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ORIGINAL ARTICLE

Bone Augmentation by Implantation of an FGF2-loaded Collagen Gel-sponge Composite Scaffold

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SYNOPSIS

Fibroblast growth factor-2 (FGF2) plays a critical role in osteoblastic cell proliferation. Collagen gel-sponge composite is an effective scaffold for tissue engineering. The purpose of this study was to evaluate whether addition of FGF2 to collagen gel-sponge composite promotes bone augmentation in rats.

The rats were assigned to groups designated F0, F3 and F15, and received implantation of collagen gel-sponge composite containing 0, 3 and 15 μg FGF2, respectively, into a cranial bone defect. Specimens were prepared 1, 2 and 5 weeks after surgery for histologic and histomorphometric analysis.

Newly formed bone area in groups F3 and F15 was significantly greater than that in group F0 at all stages. These results suggest that FGF2-loaded collagen gel-sponge composite scaffold stimulates bone augmentation and might provide a more effective bone engineering approach.

***Key words**: FGF2, collagen gel-sponge composite scaffold, bone augmentation*

INTRODUCTION

Growth factors are important elements for promoting differentiation and proliferation of cells in regenerative therapy. Many investigators have shown that fibroblast growth factor-2 (FGF2) enhances fibroblast and vascular cell growth in relation to wound healing[1]. FGF2 also stimulates proliferation of osteogenic cells, such as osteoblasts and bone marrow stromal cells[2-4]. Tabata *et al*.[5] reported that implantation of FGF2-incorporated gelatin hydrogels facilitates bone regeneration in cranial bone defects in monkeys. Murakami *et al*.[6] found that FGF2 application with gelatin facilitates regeneration of alveolar bone in class II furcation bone defects in beagle dogs. Thus, FGF2 in combination with an optimal and effective regenerative scaffold might be sufficient to initiate bone formation.

Three-dimensional scaffold, a major element of bone tissue engineering, may provide the environment and space for repopulation and specialization of cells and extracellular matrix[7]. In previous studies, several types of scaffolds for bone tissue engineering have been utilized: collagen[8], gelatin[9], calcium phosphate[10], hydroxyapatite[11], and synthetic polymer[12]. Type I collagen, which is a natural biomaterial that plays a major role in tissue formation, has

good biocompatibility and mechanical stiffness by using different cross-linking methods[13]. Shimoji *et al*.[14] reported that placement of sponge-type collagen onto rat femurs stimulated bone augmentation. However, regenerative cells were frequently detected in the margin of the collagen sponge, not in the inner part, suggesting that infiltration of tissue-forming cells to the sponge, particularly the central region, may be limited.

To achieve a high level of cell proliferation into the collagen sponge, we developed a collagen hydrogel-sponge composite in which collagen hydrogel was injected into the spaces in the collagen sponge. Ishikawa *et al*.[15] succeeded in preparing a highly bio-safe collagen hydrogel with a different cross-linking method in which collagen solution was incubated with ascorbate and copper ion. Miyaji *et al*.[16] reported that many fibroblastic cells had proliferated on the inner side of the implanted collagen hydrogel in early periodontal healing in vivo. Tokunaga *et al*.[17] performed a study in which collagen hydrogel-sponge composite was implanted into cranial bone defects. The authors observed that the collagen composite induced more cell repopulation and bone tissue replacement. Therefore, we hypothesized that collagen hydrogel-sponge composite with the addition of FGF2 would greatly promote early osteogenic cell proliferation into scaffold and, subsequently, bone augmentation. The aim of this in vivo histological study was to examine whether the addition of FGF2 to collagen composite scaffold promotes bone formation in cranial bone defects in rats.

MATERIALS AND METHODS

Preparation of collagen hydrogel and sponge

Collagen hydrogel was prepared from atelocollagen powder (Koken, Tokyo, Japan) by adding 5 mM HCl, 1 mM L(+)-ascorbic acid and 0.1 mM $CuCl_2$, and adjusted to a final concentration of 1.5%. Collagen sponge was provided by Olympus Terumo Biomaterials Corp. (Tokyo, Japan), which had been prepared as described previously[15, 18]. Atelocollagen in a dilute HCl solution (0.3%; pH 3) was neutralized by adding concentrated phosphate buffer in NaCl to a final concentration of 0.1% collagen, 30 mM Na_2HPO_4 and 0.1 M NaCl. This collagen solution was incubated at 37°C for 4 h. The resulting fibrous precipitate was referred to as fibrillar collagen (FC). Heat-denatured collagen (HAC) was prepared from atelocollagen in a dilute HCl solution by heating the preparation to 60°C for 30 min. A composite of FC (0.3%) and HAC (1%) was prepared by mixing the two at a ratio of 9:1 (w/w), respectively. This composite material was adjusted to a final concentration of 4% and was made in the form of a sponge by lyophilization at -30°C. The sponge was dehydrothermally cross-linked at 110°C for 2 h and used as a collagen sponge. In this study, 72 pieces of collagen sponge of size 6×6×3 mm were used.

