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Effects of FGF-2 on metaphyseal fracture repair in rabbit tibiae

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Abstract Fibroblast growth factor-2 (FGF-2) has been found to have stimulatory effects on fracture repair at diaphysis, while its effect on metaphyseal fracture repair, where spongiosal bone is dominant, has not been studied. This study was conducted to investigate the effect of FGF-2 on metaphyseal fracture healing in a rabbit proximal tibial metaphyseal model. The proximal tibial metaphysis of 6-month-old Japanese white rabbits was osteotomized bilaterally. Then 400 μg of FGF-2, mixed with gelatin hydrogel, and gelatin hydrogel alone (the control) were injected to each osteotomy site of the rabbit proximal tibiae, and the osteotomies were fixed with staples. One and 2 weeks after surgery, the osteoid area in the repairing spongiosal bone at the fracture site was significantly larger in the FGF-2 group than in the control group ($P < 0.05$). On immunohistochemistry, proliferating-cell nuclear antigen-positive cells had a tendency to show greater numbers in the FGF-2 group. After 4 and 8 weeks, values for bone mineral density and the cancellous bone area in the healing region of the fracture site were significantly larger in the FGF-2 group ($P < 0.05$). These data suggest that local application of FGF-2 may have an accelerating effect on the repair of metaphyseal fractures. Exogenous recombinant human rhFGF-2 may have potential clinical applications in metaphyseal fracture treatment.

Key words fibroblast growth factors (FGFs) · metaphysis · fracture repair · spongiosal bone · bone induction

Introduction

The healing of fractures is a complex multistep process, involving periosteal and endosteal reactions. In the healing of diaphyseal fractures, the most important response is that of the periosteum, where intramembranous and endochondral ossifications combine to complete the healing process. However, the repair process of metaphyseal fractures is different from that of diaphyseal fractures. At the metaphysis or epiphysis, fracture healing is mainly medullary, and new appositional bone formation occurs in the existing trabecular bone tissue. External callus formation either does not occur or only plays a subsidiary role [1]. Clinically, metaphyseal fractures have several unique problems when compared with diaphyseal fractures. For example, because the fracture site is close to the end of the long bone or joint, internal fixation is difficult. In addition, bone defects are commonplace, and thus bone grafts are often necessary. Furthermore, the resultant irregularity of the articular surface causes secondary osteoarthritis. Therefore, treatments to enhance fracture healing may be even more beneficial and needed for metaphyseal fractures than for diaphyseal fractures. Although numerous studies using various animal models have been performed on fracture healing and treatment, most address the diaphyseal fractures of long bones. Metaphyseal fractures of long bones have not been well investigated [2].

Treatments to enhance fracture healing and bone formation have been developed using physical and biological methods, such as mechanical stimulation, low-intensity pulsed ultrasound; tissue engineering techniques; and the use of osteoconductive biomaterials [24]. Fracture healing consists of a series of cellular responses, which are under the control of specific paracrine and autocrine intercellular signaling pathways. Growth factors, which are now known to play roles in cell division, migration, differentiation, and enzyme production, have been found to appear at different stages to regulate the proliferation, maturation, and differentiation of mesenchymal cells, osteogenic cells, and osteoblasts. The application of growth factors to stimulate fracture healing has aroused considerable

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interest recently. For example, the application of fibroblast growth factor (FGF)-1 was reported to be successful in enhancing fracture repair in vivo [3]. Recently, treatment of open tibial fractures in 450 patients, using recombinant human bone morphogenetic protein (BMP)-2, has also been documented [4]. Other growth factors, such as transforming growth factor-β [5], other FGFs [6,7], other BMPs [8,9], insulin-like growth factor [10,11], and parathyroid-related protein [12], have been reported to regulate bone metabolism.

