

ORIGINAL ARTICLE

Effect of basic fibroblast growth factor (FGF-2) in combination with beta tricalcium phosphate on root coverage in dog

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Abstract

Objective. In root coverage treatment, periodontal regeneration in gingival recession-type defects is an important challenge for the periodontist. The aim of this study was to histometrically investigate the effect of combined use of basic fibroblast growth factor (FGF-2) and beta tricalcium phosphate (β -TCP) on root coverage in dogs. **Materials and methods.** Sixteen adult beagle dogs were used. Buccal gingival recession defects were surgically created bilaterally in the maxillary canines. The defects in each animal were randomly assigned to: (1) an FGF-2 alone (control) group or (2) FGF-2/ β -TCP (experimental) group. At 2, 4 or 8 weeks following surgery, specimens were obtained and subjected to microscopic examination and histometric assessment. **Results.** Inhibition of epithelial down-growth was observed in both groups. At week 2, in the newly formed connective tissue at the coronal portion, the FGF-2/ β -TCP group showed significantly greater numbers of proliferating cell nuclear antigen-positive cells than the FGF-2 group (55.8 ± 4.8 vs 12.0 ± 1.4 , $p < 0.01$). In the FGF-2/ β -TCP group, new attachment was observed at 8 weeks and the extent of new bone and cementum formation was significantly greater in the FGF-2/ β -TCP group than that in the FGF-2 alone group. In both groups, the dentin surface beneath the new cementum presented minor irregularities, but no replacement resorption was observed. **Conclusions.** FGF-2 used in combination with β -TCP enhances formation of new bone and cementum without significant root resorption in root coverage in this dog model. This combination warrants further investigation in periodontal regeneration in root coverage treatment.

Key Words: *animal studies, fibroblast growth factor-2, beta-tricalcium phosphate, dental cementum, periodontal regeneration, wound healing*

Introduction

Recently, the application of various techniques to root coverage has made long-term, predictable results obtainable [1,2]. The ultimate histological goal of such treatment is new attachment to the previously denuded root surface. Nevertheless, even with techniques such as guided tissue regeneration (GTR), regeneration of a buccal surface in gingival recession defects is difficult [3] and poses a significant challenge for the periodontist.

Today, tissue engineering has made it possible to achieve favorable results with less invasive procedures. Signaling molecules have been receiving particular attention as a factor in bioregeneration in periodontal treatment [4]. Basic fibroblast growth factor (bFGF, FGF-2) is a member of the heparin-binding growth

factor family. Believed to be involved in the wound-healing process, it exerts diverse physiological effects [5–7]. Fibroblast growth factor-2 potently stimulates the angiogenic and mitogenic activities of mesenchymal cells. Previous studies employing animal models with artificial defects of the periodontal tissue or surgically-induced periodontitis revealed that FGF-2 was effective in regenerating periodontal tissues [8–10]. Recently, a large-scale multi-center randomized clinical trial in Japan reported that application of FGF-2 was efficacious in the regeneration of human periodontal tissue [11]. Given the reported effects of FGF-2 on periodontal tissue, we hypothesized that its application to root coverage might also yield favorable results.

Beta tricalcium phosphate (β -TCP) is a porous compound similar to that found in natural bone

and comprises purified calcium phosphate multi-crystals and CaPO_4 . When used as an adjunct material in GTR, β -TCP prevents the collapse of barrier membrane onto the root surface [12]. It also stabilizes blood clots [13]. One clinical trial revealed that the combination of β -TCP and recombinant human platelet-derived growth factor (PDGF)-BB increased clinical attachment level and linear bone growth and facilitated bone filling in severe intra-bony defects [14]. It has also been reported to be effective as a scaffold for periodontal regeneration in humans [15]. In an earlier study from our research group, it was shown that the combined use of FGF-2 and β -TCP enhanced periodontal regeneration in intra-bony defects in dogs [16]. However, little information is available on the use of FGF-2 and β -TCP in root coverage.