FGF2 / Collagen hydrogel-sponge construct

FGF2 solution (Fiblast spray® 500, Kaken Pharmaceutical Co., Tokyo, Japan) was added to the collagen hydrogel. For collagen hydrogel penetration into the sponge, collagen sponges were immersed into FGF2-loaded collagen hydrogel at 6°C for 1 week. FGF2/Collagen composite consisted of two groups: one group containing a loading volume of 3 μg FGF2 per piece and the other containing 15 μg FGF2 per piece. As a control, collagen sponges were immersed into only the collagen hydrogel.

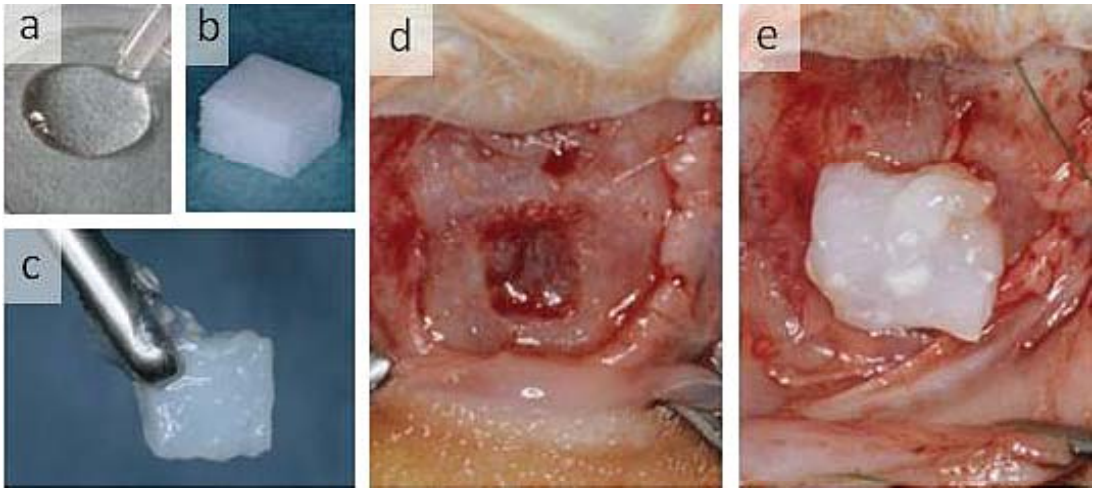


Fig. 1
a) Collagen hydrogel. b) FC-HAC sponge. c) FGF2-loaded collagen gel-sponge composite scaffold. d) Bone defect created in the cranial bone in rat. e) FGF2-loaded collagen gel-sponge composite implanted into the bone defect.

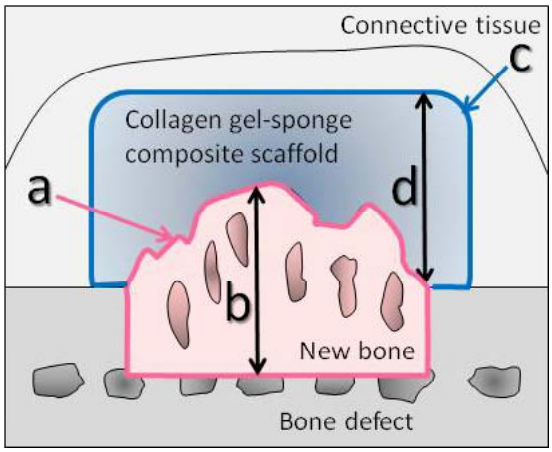


Fig. 2
Histomorphometric measurement regions.
a New bone area.
b New bone height.
c Residual scaffold area.
d Residual scaffold height.

Surgical procedure

The experimental protocol followed institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No. 08-0301). Seventy-two 10-week-old male Wistar rats weighing 300~330 g were given general anesthesia by inhalation of diethyl ether and intraperitoneal injections of 0.6 ml/kg sodium pentobarbital (Somnopenthyl, Kyoritsu Seiyaku, Tokyo, Japan), as well as a local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine (Xylocaine Cartridge for Dental Use, Dentsply-Sankin K.K. Tokyo, Japan).