Fibroblast growth factor-2 (basic FGF; FGF-2) is one of the 23 members of the FGF family, which together participate in many different cell and organ systems. FGF-2 stimulates smooth muscle cell growth, wound healing, and tissue repair. FGF-2 also plays an important role in the differentiation and function of the skeletal and nervous systems [13–15]. Recent reports have shown that the addition of exogenous FGF-2 to a fracture site or bone defect during the early healing stage accelerates fracture repair and bone formation [7,16,17]. However, these reports were based on a diaphyseal fracture model. Noting the anatomic differences, there is the possibility that response to the exogenous application of biotic factors may be different at the diaphysis and at the metaphysis. Nakamura et al. [16] reported that a single local injection of FGF-2 into the distal femoral spongiosa stimulated bone formation. To date, no experimental study has been performed to investigate the effect of FGF-2 on metaphyseal fractures.

In this study, we established a metaphyseal fracture model at the proximal bilateral tibia of the rabbit, and attempted to evaluate the effects of FGF-2 on metaphyseal fracture healing by a single administration of FGF-2 into the osteotomy site.

Materials and methods

Twenty-one 6-month-old Japanese white rabbits were used in the study. The study was reviewed by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Kyushu University, and was carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University, and the Laws (no. 105) and Notification (no. 6) of the Government of Japan. The animals were anesthetized with sodium pentobarbital (25 mg/kg body weight; Abbott, Chicago, IL, USA) intramuscularly. Recombinant human FGF-2 was obtained from Kaken Pharmaceutical (Tokyo, Japan). Biodegradable gelatin hydrogel, used as the carrier for FGF-2, was prepared through the glutaraldehyde crosslinking of acidic gelatin with a pI (isoelectric point) of 5.0, as previously reported [18].

Fracture model and FGF-2 injection

A 4-cm longitudinal skin incision was made medial to the patella and patellar ligament. A 23-gauge needle was inserted into the joint to confirm the tibia joint level. An osteotomy using a sagittal blade (Striker, Kalamazoo, MI, USA) was then performed on the bilateral tibiae at the proximal metaphysis. FGF-2, at a dose of 400 μg in 100 μl gelatin hydrogel, was injected into the osteotomy site of the left tibia (FGF-2 group) immediately after osteotomy, while 100 μl gelatin hydrogel alone was injected to the right tibia as the control group. The osteotomy site was then fixed with a staple. The staple was made from Kirschner wire 1.0 mm in diameter, 10 mm in width, and 7 mm in depth.

After surgery, the animals were returned to their individual cages for recovery. At 1 ($n = 4$), 2 ($n = 5$), 4 ($n = 5$), 6 ($n = 4$), and 8 weeks ($n = 3$) postoperatively, the animals were killed with an overdose of sodium pentobarbital. The bilateral tibiae were then harvested and fixed with 10% formalin for histologic and immunohisotochemical studies.

Radiological analysis and measurement of bone mineral content and density

Soft X-ray radiograms (Softex C-SM, Softex, Tokyo, Japan) of the bilateral tibiae were taken after the animals had been killed. Bone mineral density in the healing region of the fracture site was measured transversely with a bone mineral analyzer, using dual-energy X-ray absorbtiometry (DEXA; Dichroma Scan DCS-600R; Aloka, Tokyo, Japan). The measurement was done 4 mm from each of the proximal and distal sides of the fracture line, and was one-third of the medial part in width.

Morphological analysis

The tibia were cut coronally in to anterior and posterior parts. Both parts were demineralized in ethylenediamire tetraocetic acid (EDTA) for 2 weeks, embedded in paraffin, and cut into 4-μm-thick sections. The sections of the anterior part were stained with hematoxylin-eosin and Masson's trichrome. Serial sections were used for immunohistochemistry. Osteoid staining was performed on the posterior part according to the Goland-Yoshiki method [34].

The total area of cancellous bone in the Masson-trichrome-stained sections was measured using an image analysis computer system (Olympus Image Analysis System, SP-1000; Olympus, Tokyo, Japan). The areas measured were $6 \times 3\,mm^2$ in size, which was 3 mm from both the proximal and distal sides of the osteotomy line, and 3 mm from inner margin of cortical

bone. Evaluation of the osteoid area in the repaired spongiosal bone at the fracture site was accomplished using a bone morphologic parameter, the fractional formation surface (total formation surface/total trabecular surface; FrFS), calculated on the osteoid staining sections with Scion (NIH) Image and Adobe Photoshop. The total measured area was $2.6 \times 4.0\,mm^2$ ($2.6 \times 2.0\,mm^2$ from each of the proximal and distal sides of the osteotomy line).