In the present study, we histometrically investigated the effect of FGF-2 used in combination with β -TCP on root coverage in dog.

Materials and methods

Animal model and surgical procedures

Sixteen female beagle dogs (12-months-old, 10–12 kg each) with intact maxillary and mandibular teeth and showing good systemic and periodontal health were used for the study.

All experimental procedures were performed in accordance with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College (study no. 222203).

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, 1:80000 adrenaline) was also used. Critical-sized periodontal defects (6 × 5 mm: vertical × horizontal) were surgically created bilaterally in the maxillary canines. A metallic strip of matrix was placed over each defect and left in place for 10 weeks to promote plaque accumulation. At 2 weeks after removal of the strip, the hygiene status of the area of the defect returned to normal levels (Figure 1A). Full-thickness flaps were raised and the root surfaces scaled and planed (Figure 1B). A coronal notch (Figure 1B-a) at the cemento-enamel junction and an apical notch (Figure 1 B-b) were made at the obtained defect (bone level).

The defects in each animal were randomly assigned to one of the following treatment groups: (1) an *FGF-2 alone* (control) *group*, treated with 200 µg of 0.3% FGF-2 (Scios Nova, Mountain View, CA) plus hydroxypropyl cellulose (HPC) solution; or (2) an *FGF-2/β-TCP* (experimental) *group*, treated with the FGF-2 solution in combination with β -TCP. The β -TCP used in this study (OSferion, Olympus

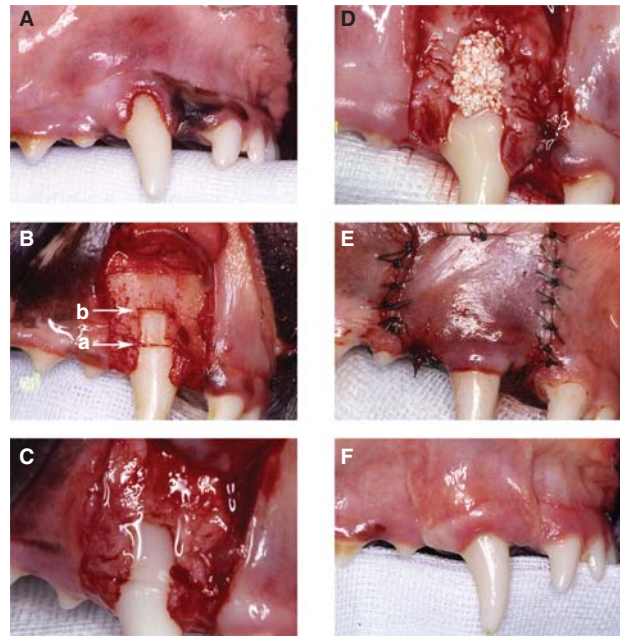


Figure 1. Surgical procedures. (A) Clinical aspect of gingival recession. (B) Defect on root after raising flap. (a) CEJ, (b) bone level. (C) Defect was filled with FGF-2. (D) Defect was completely filled with β -TCP particles and FGF-2. (E) Flaps were replaced and sutured. (F) At 8 weeks after root coverage of canine, healing was uneventful.

Terumo Biomaterials Corp, Tokyo, Japan) was whitish in color, with a macropore size of 200–400 µm, a diameter of 0.5–1.5 mm and 75% porosity. Before use, the β -TCP was placed in a dappen dish and hydrated with FGF-2 solution for 10 min. In the *FGF-2/β-TCP group*, FGF-2 was first applied to the root surfaces (Figure 1C), after which β -TCP (pre-treated with FGF-2) was placed in the defects and lightly compressed (Figure 1D). In the *FGF alone group*, no β -TCP was placed. Finally, the flaps were replaced and sutured coronally with vertical or horizontal mattress sutures (Figure 1E). 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 (eight dogs each) 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 4 months, after which they were dehydrated in

ethanol. Paraffin-embedded samples were subjected to routine histologic processing. Sections were cut along the bucco-lingual plane, parallel to the long axis of the root. Semi-serial 3- μ m histologic sections were stained with hematoxylin and eosin.

