Fibroblast Growth Factor-2 Stimulates New Bone Formation on Allografts of Demineralized Bone Matrix in Segmental Bone Defects in Rabbits

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Abstract: We studied whether fibroblast growth factor-2 stimulates new bone formation on allografts of powdery demineralized bone matrix (DBM) in segmental bone defects of Japanese white rabbit ulnae. A 1.5 cm bone defect was created on the distal 1/3 of the rabbit ulnar diaphysis and grafted with a 50 mg DBM that had been saturated with 0, 20, or 200 μg of recombinant human fibroblast growth factor-2 (rhFGF-2) in 200 μl of saline solution. As for new bone formation at the bone defect, radiographic film 3 weeks after surgery revealed marked calcification to be more pronounced in the group with 200 μg of rhFGF-2 than in the group with DBM alone. Histologically, the use of 200 μg of rhFGF-2 resulted in abundant new bone formation as if to bridge both end-sides of the resected part. FGF-2 was found to be effective for early new bone formation in DBM grafting to the bone defect.

Key words: Fibroblast growth factor-2, Demineralized bone matrix, Bone formation, Segmental bone defects, Rabbit

INTRODUCTION

Autografts are currently the most commonly used bone graft material for orthopaedic surgery. However, the use of an autograft has limitations, including donor site morbidity, limited shapes and sizes of available grafts, and the potential for intraoperative and postoperative complications. In comparison, demineralized bone matrix (DBM) is advantageous in that there is no quantitative limit, the shape and size can be selected as needed, it has both osteoconductive and osteoinductive properties and it has little antigenicity. The usefulness of DBM has been reported in experiments using DBM implants in long bone defects in rodents, in which early new bone formation was noted.

However, several investigations have shown that the usefulness of DBM in primates is doubtful. We reported the effects of grafting with DBM during lengthening of the tibia in rabbits. The results were satisfactory, but callus formation was insignificant at three weeks after surgery.

Fibroblast growth factor-2 (FGF-2, basic FGF) acts as a potent mitogen for mesenchymal cells. In osteogenic tissues, FGF-2 is produced by osteoblasts and stored in the bone matrix, facilitating the proliferation of osteoblasts. It has been reported, using a fracture model of rat fibulae, that a single local injection of FGF-2 to fracture sites facilitates the formation of callus, and that a single local injection of FGF-2 in bone stimulates the formation of intraosseous bone in rabbits. However, there has been no report on the effectiveness of a sin-
gle local administration of FGF-2 on DBM grafting to segmental bone defects. In the present study, we examined whether new bone formation is stimulated by the combined use of DBM and FGF-2 compared with grafting DBM alone to segmental defects of rabbit ulna, and also whether powdery DBM can be used as a carrier for FGF-2.

**MATERIALS AND METHODS**

Thirty male Japanese white rabbits weighing 3–3.5 kg (15-20 weeks of age; Japan SLC Inc., Shizuoka Prefecture, Japan) were used for this experiment. The powdered DBM was made from frozen cortical bone harvested from ten rabbits and processed for preparation as described previously10). The FGF-2 used was recombinant human FGF-2 (rhFGF-2; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan).

Surgery was performed on 20 rabbits. The rabbits were anesthetized with intravenous pentobarbital. The left forearm was shaved, and the skin was sterilized with a povidone-iodine solution and draped with sterile towels. A longitudinal incision was made on the forearm. A longitudinal incision was made to expose the ulnar diaphysis. A handsaw was then used to create a 1.5 cm defect (gap) in the periosteum and distal third of the ulna. After rinsing the bone defect with physiological saline solution, 20 rabbits were randomly divided into four groups of 5 rabbits each.

Grafting was performed on the bone gaps of each group as follows.

- **Group A**: No grafting
- **Group B**: 50 mg DBM + 200 µl saline solution
- **Group C**: 50 mg DBM + 20 µg rhFGF-2
- **Group D**: 50 mg DBM + 200 µg rhFGF-2

The DBM was saturated in rhFGF-2 and saline solution in amounts of 20 µg/200 µl and 200 µg/200 µl. In all groups, the periosteum and skin were closed after grafting using 5-0 nylon sutures. After surgery, all experimental animals were allowed to move freely within the cage. They were fed a standard diet of RM-4 (Funabashi Farm, Chiba, Japan) and water. This experiment was conducted in accordance with the guidelines for Animal Experiments established at Yamanashi Medical University.