Immunohistochemistry of proliferating-cell nuclear antigen (PCNA)

Immunohistochemistry of PCNA was performed on the demineralized sections using antibodies against PCNA (Sigma Chemical, St. Louis, MO, USA), as described previously [19]. Under a light microscope, two visual fields were chosen randomly near the fracture line from each of the proximal and distal side areas. The total number of cell nuclei and the number of positive-stained cells in the chosen field were then counted, and the rate of positive staining was calculated.

Statistical analysis

Differences between experimental groups were evaluated by performing analysis of variance (ANOVA), and by using a post-hoc test with Fisher's protected least significant difference (PLSD). Values for the quantitative data obtained are expressed as means ± SEM.

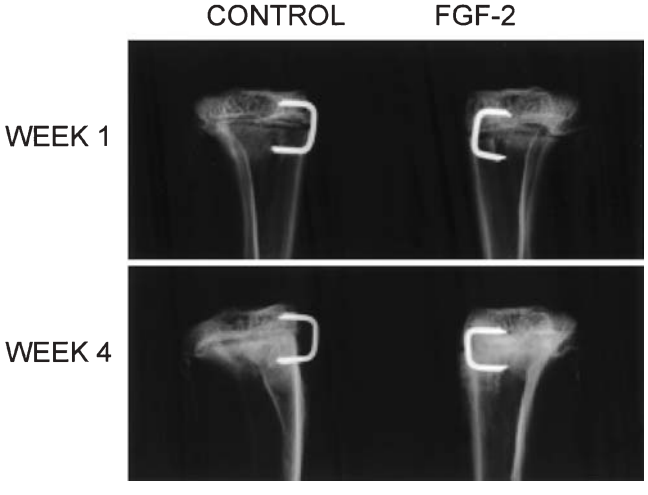

Fig. 1. Radiological features of bilateral proximal tibiae after osteotomy. At week 4, the sample injected with fibroblast growth factor-2 (*FGF-2*) showed intense ossification and more trabecular connection at the osteotomy site, indicating progressing synostosis

Results

Radiology

Radiographs of the osteotomy site showed earlier cancellous continuity or union in the FGF-2-treated tibiae than in the control tibiae, 4 weeks after operation (Fig. 1). External callus was small in both control and treated tibiae.

Bone mineral content and density

The bone mineral density at the osteotomy site was higher in the FGF-2 treated tibiae than in the controls (Fig. 2). There was a significant difference in bone mineral density between the two groups 4 and 8 weeks after operation ($P < 0.05$).

Histology

At 1 week postoperatively, histological examinations showed that there was no bony connection at the osteotomy site in either of the groups (Fig. 3). However, in the FGF-2 group more cells appeared at the osteotomy site and in the adjacent periosteum. Four weeks after the operation, thick trabecular bone was more abundant at the osteotomy site in the FGF-2-treated tibiae than in that of the control. In the external callus, more cartilage tissue was observed in the FGF-2-treated tibiae compared with the controls. Eight weeks after the operation, the trabecular bone area was still small, due to bone remodeling, in both groups.

Measurement of proportion of cancellous bone

The proportion of cancellous bone area in the healing site was significantly larger in the FGF-2-treated tibiae

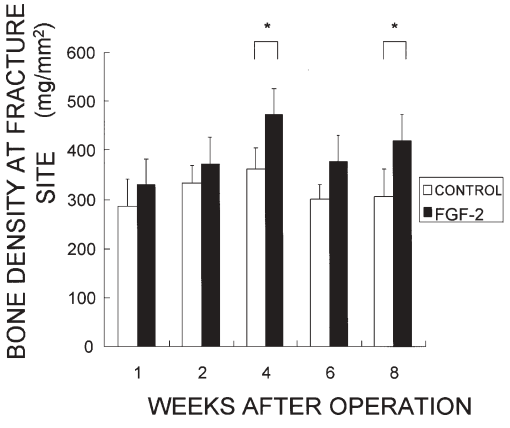

Fig. 2. Bone mineral density (BMD) in the healing region of the osteotomy site. Values are means ± SEM for bone density at fracture sites. The BMD in the FGF-2 treated tibiae was significantly higher than that in the controls, 4 and 8 weeks after operation. *$P < 0.05$

