

Effect of Control-released Basic Fibroblast Growth Factor Incorporated in β-Tricalcium Phosphate for Murine Cranial Model

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**Background:** β-Tricalcium phosphate (β-TCP) is used clinically as a bone substitute, but complete osteoinduction is slow. Basic fibroblast growth factor (bFGF) is important in bone regeneration, but the biological effects are very limited because of the short half-life of the free form. Incorporation in gelatin allows slow release of growth factors during degradation. The present study evaluated whether control-released bFGF incorporated in β-TCP can promote bone regeneration in a murine cranial defect model.

**Methods:** Bilateral cranial defects of 4 mm in diameter were made in 10-week-old male Sprague-Dawley rats treated as follows: group 1, 20 μl saline as control; group 2, β-TCP disk in 20 μl saline; group 3, β-TCP disk in 50 μg bFGF solution; and group 4, β-TCP disk in 50 μg bFGF-containing gelatin hydrogel (n = 6 each). Histological and imaging analyses were performed at 1, 2, and 4 weeks after surgery.

**Results:** The computed tomography value was lower in groups 3 and 4, whereas the rate of osteogenesis was higher histologically in group 4 than in the other groups. The appearance of tartrate-resistant acid phosphate–positive cells and osteocalcin-positive cells and disappearance of osteopontin-positive cells occurred earlier in group 4 than in the other groups.

**Conclusions:** These findings suggest that control-released bFGF incorporated in β-TCP can accelerate bone regeneration in the murine cranial defect model and may be promising for the clinical treatment of cranial defects. (Plast Reconstr Surg Glob Open 2014;2:e126; doi: 10.1097/GOX.0000000000000063; Published online 26 March 2014.)
tion, scaffold materials are important to support bone regeneration in the presence of bFGF under clinical conditions. The present study evaluated the efficacy of our new material for bone regeneration, β-TCP incorporating bFGF-impregnated AGHMs, in a murine cranial defect model.

MATERIALS AND METHODS

Preparation of Control-released bFGF-impregnated AGHMs

AGHMs with a mean diameter of 59.4 ± 19.5 μm were prepared from gelatin (Nitta Gelatin, Osaka, Japan) as described previously. AGHMs with 95.2% water content, which degrade in approximately 14 days, were used. To obtain bFGF-impregnated AGHMs, 50 μg bFGF (human recombinant bFGF, Kaken Pharmaceutical, Tokyo, Japan) in 10 μl phosphate-buffered saline (PBS) was dropped onto 1 mg of dried AGHMs and mixed. Similarly, only 50 μg bFGF in 10 μl PBS without gelatin was prepared as a control.

Experimental Design

All procedures were carried out in accordance with the Japanese Association for Laboratory Animal Science Guidelines on the Care and Use of Animals and an animal study protocol approved by Juntendo Casualty Center Animal Care and Use Committee. A total of 36 male Sprague-Dawley rats weighing 310–330 g (10 weeks old) were used in this study. The rats were anesthetized with 3.0% halothane in 50% N2O/50% O2. Bilateral cranial defects of 4 mm in diameter were made with a trephine bar, without injuring the underlying dura mater. The cranial defects were repaired by cranioplasty using β-TCP disks of 4 mm in diameter and 1.5 mm in thickness (Olympus Biomaterial, Tokyo, Japan). The porosity of the block was 75% and the pore size was 100–400 μm. Bilateral cranial defects without gelatin was prepared as a control.

β-TCP disks in 50 μg bFGF solution (group 3) and β-TCP disk in 50 μg bFGF-impregnated AGHMs (group 4) (n = 6 for each group).

Quantitative Analysis of β-TCP with Three-dimensional Computed Tomography

β-TCP resorption was measured as the computed tomography (CT) value. CT scans were obtained at 1, 2, and 4 weeks after the operation at a slice thickness of 0.625 mm using a Light Speed VCT (GE Healthcare Japan, Hino, Tokyo, Japan). DICOM data of the tomogram were analyzed using the medical image viewer OsiriX on an axial view of the implanted β-TCP, and the CT value at the volume of interest of 0.12 cm² was measured with this software.

