Changes in Bone Regeneration by Trehalose Coating and Basic Fibroblast Growth Factor after Implantation of Tailor-Made Bone Implants in Dogs

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ABSTRACT: In this study, we aimed to determine the effect of trehalose coating and the optimal dose of basic fibroblast growth factor (bFGF), an osteoinductive protein, loaded onto tailor-made bone implants for implant-induced bone formation in vivo. We fabricated tailor-made α-tricalcium phosphate bone implants (11 mm diameter with 2 parallel cylindrical holes). bFGF 0, 1, 10, 100 or 200 µg/implant was incorporated into implants with and without a trehalose coating, and these were subsequently implanted into dogs to correct temporal bone defects of the same size and shape. Four weeks after implantation, we analyzed the bone implants and surrounding tissues by using micro-computed tomography imaging and histological analyses, as well as gross evaluation. No significant difference in new bone formation was observed between implants with and without a trehalose coating at any of the bFGF doses. Bone implants with 100 and 200 µg bFGF showed significantly more new bone formation at the implant site and within the cylindrical holes of the implants than those without bFGF (P<0.05). However, heterotopic bone formation on the skull near the implant was observed in the group that received 200 µg bFGF. These results suggest that 100 µg bFGF is the optimal dose for this implant in dogs, and that the trehalose coating may not be necessary in vivo, probably due to the presence of blood proteins and electrolytes at the implant site.

KEYWORDS: bFGF, bone graft, in vivo, osteoinductive, tailor-made bone implant.


Autologous bone grafts are considered the “gold standard” for repairing bone defects. However, problems, such as surgical invasion at the donor site, and limited sources and morbidity of bones, have also been reported [1, 24]. To resolve these problems, artificial bones made of hydroxyapatite or tricalcium phosphate (TCP) have been developed as an alternative to bone grafts [11, 17]. Recently, we developed tailor-made bone implants fabricated from α-TCP using three-dimensional (3D) ink-jet printer based on CT data of bone defects. These implants closely matched the shape of the bone defect and could easily be implanted at the defect site, because it was fabricated from CT data of patients. In addition, the internal structure of the implant can be freely designed; for example, a cylindrical hole can be added for improved osseointegration [4, 13].

Basic fibroblast growth factor (bFGF), also referred to as FGF-2, is known to promote proliferation and differentiation of osteoblastic cells in vitro [8, 9] and to induce earlier bone healing at the defect site in vivo [5, 6]. A single injection of bFGF increases bone mass [29], but an injection of bFGF alone into bone defects was found to induce significantly less bone formation than bFGF injected in combination with a carrier [26]. Therefore, various carriers were studied to improve the efficacy of bFGF by controlling its release rate [2, 10].

Calci-um phosphate is known to have low bFGF kinetics under physiological conditions, possibly due to its high affinity for bFGF, which limits its capacity to act as a carrier [16, 21]. Trehalose, a natural disaccharide, is widely used as a cryoprotectant in clinical practice [7, 14]. Given the protective effects of trehalose on living systems, we previously studied the application of trehalose to the controlled release of bFGF from α-TCP in vitro, and we confirmed that bFGF was released from a trehalose-coated tailor-made bone implant made of α-TCP that retained its biological activity [3]. However, the efficacy of the trehalose-coated bone implant on in vivo bone formation was not clarified.

Compared with rodents, larger mammals may need larger doses of bFGF to promote bone regeneration. The optimal dose of bFGF is also influenced by the type of carrier used. Nakamura et al. reported that a single injection of 200 µg bFGF accelerated fracture healing in dogs [20], and that 200 µg bFGF combined with β-TCP also promoted bone regeneration in dogs [12]. In addition, a gelatin carrier administered along with 30 µg bFGF stimulated bone growth in dogs [18]. However, the optimal dose of bFGF in combination with tailor-made artificial bone to promote bone regeneration has yet to be clarified.