The skin was incised and the periosteum of the calvarium was ablated. A bone defect sized 4×4×0.2 mm was created in front of the sagittal suture in the cranial bone using a rotating round bur under water irrigation (Fig. 1-4). Subsequently, rats were divided into three groups; FGF2-loaded collagen hydrogel-sponge composite (loading dose: 0, 3 and 5 μg) was implanted into the bone defect, and the groups were labeled F0, F3 and F15, respectively (Fig. 1-5). Skin flaps were sutured (Softretch 4-0, GC, Tokyo, Japan) and tetracycline hydrochloride ointment (Achromycin Ointment, POLA Pharma, Tokyo, Japan) was applied to the wound.

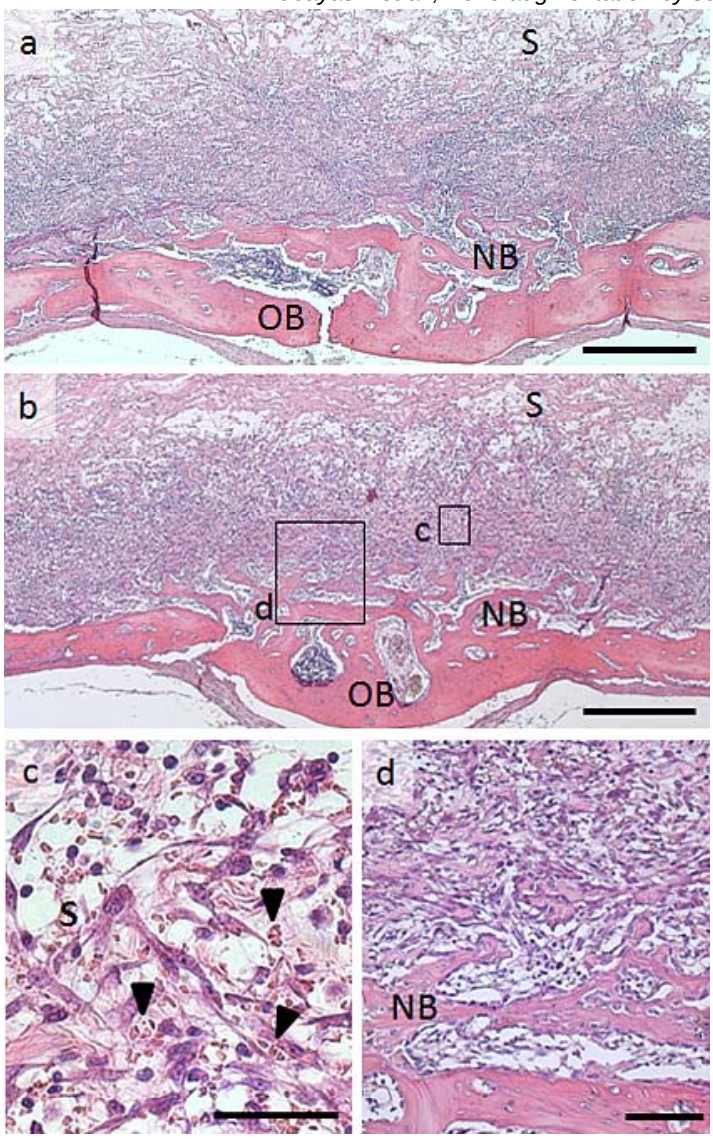


Fig. 3
Histological findings of groups F3 and F15 at 1 week post-surgery (HE staining). a) Group F3. b) Group F15. Numerous fibroblastic cells are present between the original cranial bone and the collagen scaffold. OB, original bone; NB, new bone; S, collagen gel-sponge composite scaffold; bar, 500 μm. c) Higher magnification of the framed area in c of b. Numerous blood vessels (▲) are recognized surrounding the connective tissue. Inflammatory cells are scattered. S, collagen gel-sponge composite scaffold; bar, 100 μm. d) Higher magnification of the framed area in d of b. New bone is composed of osteocytes and trabeculae lined by osteoblastic and osteoclastic cells. NB, new bone; bar, 25 μm.