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) at a dilution of 1:100. Next, each section was incubated with biotin-labeled goat anti-mouse IgG antibody as 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_2O_2 . Sections were counterstained with Mayer's hematoxylin. A brown coloration indicated a PCNA-positive reaction. A field of connective tissue in the coronal area (immediately beneath the coronal portion of newly formed periodontal tissue) or the apical area (immediately above the apical notch) was randomly selected in each section. A 0.04-mm² (0.2 \times 0.2 mm) area in the periodontal tissue was submitted to quantitative analysis as described previously [17].

Histometric analysis

From each biopsy, five sections representing the central portion of the root were used for microscopic examination and histometric assessment at $\times 40$ magnification. Histometric analysis was performed as described previously [18]. Briefly, the following features were quantitatively assessed (Figure 2): epithelial length—the distance between the apical border of junctional epithelium and the coronal notch (notch: a); cementum formation—the distance between the apical notch (notch: b) and the most coronal portion of new cementum; bone formation—measured from the apical notch to the most coronal part of new bone; and defect extension—from the apical notch to the coronal notch.

Statistical analysis

Mean and standard deviation for each measurement were calculated for each experimental group. Differences between groups were statistically analyzed using the Student's *t*-test. A *p*-value < 0.05 was considered to be statistically significant.

Results

Clinical observations

Clinically, the healing response was uneventful. No suppuration or abscess formation was observed

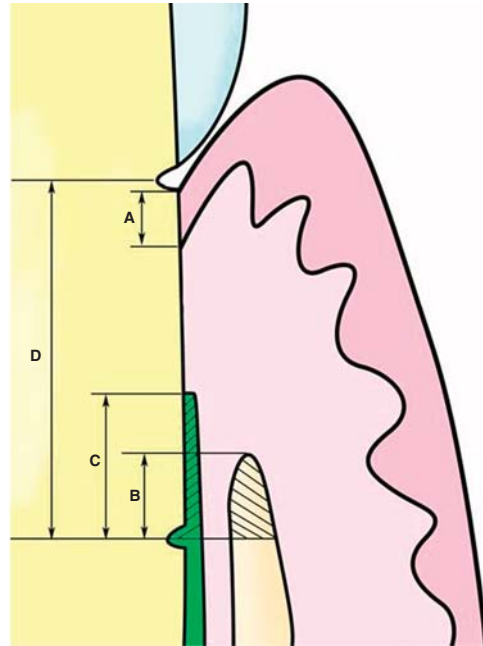


Figure 2. Linear measurements used in histometric analysis: (A) epithelial length; (B) bone formation; (C) cementum formation; and (D) defect extent.

during healing (Figure 1F). No significant differences between groups were observed in the final esthetic outcome such as color or texture of tissues.

Histological observations

At 2 weeks after surgery, no cementum formation was observed in the FGF-2/ β -TCP or FGF-2 alone groups (Figures 3A and C). In the FGF-2/ β -TCP group, most of the implanted β -TCP particles remained within the defects, surrounded by numerous fibroblasts and capillaries (Figure 3B). A few multinucleated cells adhered to the β -TCP particles, not only at the base of the defect, but also in the central region of the implanted area (Figure 3B). In the FGF-2 alone group, a small amount of new bone formation was observed at the base of the defects (Figure 3C). The bone defects were filled with newly formed connective tissue composed of fibroblasts and capillaries (Figure 3D). Maturation of granulation tissue and newly formed woven bone were observed in the defects (Figure 3D).