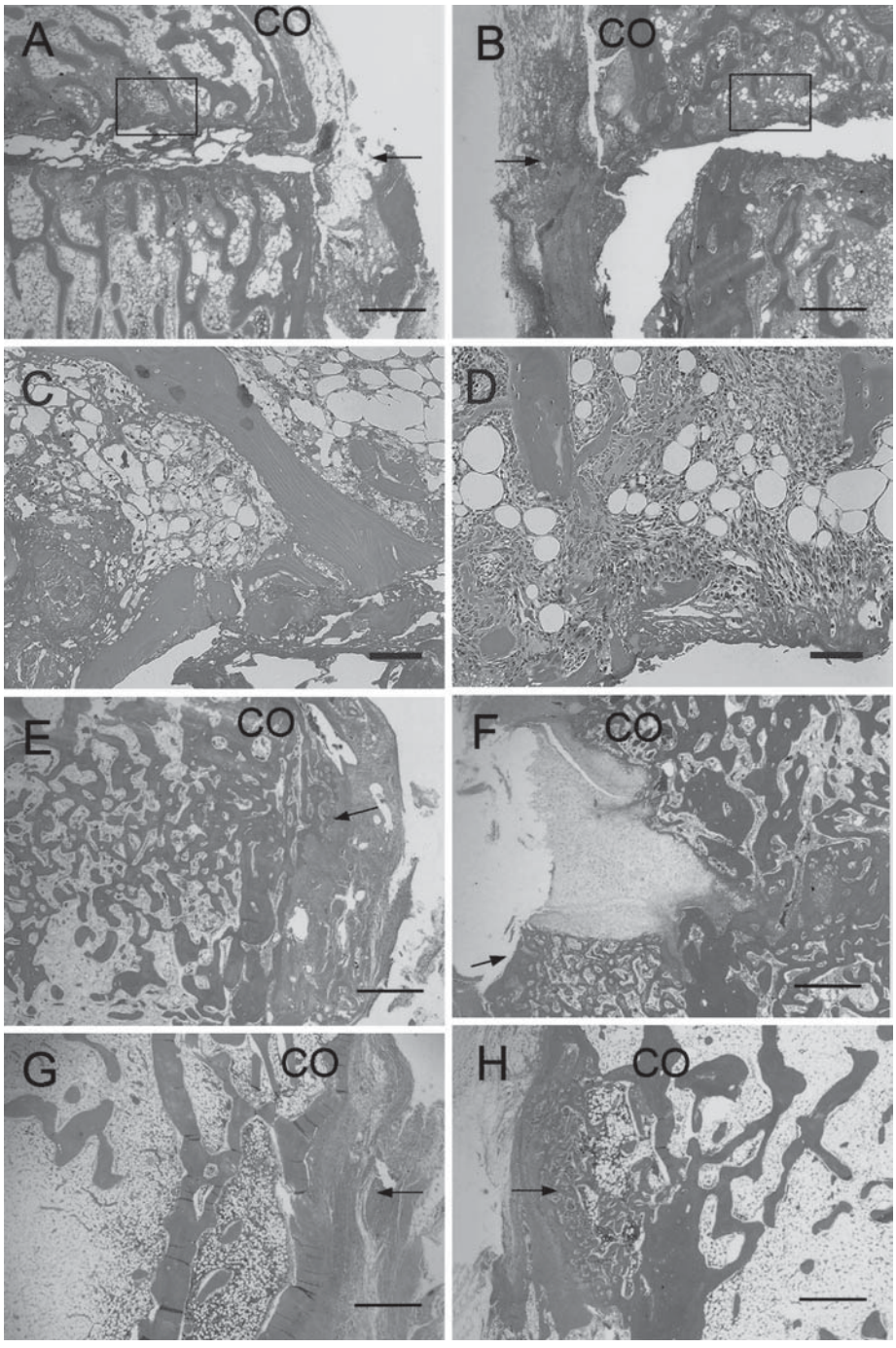

Fig. 3. Histological features of the osteotomy sites (*arrows*). **A**, **C**, **E**, **G** Control group; **B**, **D**, **F**, **H** FGF-2 treated group. **A–D** 1 Week after the operation; **C** and **D** magnified views of **A** and **B**, respectively; **E**, **F** 4 weeks after the operation; **G**, **H** 8 weeks after the operation. *CO*, cortical bone. The *bars* in **A** and **B**, and **E–H** represent 1 mm; the bars in **C** and **D** represent 100 μm. **A–H**, H&E

than in the controls, 4 and 8 weeks after operation (Fig. 4).

Osteoid staining and FrFS analysis

Osteoid staining of the osteotomy site showed large osteoid formations in the FGF-treated tibiae 1 week after the operation, and there were still large osteoid areas in the FGF-treated tibiae compared with the controls at 2 weeks after the operation (Fig. 5). Measurements of FrFS showed that it was significantly higher in the FGF-2-treated tibiae than in the controls, 1 and 2 weeks after the operation (Fig. 6).

