone; PDL, periodontal ligament. Scale bar = 200 μm.
Fig. 3. Reactivity for VIP-IR fibers (brown) and TRAP (red) in the alveolar bone in the occluded group days 1, 3 and 5, (A), (B) and (C), respectively and in the non-occluded group days 1, 3 and 5, (D), (E) and (F), respectively. (C') and (F') are insets from (C) and (F). (A) and (D) At day 1 immunoreactivity for VIP was not observed. (B) and (E) Numerous osteoclasts (arrows) covered the bone surface in the occluded group. In both groups, VIP-IR fibers (arrow heads) were identified along blood vessels usually bordering areas covered by osteoblasts. (C) Intense VIP immunoreactivity was observed in the occluded group. (C') and (F') Notice the close association between VIP-IR fibers and osteoblastic layers. ob, osteoblast; v, blood vessel. Scale bar = 100 μm.

Fig. 4. Immunoreactivity for VIP (A) and CGRP (B) on serial sections. Throughout the experimental period VIP-IR fibers (arrow heads) were observed along blood vessels, whereas immunoreactivity for CGRP was not detected (asterisk) in the alveolar bone above the furcation. v, blood vessel. Scale bar = 100 μm.

Fig. 5. Histomorphometric analyses of osteoclast number, osteoblast surface and VIP-IR fiber area, in the alveolar bone, in occluded and non-occluded groups. Each value is the mean ± SD (n = 4). (a) Significant difference (p<0.05) between the occluded group and the non-replantation group. (b) Significant difference (p<0.05) between the non-occluded group and the non-replantation group. (*) Significant difference (p<0.05) between the occluded and non-occluded groups.
occluded group was significantly increased compared to that in the non-replantation group (p<0.05). However, in the non-occluded group, osteoclast number was significantly increased compared to that in the non-replantation group only at day 1 (p<0.05). At days 3 and 5, osteoclast number in the occluded group was significantly increased compared to that in the non-occluded group (p<0.05).

At days 3 and 5, osteoblast surface in the occluded group was significantly increased compared to that in the non-replantation group (p<0.05). However, osteoblast surface in the non-occluded group was significantly increased compared to that in the non-replantation group only at day 3 (p<0.05). At day 5, compared to the non-occluded group, there was a 2-fold increase (p<0.05) in osteoblast surface in the occluded group.

VIP-IR fiber area

Since immunoreactivity for VIP-IR fibers was not observed at day 1 in the AB on both experimental groups, the results presented reflect days 3 and 5. At day 3, there were neither significant differences between the experimental groups, nor between each experimental group and the non-replantation group regarding VIP-IR fiber area. However, compared to the non-replantation group, there was a 2-fold increase (p<0.05) in VIP-IR fiber area in the occluded group at day 5. Significant differences between the experimental groups were seen at day 5; compared to the non-occluded group, there was a 2-fold increase (p<0.05) in VIP-IR fiber area in the occluded group.

Discussion

The results of this study demonstrated that tooth replantation and its association to occlusal loading induced significant changes in alveolar bone remodeling. From the results it can be suggested that tooth replantation induced the increase in osteoclast number in both experimental groups at day 1. It has been reported that the initial reaction to the injury caused by tooth transplantation is always acute inflammation. In addition, it is known that inflammatory mediators also play a role in the formation of osteoclasts. Taken together, it can be surmised that the inflammation caused by the replantation process might have participated in the increase in osteoclastogenic events in both experimental groups at day 1.

