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Revised

**Effect of basic fibroblast growth factor on root resorption following delayed autotransplantation of tooth in dog**

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Abstract

**Objectives.** To investigate the effect of basic fibroblast growth factor (FGF-2) on root resorption following delayed autotransplantation in dog.

**Study design.** Mandibular second and third premolars of beagle dogs were extracted to create sites for autotransplantation. After two months, in the experimental sites, the first and fourth mandibular premolars were extracted and air-dried prior to autotransplantation with the application of recombinant FGF-2, while control sites received teeth without FGF-2. At 2, 4, or 8 weeks after surgery, the animals were sacrificed and specimens collected and processed for histological examination.

**Results.** Autotransplantation with FGF-2 yielded formation of new periodontal ligament-like tissues with inserting collagen fibers, associated cementum and bone. The occurrence of replacement resorption in the FGF-2 treated group was significantly lower than that in the control group ($P < .01$).

**Conclusion.** It was demonstrated that topical application of FGF-2 reduced the occurrence of ankylosis and root resorption following delayed autotransplantation in this experimental model.
Key words: Basic fibroblast growth factor; Autotransplantation; Histopathology; Wound healing; in vivo
Introduction

Autogenous tooth transplantation can be an alternative treatment modality, especially in situations where dental implants and other prosthetic replacements are contraindicated.\textsuperscript{1-4} When treated with adequate indications, autotransplantation offers a fast and economically viable option for replacing missing teeth\textsuperscript{2,5-7}

Root resorption, the most common complication accompanying autotransplantation, leads to poor esthetics, the tilting of adjacent teeth, loss of function, and permanent tooth loss.\textsuperscript{8-10} Occurrence of replacement resorption is influenced by factors such as the extra-alveolar period\textsuperscript{11}, preservative solution\textsuperscript{12}, pulpal revascularization\textsuperscript{13}, and microbial contamination.\textsuperscript{14} Treatment, often complex, time-consuming, and expensive, requires a multidisciplinary approach including endodontic, periodontal, surgical, and orthodontic treatment, as well as esthetic coronal restoration.\textsuperscript{15}

Root resorption may be transitory or progressive. Replacement resorption is followed by ankylosis, and the root surface is gradually replaced with bone.\textsuperscript{16} The probability of successful autotransplantation increases when an avulsed tooth is
immediately transplanted or stored in a solution that preserves periodontal ligament (PDL) on the root.\textsuperscript{11, 14, 17, 18} PDL cells are easily injured under stressful conditions such as variable pH, osmotic pressure or dehydration, and favorable healing of the PDL depends on the quantity of viable cells preserved on the root.\textsuperscript{1} Andreasen\textsuperscript{11} showed that only 21\% of normal periodontium was histopathologically observed when extracted tooth was left in a dry condition for 60 min. prior to tooth replantation in monkeys. Autotransplantation studies using non-human primate incisors revealed that the PDL is regenerated as long as it maintains cellular activity adjacent to the cementum.\textsuperscript{19} Therefore a great deal of attention has been paid to the biological storage and maintenance of PDL cells in these teeth.

Cytokine therapy with growth factors has been suggested as a therapeutic modality for tissue regeneration due to their capacity to induce a cascade of well-regulated cellular events involving wound healing.\textsuperscript{20-22} Basic fibroblast growth factor (bFGF, FGF-2) is a heparin-binding protein with several physiological functions. It is found in ectomesenchyme during the embryonal period.\textsuperscript{23-25} FGF-2 exhibits potent angiogenic activities and mitogenic ability of mesenchymal cells within the PDL.\textsuperscript{21} Studies have
shown that application of FGF-2 enhances the healing of periodontal tissue without ankylosis, root resorption, or epithelial downgrowth in experimental alveolar bone defects in beagle dogs\textsuperscript{21} and primate models.\textsuperscript{21,26} Seshima et al.\textsuperscript{27} reported that FGF-2 promotes formation of new PDL and prevents ankylosis and root resorption following reimplantation of teeth in dogs. Recently, a large-scale multi-center randomized clinical trial in Japan reported that application of FGF-2 is efficacious in the regeneration of human periodontal tissue.\textsuperscript{28} However, the influence of FGF-2 on the periodontal tissue of transplanted teeth, remains to be elucidated. Given the reported effects of FGF-2 on periodontal tissue, we hypothesized that healing without root resorption or osseous repair might be achieved in autotransplantation of tooth with compromised periodontal ligament, through its effects on angiogenic formation and fibroblastic proliferation.

