Occlusal stimuli and the periodontal healing of replanted teeth have been reported to be related. However, the mechanism for preventing den-toalveolar ankylosis remains unclear. Basic fibroblast growth factor (bFGF/FGF-2) is considered as a key factor in wound healing. The purpose of this study was to evaluate the relationship between occlusal stimuli, bFGF, and the periodontal healing after tooth replantation. Five-week-old male rats were divided into non-occluded, occluded, and recovery groups. The right maxillary first molars were replanted in all groups, and the left maxillary first molars in the 2-week occluded group without replantation were served as non-treated. An anterior bite plate was attached to the maxillary and mandibular incisors to produce occlusal hypofunction in the non-occluded group and was then removed after 1 week in the recovery group. Histological observations were performed after 1 and 2 weeks of the experimental period. After 2 weeks, the non-occluded group had detectable ankylosis and obvious periodontal tissue stricture. Meanwhile, the occluded and recovery groups showed enlarged and thickened periodontia without ankylosis. The number of bFGF-positive cells in the occluded and recovery groups significantly increased as compared to in the non-occluded group. These results suggest that occlusal stimuli enhance the production of bFGF in the periodontal healing of replanted teeth and prevent dentoalveolar ankylosis.

Key Words: tooth replantation; occlusal stimuli; periodontal healing; ankylosis; basic fibroblast growth factor.

Introduction

Tooth transplantation is now a common procedure for replacing a missing tooth and is extensively applied in orthodontic treatment. In recent years, the prognosis of transplanted teeth has greatly improved and the success rate has increased to 90%. For successful tooth transplantation, it is necessary to preserve cell activity in the remaining periodontal ligament (PDL) of the transplanted teeth and promote the formation of new periodontal tissue around the root after transplantation.

Although the success rate of autotransplantation has increased, dentoalveolar ankylosis and root resorption are still common complications, particularly in cases of transplantation of complete teeth.
Dentoalveolar ankylosis disrupts the functional recovery of the PDL. In animal studies, some experimental models have been attempted to elucidate the periodontal healing process of transplanted teeth. Mine et al. focused on the prevention of ankylosis and reported that occlusal stimuli after tooth replantation promote PDL regeneration using a convenient method of tooth replantation. However, it is still unclear how mechanical stimuli promote periodontal healing and prevent dentoalveolar ankylosis of replanted teeth.

Attachment of the PDL of replanted teeth to the recipient alveolar bone and vascular reconstruction are essential processes for successful tooth replantation. During periodontal healing, multiple cytokines act in concert to regulate the function of various cells in the periodontium. Among these, basic fibroblast growth factor (bFGF/FGF-2) is a potent angiogenic factor whose activity is crucial for enhanced normal wound healing and is recognized as a key factor in PDL regeneration. bFGF is a member of the heparin-binding growth factor family and is highly expressed in human PDL fibroblasts and endothelial cells. It has been reported to exert versatile effects on cell proliferation, differentiation, and angiogenesis. In animal studies, the topical application of bFGF can promote periodontal healing and regeneration without epithelial down growth, ankylosis, or root resorption in a surgically created furcation. Furthermore, several studies demonstrated that mechanical stimuli enhance the production of bFGF in PDL cells and endothelial cells at both the protein and mRNA levels.

We postulate that bFGF is involved in the periodontal healing of replanted teeth, appropriate occlusal stimuli induce satisfactory periodontal healing and subsequently prevent dentoalveolar ankylosis. In this study, we aimed to investigate the relationship between periodontal healing, occlusal stimuli, and bFGF in replanted teeth.

**Materials and Methods**

**Experimental model**

Twenty-five Sprague-Dawley male rats (5 weeks old) were maintained under pathogen-free conditions. The animals were divided into 3 experimental (n = 25) groups, namely, the occluded (n = 10), non-occluded (n = 10), and recovery (n = 5) groups. The left maxillary first molars in the 2-week occluded group without replantation were served as the non-treated group (n = 5), which could provide us with useful information about the approximate normality. All animals had access to powdered fodder (Rodent Diet CE-2; Japan Clea Inc., Shizuoka, Japan) and drinking water ad libitum. All experiments were performed under anesthesia by intraperitoneal injection with ketamine hydrochloride (KETARAL 50; Sankyo Co Ltd., Tokyo, Japan) containing 20% xylazine hydrochloride (Celactal 2% injections; BAYER-Japan Co Ltd., Tokyo, Japan). The right maxillary first molar (M1) was replanted according to the method described by Kvinnsland et al. (Figure 1a).