At 4 weeks after surgery in the FGF-2/ β -TCP group, new bone formation increased and some β -TCP particles at the base of the defects were surrounded by new bone (Figure 4A). Proliferation of capillaries was observed in the area adjacent to the new bone (Figure 4B). Newly formed bone was detected around the β -TCP particles; the bone matrix stained positively with eosin and cuboidal osteoblast-like cells were observed on the surface of the new bone. Some multinucleated giant cells were in contact with the surface of the β -TCP particles (Figure 4B) in

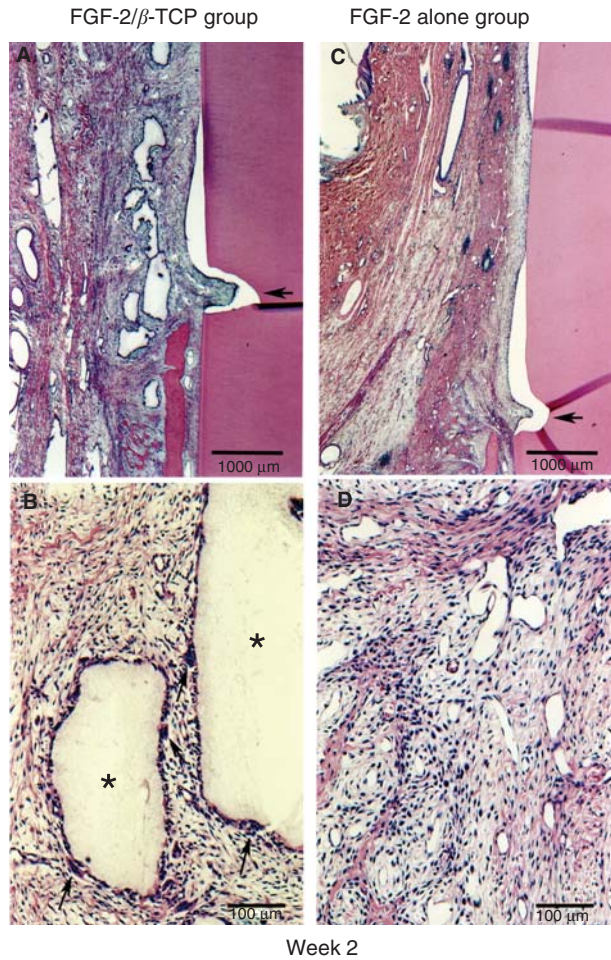


Figure 3. Representative photomicrograph at 2 weeks after surgery. (A) FGF-2/ β -TCP group. β -TCP particles and numerous fibroblasts were observed. Arrow head: apical notch. (B) FGF-2/ β -TCP group. Multinucleated cells (arrow heads) adhered to β -TCP particles (*). (C) FGF-2 alone group. Limited amount of new bone was observed. Arrow head: apical notch. (D) FGF-2 alone group. Newly formed connective tissue was composed of fibroblasts and capillaries. (Original magnification: A, C $\times 2.5$, B, D $\times 20$: hematoxylin and eosin.)

the FGF-2/ β -TCP group. In the FGF-2 alone group, new bone formation was found near the apical notch (Figures 4C and D). No significant irregularities or resorption of the root was observed in the FGF-2/ β -TCP or FGF-2 alone group.

At 8 weeks after surgery in the FGF-2/ β -TCP group, β -TCP particles were embedded within newly formed bone, which may have originated from the base of the defect (Figure 5A). New bone and cementum formation was observed to extend to the coronal portion (Figure 5B). Dense collagen fibers were observed in the PDL space between the root and the newly formed bone. No marked root resorption or ankylosis was observed. In the FGF-2 alone group, new bone formation occurred near the apical portion of the defects, while the middle and upper parts of the defects were surrounded by dense connective tissue (Figure 5C). Newly formed cementum was often

observed in the area extending from the apical notch of the root surface to the coronal portion (Figure 5D). The dentin surface underneath the new cementum presented minor irregularities, but no replacement resorption was observed.