The purpose of this study was (1) to evaluate the efficacy of a trehalose-coated tailor-made bone implant on in vivo bone formation, and (2) to investigate the optimal dose of bFGF incorporated with the tailor-made implant for bone regeneration, by using a canine bone defect model.
MATERIALS AND METHODS

Preparation of implants: Implant fabrication. The tailor-made bone implants were fabricated according to the procedure described previously [4, 13]. Each implant consisted of a round disc shape (diameter, 11 mm; height, 4 mm), and the side aspect of the implant was inclined at a 7.5-degree angle. At the side of the implant, 2 parallel cylindrical holes (2 mm in diameter) were fashioned to improve osseointegration [13]. At the top of the implant, a mark (height, 1 mm; width, 1 mm; length, 11 mm) was created to indicate the direction of the cylindrical holes (Fig. 1).

Trehalose coating. The fabricated implants were subdivided into groups with and without a trehalose coating. The coating process was the same as that used in the previous study [3]. Trehalose was purchased from Hayashibara Corporation (Okayama, Japan). Briefly, the fabricated implants were immersed overnight in 5% trehalose solution at room temperature, then washed with distilled water and autoclaved at 121°C for 30 min.

bFGF incorporation. bFGF was provided by Kaken Pharmaceuticals (Tokyo, Japan). bFGF was diluted with deionized water to make 0.1, 1, 10 or 20 µg/µl solution. The bFGF solutions with different concentrations were then inserted into the side aspects and cylindrical hole inlets of the trehalose-coated or non-coated implants using a micropipette. The total dose of bFGF incorporated into each implant was 1, 10, 100 or 200 µg. For the control group, deionized water was inserted in the same manner.

Experimental animals. Ten female beagle dogs with a mean body weight of 9.4 kg (range, 8.3–10.7 kg), aged 1–5 years, were purchased from Nosan Corporation (Yokohama, Japan). All the animals were confirmed to be clinically healthy by physical examination, radiography, hematology and blood chemistry. The experiments were conducted under the Guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo (approval number: P07-028).

Surgical procedures: Atropine sulfate (25 µg/kg SC) was injected as a preanesthetic medication, followed by midazolam (0.1 mg/kg IV). Anesthesia was induced with propofol (6 mg/kg IV) and maintained with 1.0–2.5% isoflurane and oxygen after tracheal intubation. Cefazolam (20 mg/kg IV) was injected as a preoperative antibiotic. Administration of 5–20 µg/kg/hr fentanyl hydrate (used as an analgesic) was started with a syringe pump at the time of induction of anesthesia and was continued for 24 hr post-operatively.

During surgery, hyperventilation was continued using a ventilator to maintain EtCO₂ at about 30 mmHg and prevent brain edema. To prevent seizures after implantation, the dogs were injected with phenobarbital (2 mg/kg IM) and dexamethasone (1 mg/kg IV) immediately before the end of surgery.

A modified Oklund’s bone defect model was used in this study [23]. The head was clipped and aseptically prepared with chlorhexidine and 10% povidone-iodine. The midline of the head skin and subcutaneous tissues was incised, and then the bilateral temporalis muscles and fascia were carefully bluntly dissected from the temporal bone using a periosteal elevator. Four round bone defects of 11 mm in diameter were created using a perforator (Stryker Instruments, Kalamazoo, MI, U.S.A.) to avoid injury to the dura mater and surrounding peristeum. Two defects were located cranially and 2 caudally at 4 and 2 cm from the external occipital protuberance, respectively, and bilaterally 2 cm from the external sagittal crest. Four differently prepared implants were assigned to each of the defects in each of the dogs randomly (n=4; Fig. 2).

Routine closure of the surgical wound was performed. After surgery, buprenorphine (15 µg/kg IM) and cefazolam (20 mg/kg SC) were administered by injection twice daily for 3 days. To prevent seizures and brain edema after implantation, dexamethasone (0.5 mg/kg on the 1st day, 0.25 mg/kg on the 2nd day and 0.13 mg/kg on the 3rd day IV), mannitol (0.5 g/head IV) and phenobarbital (2 mg/kg IM) were administered for 3 days after implantation.