The tooth was extracted with a tissue forceps, rotated once anteriorly so that all the roots came out of the socket while leaving a part of the mesial gingiva attached, and then immediately repositioned. No postoperative splinting was used. In the non-occluded group, an anterior metal bite plate and a metal cap constructed from stainless band steel (4.572 mm × 0.127 mm; Rocky Mountain, Colorado, USA) were attached to the maxillary and mandibular incisors, respectively, using a light-curing composite resin (Clearfil Liner Bond II, Kuraray Co. Ltd., Okayama, Japan) according to the method developed by Suhr et al. (Figure 1b). These appliances prevented occlusion at the molar region, whereas occlusal contact was maintained in the occluded group. In the recovery group, the appliances were removed after 1 week of hypofunction, and the rats were sacrificed 1 week after the removal of the appliances. All procedures were carried out in keeping with the guidelines of the Animal Ethics Committee of Tokyo Medical and Dental University.

**Tissue preparation**

After administering inhalant anesthesia, all the animals were perfused intracardially through the left ventricle with 4% paraformaldehyde in a 0.1 M phosphate-buffered saline (PBS) solution (pH 7.4) at 1 and 2 weeks after replantation. The maxillary specimens were removed en bloc, immersed in the same fixative at 4°C for 24 hours, and decalcified in 10% EDTA solution for 4-5 weeks; the specimens were then embedded in paraffin by using conventional methods. Serial sagittal sections (5-μm thick) were cut (RM2155; LEICA Co Ltd, Nussloch, Germany) parallel to the long axis of the mesial and distal roots of the M1 (Figure 2a) and mounted on glass slides coated with poly-L-lysine (Matsunami Glass, Osaka, Japan). The distalapical root in Figure 2b was selected for observation because our preliminary experiments showed that it
had obvious morphological differences following replantation. In order to examine the changes in the periodontium and in bFGF expression, hematoxylin and eosin and immunochemical stainings of the sections were performed; the stained sections were then observed by light microscopy.

**Immunohistochemistry**

The deparaffinized sections were immersed in methanol containing 3% hydrogen peroxide for 30 minutes at 37°C to block endogenous peroxide activity and rinsed with 0.01 M PBS solution. After pretreatment, the sections were incubated with the rabbit polyclonal antibody to bFGF (diluted at 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS solution containing 0.5% normal goat serum for 30 minutes at 37°C and visualized using the horseradish peroxidase (HRP) method (DAKO LSAB 2kit; Dako, CA, USA). Finally, the sections were stained with 3,3'-diaminobenzidine.

**Quantitative analysis**

The observation area was photographed using a light microscope (Nikon Microphoto-FXA, Nikon, Tokyo, Japan) equipped with a digital camera (DXm1200, Nikon, Tokyo, Japan), and the images were stored in the 24-bit true color TIFF format. The length of the dentoalveolar ankylosis was measured at both the mesial and distal regions of the distopalatal root by using image analysis software (NIS elements, Nikon, Tokyo, Japan). According to the histological observations and preliminary data, the PDL area and number of bFGF-immunoreactive cells per unit area were recorded at the distoapical region that was 1000-1500 μm from the furcation of the distopalatal root by using image analysis software (Scion Image Beta 4.02; Scion Corporation., Frederick, Maryland, USA) (Figure 2a, b).

**Statistical analysis**

Data were expressed as mean ± SD. In order to analyze the individual difference in the 2-week non-treated
and occluded groups (Figure 4a; Figure 6a, c) the Mann-Whitney U test was adopted then the 2-week experimental groups were analyzed by the Bonferroni test (Figure 4b, c; Figure 6b, d) for multiplex comparison. The significance level was set at \( P < 0.05 \) by using statistical analysis software (SPSS for Windows, version 14.0; SPSS Inc., Chicago, Illinois, USA).

**Results**

The body weight of the animals in all the experimental groups increased during the study period, and no significant difference in mean body weight was detected between all groups (data not shown).

**Hematoxylin and eosin staining**

As compared to the 2-week occluded group, the width of the PDL in the non-occluded group decreased after 2 weeks of hypofunction (Figure 3c, e; Figure 4b).

Dentoalveolar ankylosis is defined as the attachment of the alveolar bone to dentin (Figure 3e, black arrow head) and was clearly observed in the non-occluded group after 2 weeks, while it was not observed in the occluded and recovery groups. (Figure 3c, e, f; Figure 4c). Pseudo ankylosis is defined as the attachment of the alveolar bone to cementum and was observed in the 1-week non-occluded group (Figure 3d, white arrow head); it decreased in the recovery group with the application of occlusal stimuli to the replanted teeth for 1 week following replantation (Figure 3f; Figure 4c). Root resorption was observed in the 2-week occluded group (Figure 3c, arrow head). Contrastingly, there was only partial slight root resorption in the recovery group (Figure 3f).

**Immunostaining for bFGF**

In our experimental model, dentoalveolar ankylosis was observed in the distoapical region that was 1000-1500 \( \mu \text{m} \) from the furcation toward the root apex of the distopalatal root (Figure 3e). Therefore, the distoapical region of the distopalatal root was selected to investigate bFGF expression. During the experimental period, the number of bFGF-immunoreactive cells in the occluded and recovery groups increased, while that in the non-occluded group decreased (Figure 5c, e, f; Figure 6b, d).