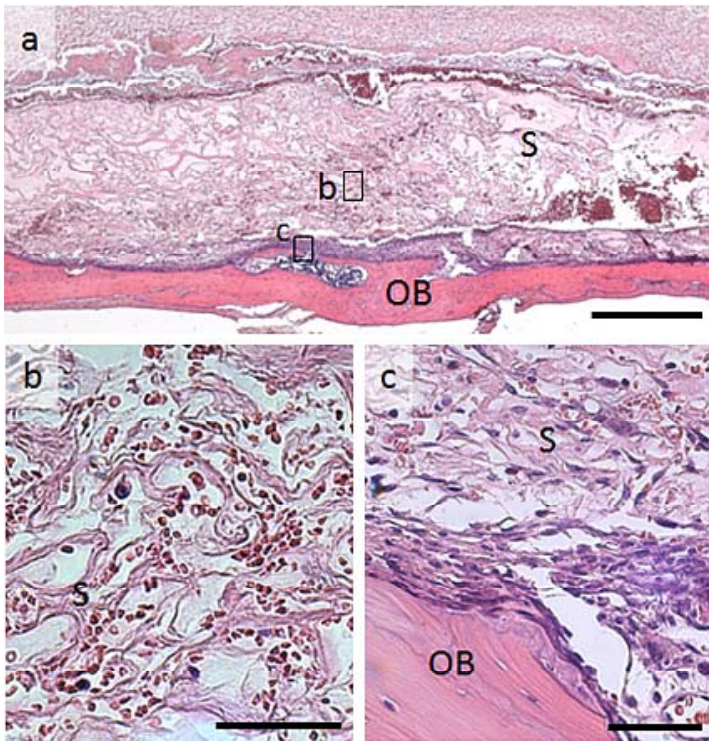


Fig. 4
Histological findings of group F0 at 1 week post-surgery (HE staining). a) Collagen composite scaffold remains present on the bone defect. S, collagen gel-sponge composite scaffold; bar, 50 μm. b) Higher magnification of the framed area in b of a. Fibroblast-like cells not observed in the scaffold. OB, original bone; S, collagen gel-sponge composite scaffold; bar, 50 μm. c) Higher magnification of the framed area in c of a. Infiltration of fibroblast-like cells observed in the surface layer of the scaffold. S, collagen gel-sponge composite scaffold; bar, 50 μm.

Histological procedure

Rats were killed using an overdose of diethyl ether. Specimens were collected from the wound 1, 2 and 5 weeks post-surgery. The tissue blocks including the cranial bone and surrounding soft tissue were fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded along the frontal plane in paraffin wax. Five-micrometer-thick sections were serially prepared and stained with hematoxylin-eosin (HE).

Histomorphometric analysis

Three of the stained sections were selected for histomorphometric measurements; one was approximately in the central area of the defect and the other two were at 300 μm from either side of the central area. Microscope images were assessed for new bone area, new bone height, residual scaffold area and residual scaffold height using image analysis software (ImageJ 1.41, National Institutes of Health, Bethesda, MD, USA) (Fig. 2).

Statistical analysis

The means and standard deviations of each parameter were calculated for each group. Differences among the groups were analyzed using the Two-way ANOVA / Bonferroni post-test. P-values <0.05 were considered statistically significant. All statistical procedures were performed using a software package (SPSS 11.0, SPSS Japan, Tokyo, Japan).

RESULTS

Histological observations

One week after operation

In groups F3 and F15, new connective tissue containing numerous fibroblastic cells was demonstrated between the original cranial bone and the collagen scaffold (Fig. 3-a, 3-b). A large number of fibroblastic cells and blood vessels had infiltrated the inner side of the collagen composite scaffold (Fig. 3-c). Bone formation was evident in the bone defect, and new bone was composed of osteocytes and trabeculae that were lined with osteoblastic and osteoclastic cells (Fig. 3-d). In group F0, although the collagen composite scaffold was retained on the bone defect (Fig. 4-a), infiltration of fibroblast-like cells into the collagen composite scaffold was insufficient when compared to the FGF2 groups (Fig. 4-b). New bone in group F0 was only slightly formed at the bottom of the artificial defect (Fig. 4-c). In all groups, inflammatory cells were scattered in the tissue around the new bone and around remnants of the collagen composite scaffold.