**EVALUATION**

Three weeks after surgery, the animals in all groups were killed with lethal doses of pentobarbital, and radiographs were taken using an X-ray apparatus (RF-500-125, Hitachi Medico, Tokyo). Specimens were excised and fixed in a 10 % formalin solution. Following decalcification in 15 % EDTA, each specimen was embedded in paraffin. Sections were cut into coronal slices of 7 µm thickness and were stained with hematoxylin and eosin (HE) for histological evaluation. Histological pictures of the area of newly formed bone in groups B, C and D were measured by an image processing analyzer (Mac SCOPE, Mitani Corp., Fukui, Japan) and normalized to the area of the 1.5 cm gap defect (new bone area/defect area). The gap defect was measured as being the area between the two cortex outer edges at the osteotomy site. The mean and standard deviation of the new bone area/defect area values were calculated. Analyses were performed by one-way analysis of variance. When the test indicated significance, the differences between the groups was determined by Tukey’s test. A test of significance was performed at the 95 % confidence interval compared to the group B.
RESULTS

Radiographic studies

At three weeks after surgery in group A, only faint, irregular bone formation was observed at the ends of the resected part. Also, no new bridging bone formation was found in any of the five rabbits. New bridging bone formation was found in every rabbit in groups B, C and D. Radiologically, calcification was more prominent in group D than the other groups (Fig. 1).

Histological examination

In group A, slight bone formation was noted at the ends, but most of the area of the bone defect was filled with fibrous connective tissues and no new bridging bone formation was found. In groups B, C and D to which DBM was grafted, new bridging bone formation was found at both ends, and this was more abundant in group D than in groups B and C (Fig. 2). High power magnification was used to look at the area showing new bone formation in group D, revealing active new bone formation rimmed with osteoblasts surrounding the DBM (Fig. 3). Figure 4 shows the effects of FGF-2 on the formation of bone 3 weeks after surgery. The addition of FGF-2 increased the bone volume on the defect in a dose-dependent manner, with significant effects at concentrations of 200 μg.

Fig. 1. Radiographs of animals, 3 weeks after surgery. Group A (A) without grafting, group B (B) with 50 mg DBM + 200 μg saline solution grafted, group C (C) with 50 mg DBM + 20 μg rhFGF-2 grafted, and group D (D) with 50 mg DBM + 200 μg rhFGF-2 grafted. New bridging bone formation is prominent in group D.
Fig. 2. Histological findings at low magnification (×4) at the site of grafting, 3 weeks after surgery. A, B, C and D represent groups A, B, C and D as described in Fig. 1. The area surrounded by small arrows shows new bone formation. Big arrows show the ends of the resected part. R = radius, U = ulna

Fig. 3. Histological findings at high magnification (×100) at the site of grafting in group D three weeks after surgery. The arrows show the grafted DBM.
DISCUSSION

The mechanism of FGF-2 accelerating bone formation has yet to be determined. Andreshak et al.\textsuperscript{14} were the first to report on the effects of FGF-2 in the healing of a grafted segmental defects. In their experiment, a 4 mm segmental defect of rat tibia was grafted with Gelfoam saturated in coralline hydroxyapatite and 1 \textmu g of FGF-2. They reached the conclusion that FGF-2 assisted with hyaline cartilage formation at an early stage (2 weeks after surgery) but was ineffective for strengthening or accelerating healing.

Wippermann et al.\textsuperscript{15} also reported that neither FGF-2 alone or in combination with ceramics or autologous bone marrow stimulated the healing of such a long bone defect. According to the results of the present study, however, new bone formation was better in the FGF-2 combination group than the control group. This discrepancy may be due to hydroxyapatite or ceramics having no osteoinductivity, or to a problem with the FGF-2 carrier. Aspenberg and Lohmander.\textsuperscript{16} have reported the effects of adding FGF-2 to DBM grafts in rats. This is an experiment in which the marrow canals of rat femoral diaphyses were filled with carboxymethyl cellulose gel (CMC) containing 75ng of FGF-2, before being implanted intramuscularly to examine the heterotopic bone formation. They reported increased bone formation with the use of CMC containing FGF-2, noting its sustained release effects, but that there was no benefit when FGF-2 was used in DBMs as a phosphate-buffer solution. In our experiment, the DBM was saturated with FGF-2 solution and the DBM itself was used as a carrier. As a result, satisfactory new bone formation was achieved with the use of 200 \textmu g of rhFGF-2. The difference between our results and those of Aspenberg and Lohmander.\textsuperscript{16} may be attributed to the following: the DBM used was powdery, it was used in segmental defects with no bone tissue remaining, and there was an approximate 2,500-fold difference (200 micrograms compared to 75 nanograms) in the amount of rhFGF-2 used. In addition, Nakamura et al.\textsuperscript{15} have reported that a single local injection of FGF-2 into the distal rabbit femur stimulated bone formation through intraosseous injection. In a rabbit tibial fracture model, Kato et al.\textsuperscript{17} observed accelerated callus formation despite less than 10 \% of the administered FGF-2 dose remaining 24 hours after the injection. Judging from these reports and our results, the release of FGF-2 over many hours may not be necessary. Using DBM as the carrier, a single local administration of FGF-2 at doses of about

![Fig. 4. Dose response of the effects of fibroblast growth factor-2 (FGF-2) on new bone area/defect area (%) three weeks after surgery. Data are means ± SD for 5 rabbits in each group. *P < 0.01, significantly different from the group B.](image-url)
200–400 μg may be effective for bone formation such as that of the rabbit bone defect. The present study has shown that FGF-2 acts effectively to stimulate new bone formation in DBM grafting of bone defects.

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REFERENCES