Immunohistochemistry

PCNA immunostaining of the osteotomy site showed many positive cells, presumably osteoblasts, around the newly formed trabecular bone, especially in the FGF-2-treated tibiae, 1 week after operation (Fig. 7).

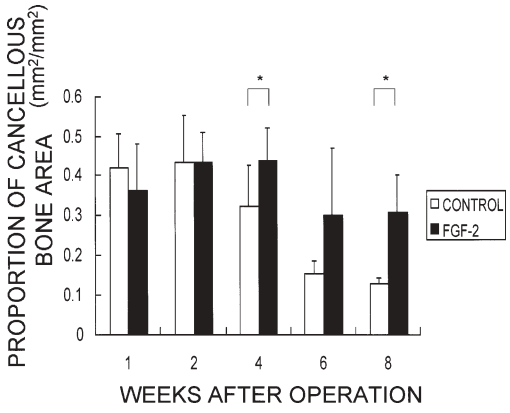

Fig. 4. Cancellous bone area in the healing region of the osteotomy site. Values are means 6SEM for cancellous bone area at the fracture sites. The proportion of cancellous bone area was significantly larger in the FGF-2 treated tibiae (*black bars*) than in the controls (*white bars*), 4 and 8 weeks after operation. $*P < 0.05$

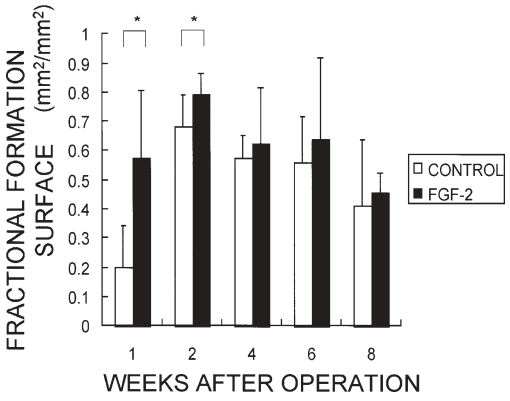

Fig. 6. Ratio of osteoid surface area (fractional formation surface; FrFS) in the osteotomy area. Values are means 6SEM for FrFS at the osteotomy sites. The FrFS in the FGF-2 treated tibiae (*black bars*) was significantly higher than that in the controls (*white bars*) 1 and 2 weeks after the operation. $*P < 0.05$