Two weeks after operation

In groups F3 and F15, bone formation was advanced in the bone defect (Fig. 5-a, 5-d). New trabecular bone contained numerous osteocytes, osteoblasts and osteoclasts (Fig. 5-b, 5-c, 5-e). Bone stroma that was occupied with marrow cells and osteoclastic cells was more developed in group F15 than in group F3. In all of the specimens where new bone was augmented above the original bone level, the bone cavity was almost completely repaired by woven bone. Cell- and blood vessel-rich connective tissue frequently encapsulated newly formed bone. Most of the collagen composite scaffold had disappeared. A large number of fibroblastic cells were present in remnant composite scaffold around the newly formed bone. In group F0, collagen composite scaffold frequently remained (Fig. 6-a, 6-b). New bone formation was observed in the bone cavity in group F0; however, the volume was low compared to the FGF2 groups (Fig. 6-c). In all groups, inflammatory cells were rarely seen around the bone or around remnants of the collagen composite scaffold.

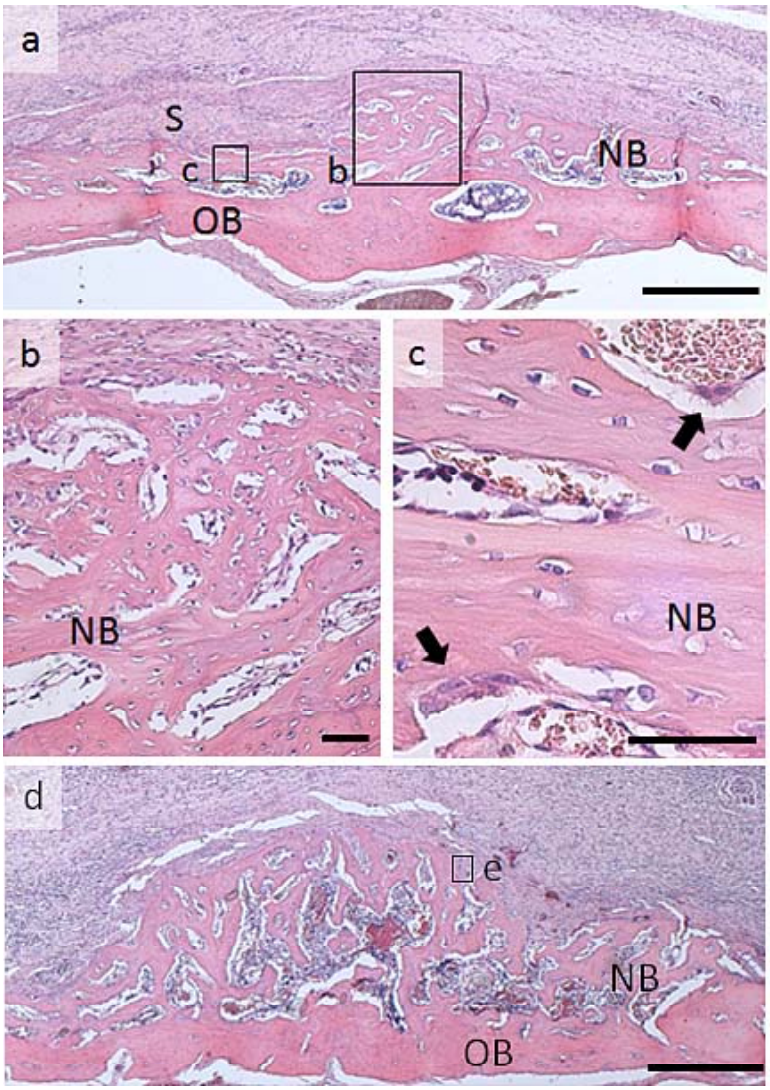


Fig. 5
Histological findings of group F3 (a-c) and group F15 (d, e) at 2 weeks (HE staining). a) New bone formation is evident. OB, original bone; NB, new bone; S, collagen gel-sponge composite scaffold; bar, 500 μm. b) Higher magnification of the framed area in b of a. New bone consists of trabecular bone. NB, new bone; bar, 50 μm. c) Higher magnification of the framed area in c of a. New bone contains numerous osteocytes and osteoclasts (arrows). NB, new bone; bar, 50 μm. d) A large amount of new bone formation is evident. OB, original bone; NB, new bone; bar, 500 μm. e) Higher magnification of the framed area in e of d. Osteoclasts are indicated (arrows). NB, new bone; bar, 50 μm.