Immunohistochemistry

At 2 weeks after surgery, newly formed connective tissue in the FGF-2/ β -TCP group showed more PCNA-positive cells in both the coronal and apical aspects of the bone defect (Figures 6A and B). In the FGF-2 alone group, however, PCNA-positive cells were observed more in the apical portion than in the coronal portion of the bone defect (Figures 6C and D).

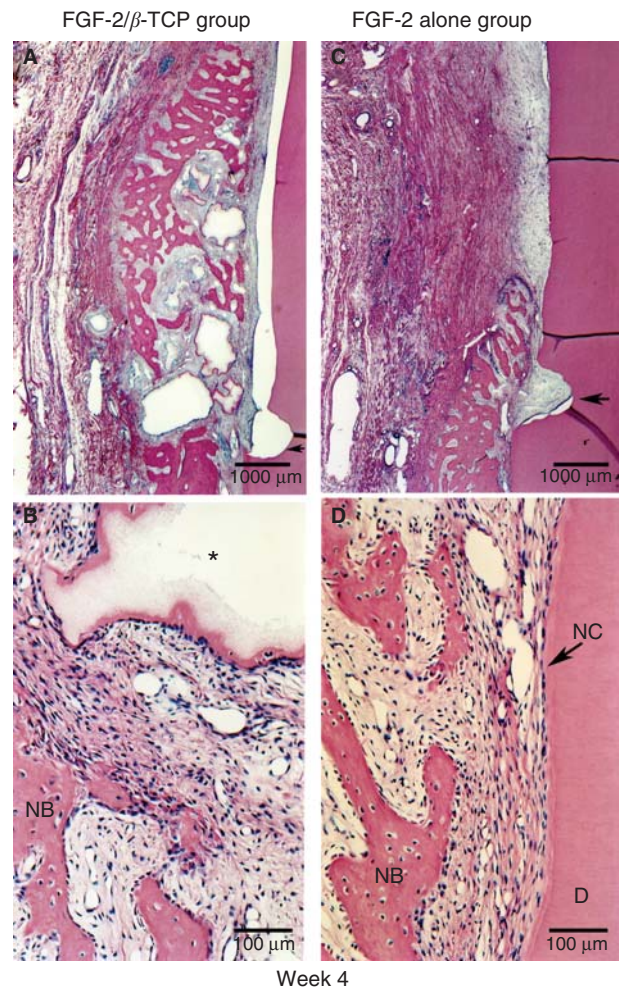


Figure 4. Representative photomicrograph at 4 weeks after surgery. (A) FGF-2/ β -TCP group. Defects contained β -TCP particles surrounded by new bone. Arrow head: apical notch. (B) FGF-2/ β -TCP group. Active new bone formation with numerous osteoblast-like cells was observed on the surface of β -TCP particles. * β -TCP, NB: new bone. (C) FGF-2 alone group. Limited new bone formation was observed within the defect. Arrow head: apical notch. (D) FGF-2 alone group. New cementum formation was observed on dentin surface. (Original magnification: A, C $\times 2.5$, B, D $\times 20$: hematoxylin and eosin.) NC: new cementum, D: dentin, NB: new bone.