Taken the results, it can be also suggested that occlusal loading induced enhance in osteoclastogenic events at days 3 and 5, and enhance in osteoblast formation at day 5. Therefore, occlusal loading significantly increased alveolar bone remodeling above the furcation after tooth replantation. The effect of occlusal loading in osteoclastogenic events in this study is in agreement with previous studies which have demonstrated that compressive forces induce osteoclast activity. In this study, the fact that there was no significant difference in osteoclast number between the experimental groups at day 1, may be due to the fact that the replantation procedure damaged the PDL with a disruption of PDL continuity. The first signs of PDL re-arrangement on day 3 coincided with an increase in osteoclast number in the occluded group, indicating that forces due to occlusal loading were transduced to the bone, thus enhancing osteoclastogenic events. Even though the mechanical properties of the PDL were reduced after tooth replantation, it can be assumed that the forces from occlusal loading were transduced to the bone and evoked an increase in osteoclastogenic events in the occluded group. It is important to emphasize that in bone, osteocytes and osteoblasts are considered to exhibit mechano-sensation properties and therefore might have also become sensitized to mechanical stimuli by occlusal loading.

In addition, it can be suggested that an increase in osteoblast formation was induced by tooth replantation in both experimental groups at day 3, and by occlusal loading in the occluded group at day 5. In both circumstances, the increase in osteoblast formation occurred following an increase in osteoclast formation. It has been reported that in bone remodeling, osteoblastic bone formation occurs in a programmed precise and quantitative manner following osteoclastic bone resorption. Therefore, it can be surmised that the increase in the number of active osteoclasts might have caused enhance in bone resorption and might have at least partially induced enhance in osteoblast formation in order to repair the resorbed bone surface.

It has been reported that traumatic occlusion of the graft after tooth transplantation is significantly related to root resorption activity. In this study, occlusal loading was also significantly related to the enhance in alveolar bone remodeling after tooth replantation. Taken the effects of mechanical loading on root resorption activity and on bone remodeling, it would be advisable to avoid excessive loading of the grafts on early stages after tooth transplantation. In contrary, it is known that masticatory stimuli reduces ankylosis after experimental tooth replantation therefore, further studies on
the amount of forces and the proper time of force application are necessary to achieve optimal results after tooth transplantation.

The influence of VIP on bone tissue has been the subject of several studies. VIP-IR fibers, which are of sympathetic origin at the head, have been observed in areas with high osteogenic activity. In addition to the fact that chemical-induced sympathtectomy resulted in the loss of nerve fibers containing VIP with a concomitant 45.5% increase in osteoclast number, bone apposition and the mineralization rate at the mandible were significantly lower following surgical sympathtectomy. These changes were concurrent with a significant increase in the number of osteoclasts per bone surface of the molar root socket. VIP-specific binding sites and VIP receptors have been described on osteoclasts and osteoblasts. Furthermore, it has been suggested that VIP contributes to the formation of committed osteoblasts and the regulation of osteoblastic differentiation. VIP has also been reported to stimulate bone formation in vitro, by up-regulating alkaline phosphatase activity in osteoblasts. Moreover, VIP inhibited the motility of differentiated osteoclasts and osteoclast formation, probably by regulating the expression of RANK, RANKL and OPG. In this study, the fact that VIP-IR fibers were preferentially distributed bordering osteoblastic layers and the increase in the occluded group on day 5, which coincided with an increase in osteoblast surface, might indicate that VIP plays a functional role in the regulation of osteoblast formation.

An anabolic effect of CGRP on bone remodeling through a decrease in bone resorption and the stimulation of bone formation has been described in vitro and in vivo. Whereas the participation of CGRP-IR fibers in osteoclastogenesis after experimental tooth movement has been described in dental tissue in rats. In this study, CGRP-IR fibers were not found on the AB above the furcation and there were no changes in their distribution throughout the experimental period, indicating that CGRP might not participate in bone-regulatory events in this area soon after tooth replantation.

Although bone remodeling is a complex process, which involves the activities of numerous mediators, the present results suggest that after tooth replantation, occlusal loading induced an increase in osteoclast and osteoblast formation, and that VIP might play a functional role in osteoblast formation.

Acknowledgments

This research was supported by Grants-in-Aid for Scientific Research (nos. 15592158 and 15791202) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

10. Lerner UH. Deletions of genes encoding calcitonin/alpha-CGRP, amylin and calcitonin receptor have give new and unexpected insights into the function of calcitonin receptors and calcitonin receptor-like receptors in bone. J Musculoskelet Neuronal Interact. 2006;6(1):87-95.