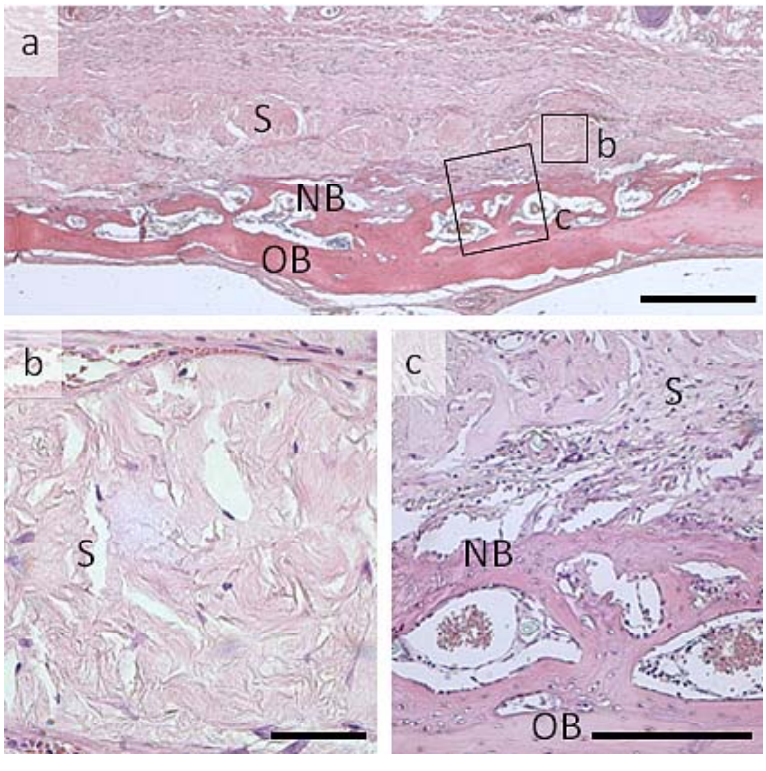


Fig. 6
Histological findings of group F0 at 2 weeks (HE staining). a) Collagen composite frequently remains. OB, original bone; NB, new bone; S, collagen gel-sponge composite scaffold; bar, 500 μm. b) Inflammatory cells are rarely seen around remnants of collagen composite scaffold. S, collagen gel-sponge composite scaffold; bar, 50 μm. c) A small amount of new bone formation is observed in the bone cavity. OB, original bone; NB, new bone; S, collagen gel-sponge composite scaffold; bar, 50 μm.