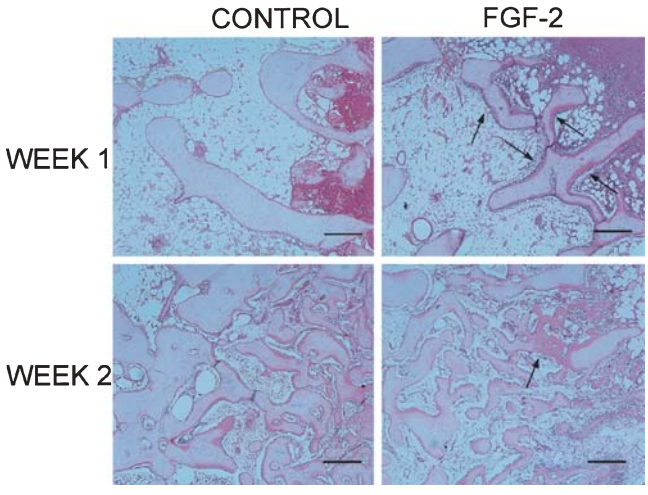

Fig. 5. Osteoid staining of the osteotomy site. In the FGF-2 treated tibiae, there was larger osteoid formation (*arrows*) compared with the control, 1 and 2 weeks after the operation. The *bars* represent 500 μm

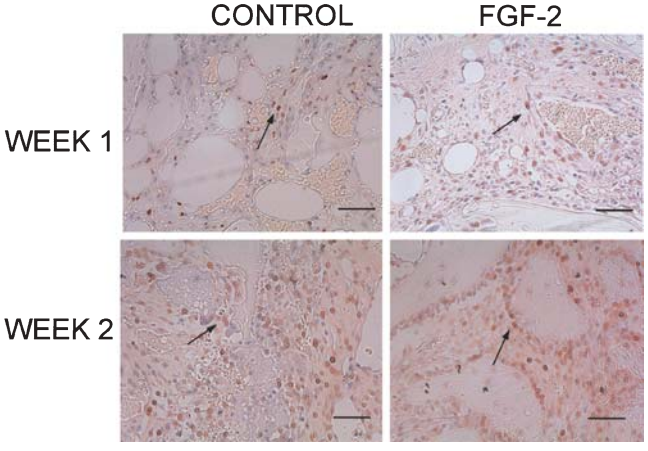

Fig. 7. Immunohistochemistry of proliferating cell nuclear antigen (PCNA) at the osteotomy site at weeks 1 and 2. Many PCNA-positive cells (*arrows*) were observed in the FGF-2 treated tibiae, especially 1 week after the operation. The *bars* represent 100 μm

There was no significant difference between the two groups throughout the observation period (Fig. 8).

Discussion

In previous models of diaphyseal fractures, the local application of recombinant human FGF-2 has been shown to accelerate fracture healing through the formation of a large external callus on the cortical bone at an early stage [7,16,20–22]. In contrast, in metaphyseal fractures, cancellous bone plays an important role, in addition to the cortical responses. The healing process of stable metaphyseal fractures has been documented in studies by Jarry and Uhthoff [1] and Uhthoff and Rahn [23]. Intertrabecular cell proliferation quickly leads to bone accretion on existing trabeculae, whereas a periosteal callus is not observed in a stable fracture. In the present study, we created an immobilized osteotomy at the metaphysis to mimic a stable metaphyseal fracture in the rabbit. In our fracture model, similar to the findings in Uhthoff's reports [1,23], bone formation was active in the intramedullary cancellous bone. In contrast, periosteal reactions were small when compared with those in fractures at the diaphysis. These results indicate that the cancellous reaction is an important event for fracture healing at the metaphysis.

Cancellous bone is considered to provide the essential elements for bone formation, including living osteo-

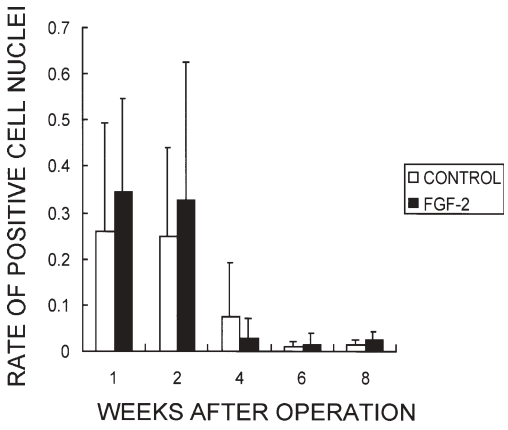

Fig. 8. Rates of PCNA-positive cells in the healing region at the osteotomy site. Values are means ± SEM for the rates of PCNA-positive cells at the fracture sites. The difference between the two groups was not significant. *Black and white bars*, as in Fig. 2