The objective of this study was to histopathologically investigate the effect of FGF-2 on root resorption following delayed autotransplantation of tooth in dog.
Materials and methods

This experiment was performed according to the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College (No 232202). Fifteen healthy female beagle dogs (12-month-old, 10-12 kg) were used for this study. Housing and feeding were according to standard animal care protocols. The animals were under sequential veterinary control during the entire experimental period.

Surgical procedure

The animals were placed under general anesthesia with sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 25 mg/kg. To reduce stress and hemorrhage in surgical areas, local infiltration anesthesia (2% xylocaine, 1:80,000 adrenaline) was also used. The second and third mandibular premolars (2P2 and 3P3) were extracted to provide space (recipient sites) for tooth autotransplantation. The dogs received daily plaque control, consisting of brushing and application of 0.2% chlorhexidine gluconate solution, in order to establish healthy gingival conditions prior
to autotransplantation.

After two months, the tooth transplantation procedure was performed as follows: One week prior to transplantation, under rubber dam isolation, root canals of each first and fourth premolar (1P1 and 4P4) were endodontally treated to prevent inflammatory root resorption from root canal infection. After endodontic access cavities were produced, the root canals were biomechanically prepared using K- and H-type files. During instrumentation, the root canals were irrigated with a 10% NaOCl and 3% H₂O₂ solutions, and later dried with sterile paper points. Then the root canals were filled with gutta-percha and hydroxyapatite -based root canal sealer (Finapec APC, Kyosera, Kyoto, Japan), using the lateral condensation method. One week later, a crestal incision was made from the first to the fourth premolar. Buccal and lingual full thickness flaps were elevated. The double-rooted 4P4 were separated to allow each root to be tested as an individual unit. 1P1 and the distal and mesial segments of 4P4 were luxated with an elevator and extracted with forceps using rotary movements. The roots were then air-dried on a glass surface for 60 min at room temperature in order to inflict damages to PDL cells.
The recipient sites were prepared using implant drills with copious saline irrigation. The size and shape of the recipient sites were made as uniform as possible. The sites were prepared approximately 2 mm wider than the size of the tooth to be transplanted, according to the method described by Katayama et al. After preparation was complete, recombinant FGF-2 (Lot No. 80HCC; Kaken Pharmaceutical, Tokyo, Japan) was diluted with distilled water and then mixed with hydroxypropyl cellulose (HPC). The prepared formulation containing 0.3% FGF-2 (30 µg/site) was applied to the root surface on the left side (P1 and P4: experimental group) of the mandible. The right side (1P and 4P: control group) was treated in an identical manner, but without application of FGF-2 or HPC. HPC was not used in the control group since the application of HPC alone did not significantly alter the healing of transplanted tooth in our preliminary experiment.

The teeth (1P1 and 4P4) were placed in the corresponding recipient sites (Fig.1). The flaps of these recipient sites were then repositioned and sutured. The transplanted roots were stabilized with a suture splint for 2 weeks. All dogs received antibiotics (Mycillin Zol KMK, Kawasaki Mitaka Pharmaceutical Co, Kawasaki, Japan; 0.05 ml/kg) for 7
days after surgery. They also received daily plaque control regimen consisting of gentle wiping of the teeth with gauze soaked with 0.2% chlorhexidine gluconate solution. The sutures were removed after 1 week.