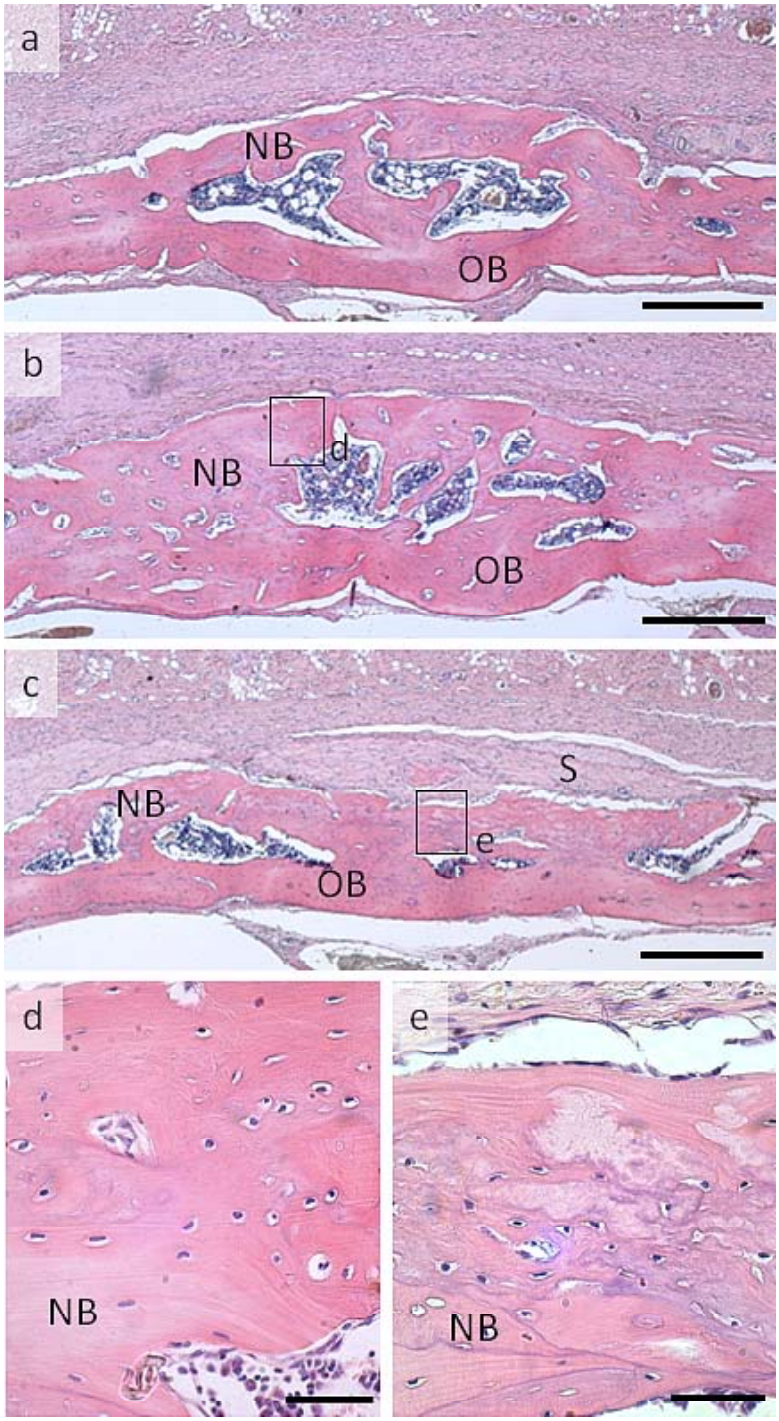


Fig. 7
Histological findings at 5 weeks (HE staining). a) Group F3, b) group F15. New bone augmentation is recognized and the thickness of the trabeculae is increased. OB, original bone; NB, new bone; bar, 500 μm. c) Group F0. New bone formation is observed. OB, original bone; NB, new bone; S, collagen gel-sponge composite; bar, 500 μm. d) Higher magnification of the framed area in d of b. Bone including osteocytes indicates a layered cement line. NB, new bone; bar, 50 μm. e) Higher magnification of the framed area in e of c. Part of the newly formed bone appears to be poor of osteocytes with an unclear cement line. NB, new bone; bar, 50 μm.

Five weeks after operation

In groups F3 and F15, bone trabeculae with a layered structure, i.e., cement line, and a distinct bone stroma were displayed (Fig. 7-a, b, d). Osteoblastic and/or osteoclastic cells were lined on the surface of the trabecula; however, the number of osteogenic cells was decreased compared to the 2-week-old specimens. Very little of the collagen composite scaffold was detected around the newly formed bone. In group F0, new bone was augmented and the thickness of trabeculae increased (Fig. 7-c). Part of the newly formed bone appeared as an unclear cement line, indicating immature tissue (Fig. 7-e). Collagen composite scaffold slightly remained.

Histomorphometric analysis

Bone formation was advanced when FGF2 was applied to the collagen gel-sponge scaffold. New bone area in groups F3 and F15 was significantly higher than that in group F0 at all experimental time points. Regarding the height of new bone, significant differences were detected between groups F0 and F15 at all stages, and between groups F0 and F3 at 2 weeks. In addition, significant differences were found between the FGF2 application groups at 1 and 2 weeks after surgery. There was an FGF2 dose-dependent increase in the amount of new bone formed.

At 1 week, the residual collagen scaffold area in groups F3 and F15 was significantly greater than that in group F0. However, at 2 weeks, the residual scaffold area was dramatically decreased in groups F3 and F15 compared to group F0, and there was a significant difference between groups F0 and F3. At 5 weeks, the collagen composite scaffold was rarely demonstrated and there were no significant differences among the groups. In the residual scaffold height, at 1 week, group F0 showed less height compared to groups F3 and F15. All groups had low scaffold volume at 2 and 5 weeks (Table 1).

DISCUSSION

The present study focuses on bone formation by the combination of FGF2 and collagen gel-sponge composite scaffold in cranial bone defects in rats. The results suggest that collagen gel-sponge composite with FGF2 can be used as a scaffold for bone tissue engineering.

Histological findings in this study revealed that FGF2-loaded collagen scaffold promptly enhances proliferation of osteoblastic and fibroblastic cells into the collagen scaffold, and significantly stimulates bone augmentation compared to composite scaffold without FGF2. We speculate that implanted FGF2 regulates the proliferation and differentiation of osteogenic cells derived from bone marrow, original bone and periosteum of rat, resulting in osteogenesis. Pitatu *et al*.[19] reported that rat stromal bone marrow cells treated with FGF2 in culture increased cAMP responsiveness, alkaline phosphatase

Table 1 Histomorphometric measurements after surgery (mean±SD)