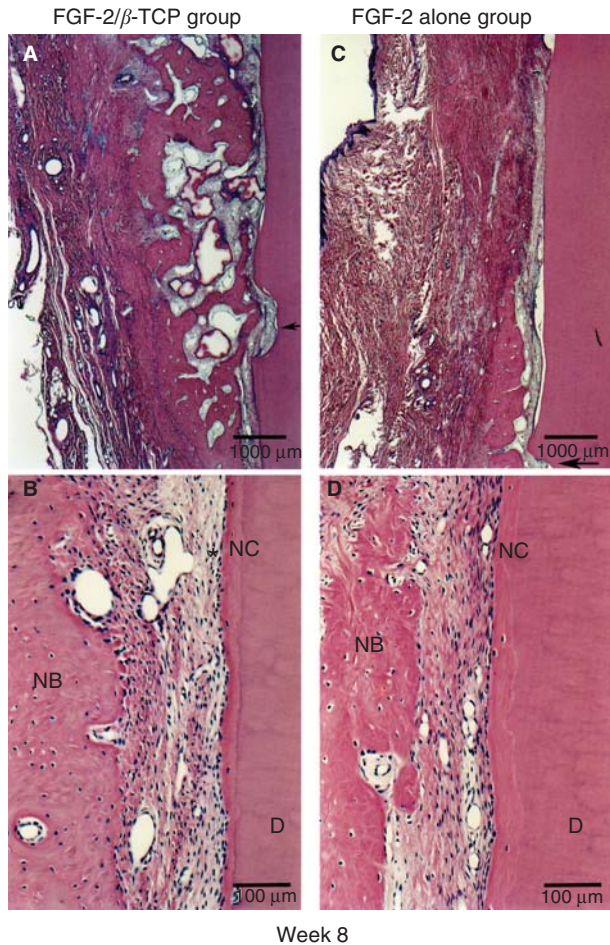


Figure 5. Representative photomicrograph at 8 weeks after surgery. (A) FGF-2/ β -TCP group. Newly formed bone filled the defect area. β -TCP particles were embedded within osteoid tissue. Arrow head: apical notch. (B) FGF-2/ β -TCP group. Dense PDL-like tissue had formed between the newly formed bone and cementum. Dentin surface beneath the new cementum shows minor irregularities, but no replacement resorption was observed. (C) FGF-2 alone group. Newly formed bone was observed extending coronally from the apical notch (Arrowhead). (D) FGF-2 alone group. PDL-like tissue was observed between new cementum and new bone. Dentin surface underneath the new cementum shows minor irregularities, but no replacement resorption was observed. NC: new cementum, D: dentin, NB: new bone (original magnification: A, C $\times 2.5$, B, D $\times 20$; hematoxylin and eosin).

Results indicating the number of PCNA-positive cells in the newly formed connective tissue of the FGF-2/ β -TCP and FGF-2 alone groups are summarized in Table I. No statistically significant difference was observed in the ratio of PCNA-positive cells in the apical portion. However, a statistically significant difference was observed in the coronal portion, showing greater numbers of PCNA positive cells in the FGF-2/ β -TCP group ($p < 0.01$).

Histometric measurements

The histometric results at 8 weeks after surgery are shown in Table II. The mean length of epithelium in

the FGF-2 alone group was significantly greater than that in the FGF-2/ β -TCP ($p < 0.05$). In the FGF-2/ β -TCP group, the amount of new cementum and bone observed was significantly greater than that in the FGF-2 alone group ($p < 0.05$, 0.01 , respectively).

Discussion

In this study, the use of FGF-2 inhibited epithelial down-growth. Taken together with those of earlier studies [19,20], the results of a preliminary experiment (data not shown) demonstrated that sites treated with coronally positioned flaps healed mainly by long epithelial attachment. In order to avoid epithelial down-growth, the principles of wound healing such as space provision, stability of the wound and primary intention healing need to be respected [21]. It was shown that FGF-2 acts differently on PDL cells and gingival epithelial cells *in vivo* in terms of proliferative response [22]. In the presence of serum, FGF-2-induced proliferation of gingival epithelial cells was inhibited, but that of PDL cells was synergistically enhanced [22]. These properties of FGF-2 may have played a role in the inhibition of epithelial down-growth observed in the FGF-2 treated groups in the present study.