Computed tomography (CT): CT was conducted to evaluate new bone formation around the implant immediately after surgery and 2 and 4 weeks after implantation under sedation with midazolam (0.3 mg/kg IM) and medetomidine (20 µg/kg IM). A 4-channel multi detector CT system (Asterton TSX-021B; Toshiba, Tokyo, Japan) was used with 0.5 mm slice thickness under conditions of 120 kV and 180 mA.

Gross evaluation: Four weeks after implantation, the dogs were euthanized with an appropriate dose of potassium chloride under deep anesthesia with thiopental sodium at 30 mg/kg. The implant and surrounding tissues were grossly observed and carefully excised using a sagittal saw (Osada Electronics, Tokyo, Japan) for micro-CT scanning and histology.

Micro-CT: A desktop X-ray micro-CT system (SMX-90CT; Shimadzu, Kyoto, Japan) was used to evaluate regenerated bone in the excised tissues. The excised tissues were scanned by a micro-focus X-ray tube at 90 kV and 110 µA. The scanned data were reconstructed along z-axis to be parallel to the cylindrical holes in the implant, and observed change of the bone formation area within the holes and on
of the surrounding of the implant by using a micro-CT analysis program (TRI/3D-BON; RATOC, Tokyo, Japan).

**Histology:** Excised tissues were fixed with 10% neutralized formalin for 5 days and decalcified with 10% ethylenediaminetetraacetic acid (EDTA; WAKO, Tokyo, Japan) solution for 4 months. Decalcified tissues were trimmed parallel to the cylindrical holes and embedded in paraffin. The tissue block was cut into 7 µm-thick sections and stained with Masson’s trichrome. The area of regenerated bone, identified as mineralized area stained in blue within the cylindrical holes and around surrounded periosteum, was measured using image J software (US National Institutes of Health, Bethesda, MD, U.S.A.).

**Statistical analysis:** The mean values and standard deviations of the area of regenerated bone were calculated. One-way analysis of variance was performed, followed by Dunnett’s test using SPSS software (17.0; SPSS Institute, Cary, NC, U.S.A.). P values <0.05 were considered statistically significant.

**RESULTS**

No abnormal clinical signs were observed in any of the dogs during the entire observation period.

**Gross evaluation:** Figure 3 shows typical gross findings of the artificial bone in all groups at 4 weeks after implantation. All the implants were well seated and attached to the surrounding bone tissues without inflammation or infection. No difference in new bone formation or bone union was discernible between the groups with and without a trehalose coating. In the groups that received 100 and 200 µg, more new bone formation was observed in the surrounding periosteum than in the control group or the groups that received less bFGF. Heterotopic bone regeneration was found in the 200 µg bFGF group (Fig. 4).

**CT images:** Displacement and breakage of the implants were not observed on CT in any dogs during the entire observation period. Figure 5 shows the typical CT images for all groups at 0, 2 and 4 weeks after implantation. Two weeks after implantation, no changes in CT images were observed for any of the groups. Four weeks after implantation, new bone formation was observed in the cylindrical holes and at the interface between the implants and skull bones. The extent of this new bone formation was greater in the groups that received 100 and 200 µg bFGF than in the other groups. There were no significant differences between the groups with and without a trehalose coating.

**Micro-CT images:** No differences in new bone formation
were observed on the micro-CT images between the groups with and without a trehalose coating. In the groups that received 100 and 200 µg bFGF, massive mineralized tissues were apparent from the surrounding periosteum to the cylindrical holes, whereas in the groups that received 0, 1 and 10 µg bFGF, only scant mineralized tissue was present within the cylindrical holes (Fig. 6).

**Histological findings:** Figure 7 shows the histological findings for all groups. There appeared to be no difference in bone regeneration at any area of the implants for those with and without a trehalose coating. In the implants that contained 100 and 200 µg bFGF, massive regenerative bone tissues were formed from the surrounding periosteum to the cylindrical holes, as was shown by micro-CT imaging.