		Group F0	Group F3	Group F15
New bone area (mm^2)	1 week	0.02±0.02	0.38±0.28[a]	0.52±0.23[a]
	2 weeks	0.23±0.23	0.97±0.48[a]	1.31±0.51[a]
	5 weeks	0.83±0.33	1.50±0.45[a]	1.42±0.58[a]
New bone height (mm)	1 week	0.09±0.07	0.19±0.10	0.36±0.11[ab]
	2 weeks	0.14±0.08	0.37±0.13[a]	0.60±0.18[ab]
	5 weeks	0.37±0.13	0.45±0.05	0.62±0.18[a]
Residual scaffold area (mm^2)	1 week	5.65±2.49	8.47±3.89[a]	7.93±2.14[a]
	2 weeks	4.52±2.74	1.01±1.05[a]	1.67±1.84
	5 weeks	0.84±1.13	0.17±0.27	0.27±0.47
Residual scaffold height (mm)	1 week	1.33±0.38	1.60±0.54[a]	1.75±0.43[a]
	2 weeks	0.58±0.27	0.58±0.27	0.62±0.42
	5 weeks	0.25±0.26	0.13±0.17	0.15±0.20

[a]Statistical difference compared to group F0 ($p<0.05$)
[b]Statistical difference compared to group F3 ($p<0.05$)

activity, osteocalcin levels, Ca^{2+} deposition and mineralized-like tissue formation.

Furthermore, during the early observation periods, many blood vessels in connective tissue in the neighboring implanted collagen scaffold were detected in the groups receiving FGF2 compared to group F0, thus suggesting that angiogenesis was facilitated by the application of FGF2. Norrby[20] and Kodama *et al*.[21] reported that FGF2 caused angiogenesis in a dose-dependent manner in rats. Fujimura *et al*.[22] reported that the amount of bone regeneration at the implant site was affected by vascularization in tissue and by oxygen partial pressure. Therefore, newly formed bone in the FGF-receiving site might be associated with the level of vascularization by FGF2.

Aspenberg *et al*.[23] reported in a study using a cellulose gel carrier system in rats that FGF2 increased the amount of bone formation in a dose-dependent manner. We also found that FGF dose-dependently increased bone induction. The new bone area in group F15 at 1 and 2 weeks was greater than that in group F3, and new bone height was significantly higher than in group F3. On the other hand, the osteogenic effect of FGF2 at high doses was dose-dependent but in a reverse manner[24]. The decision of what optimal FGF2 dose to use is an important aspect to be verified in the future.

Many osteoclastic cells were observed in histological sections of the FGF2-applied groups, especially in group F15. In general, this is known as a coupling process wherein osteoblasts and osteoclasts conserve the same spatial and temporal connection between each other. Osteoclastic potential plays a significant role in bone maturation in bone tissue engineering. Gong *et al*.[25] reported that bone maturation was not demonstrated in rats in the long term by bisphosphonate administration, which inhibited osteoclast-mediated resorption. In this examination, 5-week specimens in group F0 revealed unclear lamellar structures of new bone, suggesting that immature bone was formed. Therefore, bone remodeling related to osteoclasts may be stimulated by FGF2 at a relative high dose in this examination. In bone marrow-derived cells of ovariectomized rats, FGF2 stimulated the expression of receptor activator of nuclear factor kappa B ligand and cathepsin K, and differentiation of tartrate-resistant acid phosphatase-positive cells[26].

At the late stage, bone formation also occurred in group F0, which did not receive FGF2, suggesting that type I collagen matrix in the collagen composite scaffold was able to stimulate cell differentiation and tissue growth. Mizuno *et al*.[27] and Miyamoto *et al*.[28] also indicated that bone marrow-derived cells could differentiate into osteoblasts in culture with collagen gel. They reported that markers of the osteoblastic phenotype, such as alkaline phosphatase, bone sialoprotein and osteocalcin, of rat bone marrow stromal cells were stimulated by 3-D cultures containing type I collagen gel. Implanted collagen hydrogel and sponge containing heat-denatured collagen possessed high biocompatibility and degradability in the body. Consequently, the early exchange of space between degrading collagen with no cytotoxity and newly formed bone tissue, i.e., tissue-replacing ability, might occur and guide bone augmentation with synergistic effects to FGF2, after implantation of the collagen hydrogel-sponge composite.

It has been reported that collagen hydrogel possesses water absorption properties so that the collagen hydrogel can hold water in a 20- to 200-fold range of empty weight[15]. Although decortica-

tion was performed in the cranial bone in this study, the regenerative space may have been filled with tissue interstitial fluid and bone marrow exudate containing several growth factors that promote tissue healing[29]. It seems that water-absorbable scaffold may have an advantage in wound healing.

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