Histological processing

The animals were euthanized with an intravenous overdose of sodium pentobarbital at 2, 4, or 8 weeks following the procedure described above. The jaw of each animal was then removed and specimens containing the experimental areas placed in 20% buffered formalin for 7 days. Specimens were decalcified with 10% ethylenediamine tetraacetic acid (EDTA) (Wako, Tokyo, Japan) over 5 months, after which they were dehydrated in ethanol, embedded in paraffin, and serially sectioned to 5 µm thickness in the buccolingual direction. The sections were then stained with hematoxylin-eosin (H & E). Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was performed using an immunoperoxidase staining kit [Histofine SAB-PO (MULTI) Kit; Nichirei, Tokyo, Japan]. The sections were incubated with mouse anti-PCNA primary antibody (PC-10; DAKO Corporation, Carpinteria, CA, USA) at a dilution of 1:100.
Next, each section was incubated with biotinylated secondary antibody and streptavidin peroxidase reagents. The presence of peroxidase-complexes was visualized by 3-3' diaminobenzidine tetrahydrochloride (0.1 mg/ml) solution with 0.65% H$_2$O$_2$. Sections were counterstained with Mayer’s hematoxylin. A brown coloration indicated a PCNA-positive reaction. Magnification was set at ×100, and field of connective tissue in the middle portion of root was selected in each section, which was randomly selected from each dog. A 0.12-mm$^2$ (0.2 × 0.6 mm) area in the periodontal tissue was submitted to quantitative analysis as described previously.$^{30}$

Histomorphometric assessment

All measurements were performed with an image analysis system comprising a light microscope (Olympus BX51 Microscope, Olympus, Tokyo) with a 4× objective equipped with a digital camera (HC-2500, Fujifilm, Tokyo). A computer (Precision Work Station 220 System, Dell, Round Rock, TX, USA) employing an image processing software (Image Pro Plus v 3.0, Media Cybernetic, Silver Spring, MD, USA) was used.
The analysis of periodontal morphology was a modification of the method described previously.\textsuperscript{16,29} Briefly, the resorption patterns were classified as 1. surface resorption; small superficial resorption cavities within cementum, 2. dentin resorption; the condition where bowl-shaped areas of resorption, involving both cementum and dentin; 3. replacement resorption; condition where direct contact between the alveolar bone and the mineralized root substance (ankylosis) was the characteristic feature.

Three sections (the central part of the tooth and 200-\textmu m sections on either side of that area) from each transplanted tooth were used for morphometric evaluation. The extent of each resorption pattern on the root periphery was expressed as a percentage of the total length of the peripheral contour of the root surface; that is, the distance between the distal and mesial portions of the cemento-enamel junction. For statistical analysis, Student’s t test was used to compare differences between experimental and control groups. \textit{P}-values less than 0.05 were considered statistically significant.
Results

Clinically, the healing response was uneventful in all dogs tested, throughout the observation periods.

1. Histopathological examination: Week 2

   Experimental group:

   Newly formed connective tissue covered the roots of tooth transplants (Fig. 2a). The spaces in the bone and root surface were filled with newly formed connective tissue composed of spindle-shaped cells, which are associated with growing capillaries (Fig. 2b). A number of PCNA-positive cells were observed in the newly formed connective tissue (Fig. 2c). The number of PCNA-positive cells in the experimental group was statistically significantly greater than that in the control group (p < .001) (Table I).

   Control group:

   The root exhibited surface and/ or dentin resorption (Fig. 2d, e). Multinucleated cells were often observed on the root surface. Few PCNA- positive cells were observed in the connective tissue (Fig. 2f).
2. Histomorphometric assessment: Week 2

The results of histomorphometric assessment performed at 2 weeks after autotransplantation are presented in Table II. Root resorptions extending to cementum and/or dentin were already observed in both groups. The occurrence of dentin resorption in the experimental group was significantly lower than that in the control group ($P < .01$). Replacement resorption was not observed in the experimental or control group at this early stage.

3. Histopathological examination: Week 4 and 8

Experimental group:

At week 4, bone formation had advanced along the root surface (Fig. 3a), and as a result, PDL-like tissue was observed between the root surfaces and the new bone. Osteoblast-like and cementoblast-like cells were visible in newly formed bone and cementum, respectively. It is significant that connective tissue fibers of the newly formed PDL inserted directly into the newly formed bone.
At week 8, the newly formed alveolar bone had advanced to surround the entire root of the transplanted teeth, and PDL-like tissue was evident between the root surface and the new bone (Fig. 3b). Cementoblast-like cells were aligned along the surface of the cementum. In some areas, new cementum had formed on the original cementum. Fibers had inserted into newly formed cementum and adjacent bone. Only a few cavities indicating dentin resorption were discernible in the middle and apical portions of the root. Prevalence of replacement resorption with ankylosis was low.