Assessment of Bone Regeneration

Three rats from each group were euthanized with diethyl ether at 1, 2, and 4 weeks after the operation. The bilateral parietal bones were harvested and immersed in 15% formaldehyde. Bone specimens were decalcified in 10 wt% ethylenediamine tetraacetic acid solution at 4°C for 3 days, embedded in paraffin, and cut into 3.5-μm-thick sections. Each section was stained with hematoxylin and eosin for examination under light microscopy. The rate of bone regeneration in the area of the β-TCP disk was measured using the image analysis software KS400 (Carl Zeiss Vision GmbH, Aalen, Germany).

Tartrate-resistant Acid Phosphate Staining

To detect osteoclasts, tartrate-resistant acid phosphate (TRAP) staining was carried out according to the Kawahara method with the TRAP stain kit (Wako, Osaka, Japan). The quantity of positive staining in each field was measured as the number of TRAP-positive cells per body.

Immunohistochemical Staining for Osteoblasts

To examine the differentiation stages of bone-forming cells, consecutive deparaffinized specimens (3.5 mm) were prepared from the tissue samples. After deparaffinization and dehydration with xylene and ethanol, tissue endogenous peroxidases were blocked by treatment with 3% hydrogen peroxide in methanol at room temperature for 10 minutes, and nonspecific reactions were blocked by treatment with 1:20 diluted goat serum at room temperature for 20 minutes. The sections were incubated with 1:500 diluted mouse anti-osteocalcin monoclonal antibody (Abcam, Tokyo, Japan) and 1:1000 diluted rabbit anti-osteopontin polyclonal antibody (Abcam) separately for 16 hours at 4°C and then washed with PBS. For the immunoreaction, mouse immunoglobulin G polyclonal second antibody (biotin) was used and signals were visualized using a DAB (Innovex Biosciences, Richmond, Calif.). Counterstaining of the nuclei was performed with hematoxylin.

Statistical Analysis

All values are presented as the mean ± standard error of the mean. Statistical significance was determined as P < 0.05 using the Turkey-Kramer multiple comparison test.
RESULTS

Quantitative Analysis of β-TCP with Three-dimensional Computed Tomography

Groups 3 and 4 showed significantly lower mean CT value compared with group 2 at 2 and 4 weeks after operation ($P < 0.05$) (Fig. 1). Significant differences were seen between group 3 (1 week: 1297.5 ± 41.19 Hounsfield unit (HU), 2 weeks: 1215.25 ± 20.40 HU, 4 weeks: 1180.50 ± 58.93 HU) and group 4 (1 week: 1297.0 ± 66.09 HU, 2 weeks: 1196.50 ± 48.64 HU, 4 weeks: 1179.75 ± 70.74 HU) vs group 2 (1 week: 1484.5 ± 181.03 HU, 2 weeks: 1693.75 ± 107.82 HU, 4 weeks: 1655.00 ± 124.48 HU) at 2 and 4 weeks ($P < 0.05$). bFGF demonstrated similar findings in groups 3 and 4, with the CT value decreasing from 1 week after operation. On the other hand, group 2 showed no remarkable change in CT value at each time point (Fig. 2).

Assessment of Bone Regeneration

Bone formation occurred from both the horizontal margin of the defect and the underlying dura mater into the β-TCP beginning at 1 week and gradually developed through 2 to 4 weeks in group 4 (Fig. 3). Quantitative analysis showed statistically significant differences between group 4 (1 week: 0.70% ± 0.60%, 2 weeks: 9.49% ± 2.93%, 4 weeks: 23.35% ± 9.43%) vs group 1 (1 week: 0.00%, 2 weeks: 0.48% ± 0.09%, 4 weeks: 6.84% ± 1.23%), group 2 (1 week: 0.00%, 2 weeks: 6.11% ± 0.74%, 4 weeks: 13.46% ± 5.61%), and group 3 (1 week: 0.00%, 2 weeks: 0.61% ± 0.69%, 4 weeks: 10.53% ± 5.06%) at 2 and 4 weeks ($P < 0.05$) (Fig. 4).