At 2 weeks, there were more PCNA-positive cells in the coronal portion of the bone defect in the FGF-2/ β -TCP group than in the FGF-2 alone group. The aggregates formed by FGF-2 constitute a favorable environment for proliferation of cells and β -TCP may have contributed to the stabilization of blood clots. It was shown that β -TCP was capable of releasing pre-adsorbed PDGF-BB that could result in significant osteoblastic proliferative responses *in vitro* [23]. The potential of β -TCP as a carrier for FGF-2 has been reported [16,24]. We also speculate that β -TCP worked as an effective vehicle for FGF-2 in the present experimental model. However, the binding of FGF-2 to β -TCP and its release kinetics from β -TCP need to be investigated.

One notable finding was that new bone formation was greater in the FGF-2/ β -TCP group than that in the FGF-2 alone group. An earlier study on the root coverage with enamel matrix derivative showed only a small amount of new bone formation. Buccal alveolar bone was thin and often associated with bone resorption and the space between the flap and root surface was minimal [25]. The lack of sufficient space in this type of defect can compromise periodontal regeneration. In this regard, a β -TCP particle scaffold can be a critical component, providing a 3-dimensional structure for cell seeding, migration and growth, as well as new tissue formation.

In this study, we observed active bone formation in close proximity to the β -TCP particles at 4 weeks. Bone had formed at the defect wall in the early stage of healing and progressed towards the center of the

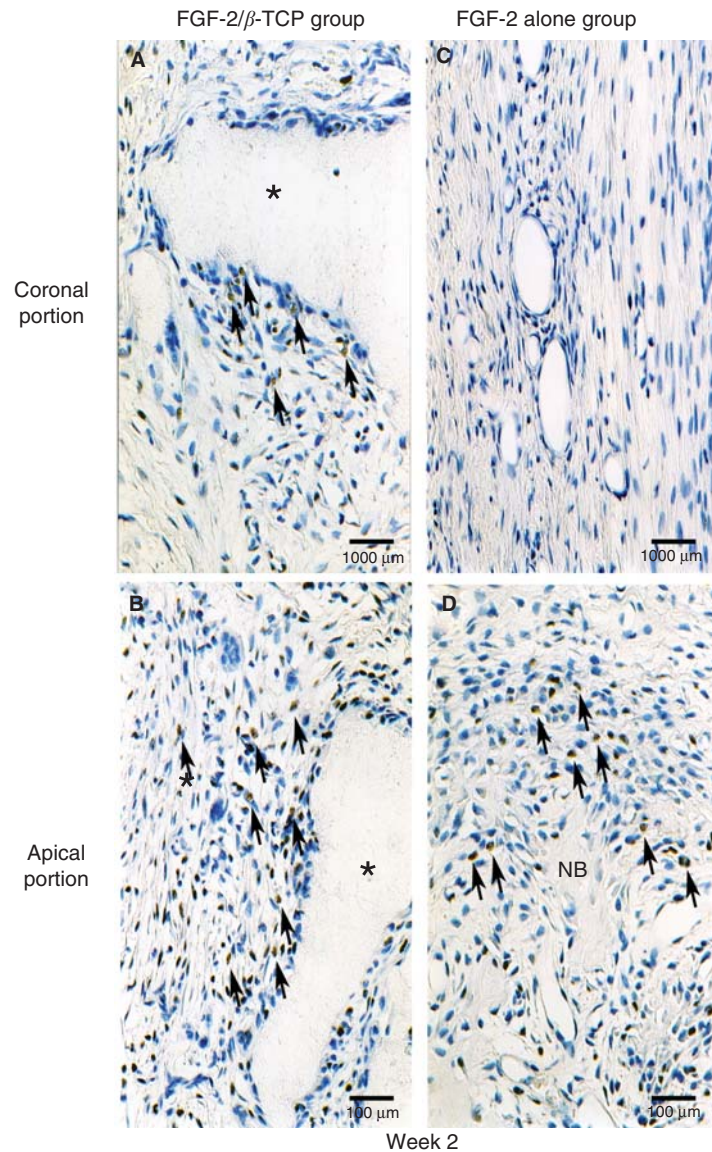


Figure 6. Representative photomicrograph of immunohistochemical staining for PCNA in the FGF-2/β-TCP group (A and B) and FGF-2 alone group (C and D) at 2 weeks after surgery. In the FGF-2/β-TCP group, PCNA-positive cells (arrow heads) were more frequently immunolocalized around β-TCP particles in the coronal portion. In contrast, in the FGF-2 alone group, they were localized around newly formed bone. * β-TCP, NB: new bone (PCNA and counterstaining with Mayer's hematoxylin stain, original magnification ×40).