Control group:

At week 4, areas of root resorption were observed (Fig. 3c). The root surface was either covered with connective tissue in which collagen fibers ran parallel to the surface or was the seat of active dentine resorption as evidenced by the presence of multinucleated cells. Periodontal ligament close to the alveolar bone was partially occupied by newly formed bone tissue that was in close proximity to or in direct contact to the dentin surface.

At week 8, the surface of the transplanted root showed signs of replacement resorption (Fig. 3d). Replacement resorption was observed in the lateral portion of all
the roots. Here, resorption was the predominant feature in the middle and apical portions of the root. In areas with ankylosis, the resorbed dentin surface was in direct contact with alveolar bone.

4. Histomorphometric assessment: Week 4 and 8

At both observation periods, the occurrence of replacement resorption in the experimental group was significantly lower than that in the control group ($P < .01$) (Table II). No statistically significant difference was found in dentin or surface resorption between the experimental and control groups.
Discussion

In the present study, the root surface treated with FGF-2 achieved a favorable healing after delayed autotransplantation, whereas the control, non-treated group exhibited high occurrence of dentin and / or replacement resorption. Uniformity of the histologic results observed within each group demonstrates that the study design was sound and is adequate for this type of study. After 4 to 8 weeks, the occurrence of replacement resorption in the control group was statistically significantly higher than that in the experimental group. This may be related to the reduction in cellular viability or activity on the root surface of the transplanted tooth.

The healing process of autotransplantation puts two different tissues in competition: the PDL on the root surface and the bone tissue of the alveolus.31 In the present study, the roots were left air-dried for 60 min. prior to transplantation, for the purpose of damaging the PDL cells. Desiccation for 60 min. or more can cause necrosis of PDL cells32 and expansion of the injury site in periodontal tissue on the root surface.33 We speculate that some PDL cells remain vital after 60 min of extra alveolar period, mainly based on the histopathological findings and data presented by Andreasen11. In a
preliminary experiment, we performed toluidine blue staining of the root immediately after extraction, and observed that most of the PDL tissue appeared to be present on the root surface. In addition to the reduction of cellular viability by desiccation, it is possible that damages to PDL and/or cementum by physical stress of extraction are responsible for the occurrence of surface or dentin resorption at 2 weeks after transplantation. In competitive wound healing of the damaged or denuded root surface, repopulation of endosteal osteoblasts would tend to be favored over the limited viable PDL fibroblasts. This may explain high occurrence of replacement resorption observed in the control group after 4 weeks.

FGF-2 is known to be deeply involved in cell proliferation and differentiation and also in control of extracellular matrix generation during the processes of tissue generation and wound healing. FGF-2 has been shown to act on a variety of mesenchymal cell types. With regard to delayed-replantation of tooth, topical application of FGF-2 was shown to be effective in prevention of replacement resorption in animal studies. The ability of FGF-2 to stimulate variety of undifferentiated cell types in periodontal tissue lead us to test its potential in enhancing healing of
damages induced during the process of autotransplantation. In the present study, significantly greater numbers of PCNA positive cells were found in the newly formed connective tissue in the FGF-2 treated group than in the control group. PCNA is a 36-kD polypeptide expressed in the late G1 to S phases of the cell cycle and is a specific marker for cell proliferation. The PCNA positive cells have been shown to be associated with blood vessels within PDL tissue, and the blastic-cells in the PDL may be supplied from cells related to these blood vessels. Collectively, it was suggested that cells derived from alveolar side and/or residual PDL of the transplanted tooth with FGF-2 proliferated in blood clots, reached the surface of the root, and initiated the formation of an intact PDL. Murakami et al., using a canine model, reported that FGF-2 expression could be detected in immature granulation tissue 1 week after the operation, and tended to decrease 2 weeks after the operation. The aggregates formed by FGF-2 are considered to constitute a favorable environment for proliferation of the cells. Endogenous FGF-2 has been shown to be induced in the early phase of wound healing in periodontal tissue. It is possible that inhibitory effect of FGF-2 on osteoclast-like cells of monocyte/macrophage lineage played a role in the suppression of dentin
resorption in the experimental group at 2 weeks.