TRAP Staining

TRAP-positive multinucleated cells, which had attached to the β-TCP, were observed at 1 to 2 weeks after operation in all groups except at 1 week in group 1 (Fig. 5A). TRAP-positive cells per unit area were higher at 1 and 2 weeks in group 4 (1 week: 9.5 ± 6.24, 2 weeks: 61.50 ± 32.70) than in group 1 (1 week: 0, 2 weeks: 1.75 ± 1.70), group 2 (1 week: 2.0 ± 1.63, 2 weeks: 32.25 ± 7.50), and group 3 (1 week: 6.5 ± 2.88, 2 weeks: 27.75 ± 13.74), although there was no statistically significant difference. Interestingly, however, TRAP-positive cells per unit area were lower at 4 weeks in group 4 (39.0 ± 10.95) than in group 2 (47.25 ± 9.46) and group 3 (61.33 ± 26.08) (Fig. 5B).

Immunohistochemical Staining for Osteoblasts

Osteopontin-positive cells appeared around small osteoid islands at 1 week only in group 4, but appeared around newly formed bone at 2 weeks in groups 2, 3, and 4. On the other hand, osteocalcin-positive cells appeared at 4 weeks only in group 4 (Fig. 6).

DISCUSSION

bFGF is a powerful angiogenic growth factor with strong effects for bone regeneration. However, daily administration is essential as the half-life of the free form of bFGF is less than 1 hour$^{13}$ and bFGF is water soluble, so is easily eliminated from the applied site by diffusion. Such a daily procedure is time-consuming and may result in patients discomfort and higher risk of infection.

Recently, a novel drug delivery system was designed that enables controlled release in vivo of either single or multiple growth factors by using...
gelatin hydrogel as a vehicle, which resulted in more effective growth factor therapy.\textsuperscript{14,15}\textsuperscript{125}I-labeled bFGF-impregnated gelatin hydrogel injected subcutaneously in a murine model was clearly shown to be degraded in 2 to 4 weeks depending on the water content of the hydrogel without causing nonspecific inflammatory reaction and had induced neovascularization around the injected site.\textsuperscript{8,9,14,15} The present study used $\beta$-TCP as a bony scaffold for the murine cranial defect model. $\beta$-TCP, a biodegradable material with prominent osteoconductive properties, is widely used clinically as a bone graft substitute.\textsuperscript{16} $\beta$-TCP has been applied to bone defects occurring after operative procedures such as bone tumor resection, bone fracture correction, and maxillary sinus floor augmentation.\textsuperscript{16–18} As in previous studies, our results also showed that the bone regeneration rate in group 2 was higher than in group 1, suggesting that $\beta$-TCP accelerates bone formation in the cranial defect model. Three-dimensional computed tomography revealed that the radiolucency of $\beta$-TCP increased in rats treated with bFGF (groups 3 and 4) and the CT value decreased over time in groups 3 and 4 compared with group 2, suggesting that the administration of bFGF may accelerate the absorption of $\beta$-TCP.

The present study also used TRAP staining to identify any increase in cells, including osteoclasts, which are related to bone remodeling. The number of TRAP-positive cells was calculated to examine the process of disintegration and absorption of $\beta$-TCP. Bone absorption caused by TRAP-positive cells and conductive bone formation occurred in a complicated manner involving both resorption of $\beta$-TCP and the remodeling phenomenon.\textsuperscript{19} Multinucleated giant cells or macrophages also adhered to implanted $\beta$-TCP in animals, indicating that these cells are central in biore sorption of $\beta$-TCP.\textsuperscript{20–25} Moreover, multinucleated cells, TRAP-positive cells, or macrophages are considered to be in contact with the $\beta$-TCP surface from 2 to 4 weeks after implantation, and these cells resorb $\beta$-TCP based on both light and electron microscopy observations.\textsuperscript{20–25} By contrast, the involvement of osteoclasts or biore sorptive cells at the early stage of biore sorption of $\beta$-TCP remains unclear. The present study detected multinucleated TRAP-positive cells around the $\beta$-TCP at 1 week af-
ter implantation in the rat skull, which was earlier than in previous studies. Two different biological resorption pathways have been proposed: a solution-mediated process and a cell-mediated process. The appearance of TRAP-positive cells at the early stage suggests that cell-mediated disintegration of β-TCP plays a central role in the bioresorption of β-TCP. The present study found that the number of TRAP-positive cells remarkably increased earlier in group 4 than in the other groups, and these cells appeared adjacent only to β-TCP, suggesting that absorption of β-TCP and leading to the remodeling phenomenon was accelerated by the control-released bFGF.