Figure 8 shows the area of regenerated bone for all groups. The area of regenerated bone tissues of the implant without trehalose coating group was 1.91 ± 2.25 mm² in 0 µg bFGF, 2.04 ± 0.63 mm² in 1 µg bFGF, 1.32 ± 0.70 mm² in 10 µg bFGF, 6.90 ± 6.51 mm² in 100 µg bFGF and 5.77 ± 5.07 mm² in 200 µg bFGF, respectively. That of the implant with trehalose coating group was 1.19 ± 1.03 mm² in 0 µg bFGF, 2.23 ± 0.80 mm² in 1 µg bFGF, 2.81 ± 1.83 mm² in 10 µg bFGF, 6.15 ± 1.98 in 100 µg bFGF and 9.31 ± 2.30 mm² in 200 µg bFGF, respectively. The area of regenerated bone in the implants with 1 and 10 µg bFGF showed no significances than that of the individuals that did not receive bFGF. In the implants with 100 and 200 µg bFGF, the area of regenerated bone was significantly higher compared with those that did not receive bFGF (P<0.05). There was no significant difference in the area of regeneration bone between the groups with and without a trehalose coating at all bFGF doses.

**DISCUSSION**

In this study, there appeared to be no difference in the extent of *in vivo* bone regeneration between implants with and without a trehalose coating, whereas we previously found that trehalose-coated implants released more bFGF *in vitro* than non-coated implants, probably due to the high affinity of α-TCP for bFGF [3]. We speculate the cause of this discrepancy as follows. When a non-coated implant is implanted *in vivo*, proteins or other blood components may non-specifically bind to the implant; this non-specific binding possibly may inhibit the binding of bFGF to the calcium phosphate, as occurs with the trehalose coating. Blood proteins, such as albumin, can easily bind to calcium phosphate [28]. Although the binding affinity of these proteins and bFGF to calcium phosphate is not known, they may displace bFGF bound to the implant. In addition, various electrolytes in the body fluid may interfere with Ca²⁺ dissolution from the implant. In the *in vivo* condition, negative electrolyte ions attached to implants may bind to Ca²⁺, and...
the dissolubility of Ca\(^{2+}\) may be increased. Therefore, bFGF bound to Ca\(^{2+}\) may be decreased through Ca\(^{2+}\) dissolubility.

bFGF has been reported to promote bone healing (even with a single injection of bFGF) in various bone fracture models of various animals [19, 20, 27]. The use of a bFGF in combination with a carrier may result in more potent bone regeneration than a single injection [22, 26]. For example, gelatin incorporating 30 µg bFGF was shown to produce more regenerated bone than gelatin alone in mandibular bone defects in dogs [18]. A collagen minipellet containing 0.15 µg bFGF has also been shown to increase bone regeneration [12]. However, the optimal dose of bFGF incorporated into the carrier may depend on the material from which the carrier is fabricated.

In this study, implants incorporating 100 or 200 µg bFGF showed significantly more bone regeneration than a control group without bFGF. There was no significant difference between dogs that received 100 and 200 µg bFGF in terms of both gross findings and histological evaluations. Moreover, heterotopically regenerated bone was observed in dogs that received implants with 200 µg bFGF. bFGF might have become supersaturated at the surface of the bone implant and flowed out to the heterotopic region of the skull in the 200 µg bFGF group. The outflow of bFGF to the heterotopic region may lead to extra new bone formation, which may be a hazard for the surrounding soft tissues and may elongate the time for bone remodeling to return the bone to its normal shape. In addition, excessive doses of bFGF may potentially inhibit osteoblast differentiation [15]. Taken together, our findings suggest that 100 µg bFGF may be optimal for the induction of appropriate bone regeneration when incorporated into a tailor-made bone implant of the specified size. Further investigations are required to determine the appropriate doses of bFGF for osteoinductive activity in bone defects of various sizes.

Histological analyses revealed that most of the regenerated bone was oriented from the periosteum around the defect sites. Given that the periosteum has bone lining cells that consist of the osteoprogenitor cells [25], the histological findings suggest that bFGF released from the implant mainly induces bone regeneration from sites rich in osteogenic cells.

In conclusion, we confirmed in vivo the biocompatibility and osteoinductivity of the bFGF-incorporated tailor-made implant; 100 µg bFGF was considered to be the optimal effective dose for incorporation.

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REFERENCES