At later stages, the FGF-2-treated sites showed formation of intact periodontal tissues (new cementum, PDL, and supporting bone formation). While the source of PDL fibroblast-like cells could be, in part, from those viable cells remaining in the PDL and/or cells on alveolar side, it could be speculated that, under appropriate inductive stimuli, progenitor cells including paravascular cells could differentiate into a PDL fibroblastic phenotype. The high incidence of PDL collagen fibers inserting directly into newly formed cementum observed in the experimental group after 4W supports this hypothesis. This finding is consistent with the previous report that the types of cell which repopulate the root surface determine the nature of attachment during healing.

FGF-2 downregulates alkaline phosphatase activity and reversibly diminishes the PDL cells’ potency for mineralized nodule formation. FGF-2 has been shown to suppress the expression of bone-related proteins, such as type I collagen, osteocalcin and bone sialoprotein, but prompted the expression of osteopontin by PDL cells. Furthermore, effects of FGF-2 on hard tissue formation and mineralization may differ between each cell type, and similarly, functions of osteopontin appear to vary depending
on the cell types despite mineralizing and non-mineralizing tissue.\textsuperscript{46} Taken together, it is suggested that topical application of FGF-2 stimulates multipotent mesenchymal cells within the residual PDL\textsuperscript{22,26} and/or near alveolar bone, and potentially decreases the occurrence of replacement resorption, possibly by its differential effects on various cell-types including those involved in bone remodeling and root resorption.

In the present study, application of FGF-2 did not completely prevent occurrence of replacement resorption. Since FGFs are structurally and functionally prone to denaturation under low pH at injury sites\textsuperscript{46}, the myriad biological effects associated with the exogenous FGF-2 applied in our study might not be sustainable. However, it was shown that in bone, FGF-2 produced by osteoblastic cells is deposited in bone matrix, and FGF-2 may be released from the bone matrix and affects bone remodeling.\textsuperscript{43} The sustainability of its biological effects may critically influence the manner in which FGF-2 modulates hard tissue remodeling.

The differences in histomorphometric outcomes between the experimental and control teeth clearly favor FGF-2 treatment in autotransplantation and could be considered clinically relevant. Clinical safety of FGF-2 has been shown when applied in
conjunction with periodontal surgery: the immunogenic potential of FGF-2 was found to be extremely low.\textsuperscript{47} The results from the present study suggest that tissue engineering to functionally restore the damaged periodontal tissue could ultimately inhibit replacement resorption.

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Figure legends

Fig. 1

Diagram illustrating transplantation.
Representative photomicrograph of transplanted tooth at week 2 [Experimental (FGF-2 treated) group, a-c; Control group, d-f] Middle portion of the root.

a, Newly formed connective tissue covered root of transplanted tooth. (H&E staining).

b, Spindle-shaped cells and blood vessels adhered to bone surface (H&E).
c. A number of PCNA-positive cells (arrows) are observed in the connective tissue adjacent to the root surface and alveolar bone side (PCNA and counterstaining with Mayer’s hematoxylin stain).

d, e Root resorption (arrow in d) extending to dentin area can be observed (H&E).

f. Only a minimal number of PCNA-positive cells (arrows) are observed in connective tissue (PCNA and counterstaining with Mayer’s hematoxylin staining).
Fig. 3
Representative photomicrograph of transplanted tooth at week 4 and week 8

[Experimental (FGF-2 treated) group, a and b; Control group, c and d]. Middle portion of the root.

a. Week 4. Bone formation is observed along the root surface. Connective tissue fibers had inserted into newly formed bone (H&E staining).

b. Week 8. PDL-like tissue had formed between the root surface and new bone (H&E).

c. Week 4. The root surface exhibits replacement resorption. Multinucleated cells are present (arrows) near the site of resorption (H&E).

d. Week 8. Bone is fused with areas of root resorption (H&E).

Table I. Quantification of PCNA-positive cells

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<td>No. of cells</td>
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Experimental; FGF-2 treated

Values given as mean (%) ± standard deviations (n = 10)

**; P < .001, Student's t-test
Table II. Histomorphometric assessment of root resorption

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Experimental; FGF-2 treated

Values given as mean (±) = standard deviations (n = 5)

*; P < .01,   NS; not significant, by Student’s t-test