Membranous ossification is a generally accepted process in the development of the cranium. Although bFGF is important in stimulating bone formation and promotes enchondral ossification during the early stage of fracture healing of long bone, our study found bone formation from both the horizontal margin of the residual cranial bone and the underlying dura mater from 1 through 4 weeks in group 4, suggesting that membranous ossification was promoted rather than enchondral ossification.

The specific mechanism of the signaling pathway induced by bFGF for osteogenesis remains to be clarified. A previous study has shown that bFGF seems to be a more potent mitogen for fibroblasts, immature osteoblasts, and mesenchymal cells than for differentiated osteoblasts. Another study has

**Fig. 5.** A, TRAP-positive multinucleated cells that adhered to β-TCP were observed at 1 week after implantation in group 4. The number of TRAP-positive cells had increased at 2 weeks compared with 1 week, but decreased at 4 weeks. B, Quantitative analysis of the number of TRAP-positive cells per β-TCP block. TRAP-positive cells appeared in the early stage in group 4 compared with groups 2 and 3, although there was no statistically significant difference.

**Fig. 6.** Immunohistochemical staining for osteopontin and osteocalcin of the specimens in group 4. Fibroblast-like cells in bone marrow were positive for osteopontin at 1 week. However, no osteopontin-positive cells were detected around the newly formed bone at 4 weeks. On the other hand, no cells expressed osteocalcin at 1 week. Osteocalcin-positive cells were abundant in the newly formed bone at 4 weeks. This phenomenon occurred earlier in group 4 than in the other groups (data not shown).
also demonstrated that the direct effect of bFGF on bone formation seems to be either the stimulation of mesenchymal cell proliferation or the recruitment of such cells from less differentiated progenitor cells.28 Because the findings of our study are similar to these reports, bone formation induced by bFGF may result from direct stimulation followed by differentiation of mesenchymal cells and adjacent precursor cells toward osteogenesis. Alternatively, bFGF possibly acts through stimulation of local production of other factors including transforming growth factor-β (TGF-β) and bone morphogenetic protein, which form a serial cascade of bone formation. Further studies are required to explore the specific mechanism through which bFGF induces bone formation.

In the present study, osteopontin-positive cells appeared around small osteoid islands earlier in group 4 than in the other groups. Previous studies have demonstrated that osteopontin-positive cells are hard to identify as osteoblasts, osteoclasts, or another cell type without using immunohistochemical staining for the ED1 protein.29 However, we considered that these mononuclear cells were osteoblasts because they adhered to newly formed bone and not to β-TCP in contrast to the TRAP-positive cells. On the other hand, osteocalcin-positive cells were found around the newly formed bone only at 4 weeks in groups 2 and 3, 2 weeks later than in group 4, which was treated with control-released bFGF. Osteocalcin is responsible for calcium ion binding and is believed to be a marker of the late stage of osteoblastic differentiation.29 The present findings suggest that control-released bFGF acted at the β-TCP implantation site in the same manner as in normal bone and accelerated osteoblastic differentiation.

Numerous studies have demonstrated that the autologous stem cells derived from bone marrow or adipose tissue can induce osteogenesis both in vitro and in vivo.30,31 Such strategies are promising but may have some drawbacks including donor-site morbidities and high cost for cell processing and isolation particularly in clinical situations. By contrast, our method simply uses growth factor and biomaterial scaffold, both of which are already commercially available.

Other growth factors may be considered to promote osteogenesis instead of bFGF. In particular, autologous platelet-rich plasma (PRP) might be a good candidate. PRP contains several growth factors including platelet-derived growth factor, vascular endothelial growth factor, epithelial growth factor, and TGF-β,32,33 which all strongly enhance bone regeneration. However, similar to bFGF, the effect of the free form of PRP disappeared within a few days in vivo.33 As PRP is also easy to obtain from the peripheral blood of the patients, controlled release of PRP has good potential for bone regeneration and remodeling. Further study is needed to explore which growth factors and scaffolds are optimal for bone regeneration.

**CONCLUSIONS**

The present study showed that control-released bFGF incorporated in β-TCP implanted into the murine cranial defect model resulted in the regeneration of bone and the remodeling phenomenon similar to that seen with normal bone. This study also showed that β-TCP resorption and bone regeneration are promoted by control-released bFGF in the murine cranial defect model. Such materials are thought to be one of the promising bone substitutes for the clinical treatment of cranial defects.

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