genic cells and a scaffold of apatite, which supports the ingrowth of newly formed bone [24]. Moreover, an extensive vascular supply exists to carry inflammatory cells, mesenchymal cells, and paracrine factors, which are sources of signaling molecules that initiate the cascades of cellular events, to the fracture site. Therefore, to investigate the effect of FGF-2 in the metaphyseal model, we focused on the changes in cancellous bone. There was a tendency for a high proportion of PCNA-positive cells in the FGF-2 group at an early stage. Osteoid staining of the osteotomy-site specimens also showed faster bone formation than that of the control group. The early response indicates that FGF-2 shows effective stimulation of related precursor cells in the cancellous region. Therefore, this report demonstrates, for the first time, that exogenous FGF-2 promotes fracture healing at the metaphysis.

FGF-2 has been shown to have an important role in the development and function of numerous organ systems. In the fracture-healing process, FGF-2 participates in the key steps of the sequential cellular and molecular cascades [6,7]. In addition, earlier reports have shown that FGF-2 displays anabolic actions on intramembranous and endochondral bone formation [18,21,22]. The promotion effect on fracture healing is likely accomplished through either the stimulation of proliferation or the recruitment of mesenchymal cells. These mesenchymal cells then differentiate or support the differentiation of precursor cells [16,21]. FGF-2 has also been reported to stimulate the expression and transactivation activity of Runx2, which is needed for all the steps of osteoblast differentiation [26], all of which use protein kinase C-signaling pathways, among others [27]. Furthermore, recent studies show that FGF-2 stimulates the differentiation of chondrogenic precursor cells [28]. It is also reported that mutations of transmembrane receptors for the FGF receptor-3 (FGFR-3) gene cause achondroplasia [28] and hypochondroplasia [29]. All these reports indicate that FGF-2 plays an important role in endochondral ossification. In our model, FGF-2-treated fractures showed more bone formation at the osteotomy site, and also more cartilage formation in the periosteal area than the controls. In addition, endochondral ossification occurred earlier than it did in the control group.

During fracture healing, the expression of FGFR-1 and FGFR-2 occurred very early and continued throughout the entire process: in early proliferating periosteal mesenchymal cells, in osteoblasts during intramembranous bone formation, and in chondrocytes and osteoblasts during endochondral bone formation. FGFR-3 had a different temporal pattern, showing expression in mesenchymal cells and chondrocytes at later stages [25,30]. A report by Britto et al. [31] also indicates that FGFR-2 and FGFR-1 are expressed in the metaphyseal periosteum. However, no other studies have shown the spatial and temporal expression of the *FGFR* gene during fracture healing. Our data suggest that FGFRs are also expressed in the trabeculae in the cancellous area.

Other reports show that FGF-2 could possibly stimulate the local production of other factors such as prostaglandins, insulin-like growth factor (IGF-1), endogenous FGFs, transforming growth factor-beta, and BMPs, which constitute a serial cascade of bone formation [7,32,33]. Previous studies showed that FGF-2 was released from gelatin hydrogel during a period of approximately 2 weeks [35]. FGF-2 may also act as a factor that initiates the cascade of events in the later stages of the bone formation process, by enhancing the secretion of sequential factors.

FGF-2 is now used clinically in the treatment of skin ulcers and as a therapeutic strategy to treat fracture healing. Our data show that the application of FGF-2 has a promoting effect not only on the cortex but also in the area of cancellous bone. Our results also suggest the possibility of FGF-2 use for the treatment of metaphyseal fractures. Moreover, FGF-2 may also be effective in the healing of other cancellous bone fractures, such as compression fractures of vertebrae.

In conclusion, we designed a metaphyseal fracture model to investigate the effect of FGF-2 on fracture healing in the rabbit. Treatment with exogenous recombinant human FGF-2 in gelatin hydrogel showed early bone formation and union at the metaphyseal osteotomy site. These results indicate the effectiveness of FGF-2 in the treatment of metaphyseal fracture healing, with other studies having shown its effectiveness in treating diaphyseal fractures.

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