defect by bridging the β-TCP particles. Our experimental design did not include a β-TCP alone group and this represents a limitation in the present study. Using a 1-wall periodontal defect model in beagle dogs, Anzai et al. [24] demonstrated that, compared

with β-TCP alone, the combined use of FGF-2 and β-TCP significantly stimulated neogenesis of PDL, alveolar bone and cementum, accompanied by insertion of collagen fascicles into newly formed

Table I. Number of PCNA positive cells in connective tissue of FGF-2/β-TCP and FGF-2 alone group at 2 weeks.

	FGF-2/β-TCP	FGF-2 alone	Difference*
Coronal portion	55.8 ± 4.8	12.0 ± 1.4	<i>p</i> < 0.01
Apical portion	53.2 ± 5.8	45.2 ± 3.6	NS

Data expressed as mean ± standard deviation.
*Student's *t*-test.
NS, not significant.

Table II. Histometric results at 8 weeks.

	FGF-2/β-TCP	FGF-2 alone	Difference*
Epithelium migration	0.76 ± 0.18	1.31 ± 0.29	<i>p</i> < 0.05
New cementum	5.09 ± 0.36	4.40 ± 0.24	<i>p</i> < 0.05
New bone	4.32 ± 0.25	2.93 ± 0.18	<i>p</i> < 0.01
Defect extension	5.86 ± 0.24	5.71 ± 0.12	NS

Data expressed as mean ± standard deviation (mm).
*Student's *t*-test.
NS, not significant.

hard tissues. Taken together with the present results, this may support the findings from previous studies on the osteoconductive properties of β -TCP particles [13,14]. On the other hand, in regard to the effect of the composite use of bFGF and TCP on bone formation, Maus et al. [26] demonstrated a negative effect using a trepanation femoral defect in a sheep model. Although differences in the animal model, types of defects or TCP, doses and application modes may explain these conflicting results, the effect of combined use of FGF-2 and β -TCP on bone formation requires further investigation.

In this study, the FGF-2/ β -TCP yielded marked new bone formation at 4 weeks after application. Significant new attachment and new bone were observed at 8 weeks. No replacement root resorption was observed. Murakami [4] suggested that, during the early stages of periodontal regeneration, FGF-2 increases the number of PDL cells while suppressing differentiation into osteoblasts and cementoblasts. During the subsequent healing processes, periodontal ligament cells begin to differentiate, inducing marked periodontal regeneration. It was also reported that FGF-2 does not simply induce angiogenesis but also increases the production of osteopontin, heparan sulfate and macromolecular hyaluronan from periodontal ligament cells [27–29]. It is possible that β -TCP further enhances the ability of FGF-2 to create a local environment suitable for regeneration of periodontal tissue.

Questions remain regarding the characteristics of periodontal regeneration observed in the present study. The defect area in the FGF-2/ β -TCP and FGF-2 alone groups often showed PDL fibers running obliquely or parallel to the root surface. This type of PDL formation has also been reported with the use of PDGF in combination with atelocollagen in a gingival recession-type defect model in dog [30]. This connective tissue/root surface relationship may be similar to that described as ‘tissue adhesion’ [31]. A longer observation period may be necessary to clarify this.

In conclusion, within the limits of this *in vivo* study, FGF-2 in combination with β -TCP enhanced formation of new cementum and bone without root resorption in root coverage. This suggests that this combination warrants further investigation in periodontal regeneration in root coverage treatment.

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