**Discussion**

The regeneration of periodontal tissue and prevention of dentoalveolar ankylosis are essential for favor-
able prognosis in tooth replantation\textsuperscript{13,15,16}. In previous reports, occlusal stimuli were shown to promote the periodontal healing of replanted teeth and prevent dentoalveolar ankylosis\textsuperscript{13,15}. However, the role of an endogenous factor in the wound healing of replanted teeth is still not well known\textsuperscript{13}. In the present study, appropriate mechanical stimuli enhanced the expression of bFGF/FGF-2 in replanted teeth and prevented dentoalveolar ankylosis and root resorption (Figure 3a, c, e, f; Figure 4c; Figure 6a, b, c, d).

bFGF induces the proliferation of PDL cells\textsuperscript{17} and accelerates desirable periodontal tissue regeneration without ankylosis or epithelial down growth\textsuperscript{22-25}. In animal studies, the suppression of bFGF has been shown to delay wound healing in traumatized tissues\textsuperscript{29,30}. In our results, dentoalveolar ankylosis and the decrease in the PDL area of replanted teeth were clearly observed in the 2-week non-occluded group. Subsequently, bFGF expression decreased significantly, which suggests that low bFGF expression could delay periodontal healing. Previous reports suggest that replanted teeth need protection from traumatic forces in the early phase of periodontal healing; subsequent exposure to appropriate occlusal stimuli promotes the periodontium regeneration and prevents the dentoalveolar ankylosis and root resorption of

\begin{figure}
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\caption{(a, b) Quantitative analysis of the PDL area. The PDL area was measured at the distoapical region that was 1000-1500 \( \mu \text{m} \) from the furcation of the distopalatal root. (c) Quantitative analysis of dentoalveolar ankylosis. The length of the dentoalveolar ankylosis was measured at both the mesial and distal surfaces of the distoapical root. All data are expressed as mean ± SD; \( n = 5 \) for each group; *\( P < 0.01 \).}
\end{figure}

\begin{figure}
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\caption{Immunostaining of bFGF. (a) Non-treated group, (b) 1-week occluded group, (c) 2-week occluded group, (d) 1-week non-occluded group, (e) 2-week non-occluded group, and (f) recovery group. Insets, higher magnification of the boxed area in Figure 5a, b, c, d, e, and f. Black arrow heads, bFGF-immunoreactive cells. Me, mesial; Di, distal; AB, alveolar bone; C, cementum; D, dentin; P, pulp; and PDL, periodontal ligament. Bar = 100 \( \mu \text{m} \).}
\end{figure}
replanted teeth. In the 2-week occluded group, there was no significant difference in comparison with the non-treated group (Figure 4a; Figure 6a, c) but root resorption occurred (Figure 3c, arrow head), which suggests that mechanical stimuli might disrupt the early phase of wound healing. On the other hand, the recovery group showed no dentoalveolar ankylosis and only slight root resorption; further, the appearance of the periodontium and the bFGF expression pattern in the recovery group were similar to those in the non-treated group (Figure 3a, f; Figure 4a, b, c; Figure 5a, f; Figure 6a, b, c, d). Mechanical stimuli up-regulates various growth factors in various cells. Occlusal stimuli may play a key role in the late phase of periodontal healing of tooth replantation by bFGF up-regulation.

Periodontal healing is a complex process involving inflammation, neovascularization, neurogenesis, bone formation, and matrix remodeling. bFGF is a potent angiogenic factor, and angiogenesis may be involved in the periodontal regeneration promoted by bFGF. bFGF exerts versatile effects on cell proliferation and differentiation in various cells and tissues. In vitro, the effect of bFGF on PDL cells gradually decreases during the course of culture. Shimabukuro et al. proposed that bFGF can act effectively on immature PDL cells at the early stage of wound healing. Recently, it has been revealed that PDL tissue possesses multipotent mesenchymal stem cells that can differentiate into osteoblasts and cementoblasts in vitro. PDL cells display various osteoblast-like properties such as the expression of bone-associated factors and the formation of mineralized nodules. However, PDL tissue is never ossified in vivo under normal conditions. In vivo studies have also shown that bFGF suppresses the alkaline phosphatase activity of PDL cells and reduces mineralized nodule formation. The molecular mechanism by which the PDL is maintained as a flexible connective tissue and not ossified during periodontal healing remains to be completely clarified. Some mechanisms that constitutively prevent unorchestrated osteogenesis by PDL cells and dentoalveolar ankylosis in transplanted teeth may exist. Thus, bFGF may play roles at different stages of the periodontal healing process of transplanted teeth due to its various biological properties, which may be enhanced by mechanical stimuli.

In summary, opportune occlusal stimuli promote PDL cell proliferation and prevent the dentoalveolar ankylosis of replanted teeth. Our study suggests that bFGF is up-regulated by mechanical stimuli and may participate in the periodontal healing of replanted teeth.

![Fig. 6. Quantitative analysis of bFGF-immunoreactive cells.](image-url)
Acknowledgments

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