Continuous local infusion of fibroblast growth factor-2 enhances consolidation of the bone segment lengthened by distraction osteogenesis in rabbit experiment

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Abstract

Experimental tibial lengthening was achieved in 61 rabbits to examine the effect of continuous local infusion of recombinant human fibroblast growth factor-2 (rhFGF-2) on bone healing of the lengthened segment. The tibial diaphysis was separated by osteotomy and was subjected to slow progressive distraction (rate: 0.35 mm/12 h) using a monolateral external fixator. There were a lag phase for 1 week, a distraction phase for 2 weeks, and a consolidation phase for 5 weeks in this experiment. At various stages of distraction, rhFGF-2 was infused continuously for 2 weeks into the lengthened segment (rate: 14.28 μg/60 μl/day) using an osmotic pump implanted under the skin. Bone healing was significantly accelerated when rhFGF-2 was infused in the beginning of consolidation phase, but not in the distraction phase or in the lag phase. Infusion of normal saline (N/S) using the same osmotic pump had no effect. Dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computerized tomography (pQCT) studies demonstrated that rhFGF-2-treated tibia had increased bone mineral density (BMD), bone mineral content (BMC) and cortical bone thickness (CBT) when compared with N/S-treated tibia. Three-point bending test demonstrated that rhFGF-2-treated bone had significantly stronger mechanical properties than N/S-treated bone. Finally, distribution of the infused materials was checked by using Indian ink or radio-opaque. The dyes distributed widely but exclusively in the lengthened segment. Based on these results, we conclude that direct delivery of rhFGF-2 into the lengthened segment can shorten the consolidation phase of limb lengthening and the method is applicable to the clinical treatment.

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Introduction

Recent advances of external fixation and newer knowledge of distraction osteogenesis have brought a revolution in surgical treatment of congenital or post-traumatic short extremities. Various types of external fixation devices have been developed to achieve limb lengthening and simultaneous correction of the complex bone deformities [1–3]. Lengthening a bone for more than 10 cm is now possible, if the proper technique is used. Principle of distraction osteogenesis has also been applied in treatment of segmental bone loss, infected nonunion and congenital pseudoarthrosis of the bone [4,5].

We have been engaged in studying the basic mechanism of distraction osteogenesis using animal models of limb lengthening [6–10]. Biological events of distraction osteogenesis are understandable if the treatment process is divided into three distinct phases, i.e., a lag phase, a distraction phase, and a consolidation phase. During the lag phase after osteotomy, blood circulation recovers and immature callus is formed around osteotomy site [8]. During distraction phase, new bone regenerate is continuously formed within the lengthened segment. During consolidation phase, the lengthened segment matures and bone union is obtained while the external fixator is still on.

The factors affecting bone healing during distraction osteogenesis may include type of osteotomy, timing and rate of distraction, stability of fixation, age of the patient and underlying disease. Although the efforts to improve osteotomy techniques and stability of fixation [8,11,12], the overall treatment time of limb lengthening still requires a long period. Healing indices, calculated by dividing the treatment time with the amount of lengthening, ranged from 28 to 36 days/cm
The patient has to tolerate wearing a bulky external fixator at least for several months until consolidation of the lengthened segment is obtained.

Several authors have attempted to promote bone formation during distraction osteogenesis by local administration of growth factors or cytokines [16–19]. Okazaki et al. [16] reported that a single-shot injection of rhFGF-2 into the regenerating bone was effective to stimulate bone healing in rabbit tibial lengthening. In the present study, we are demonstrating that continuous local infusion of a low-dose rhFGF-2 into the lengthened segment can accelerate bone healing of the lengthened segment in rabbit model.

**Materials and methods**

**Animals**

Animal experiment was carried out on 61 male Japanese white rabbits, weighing 1.8 to 2.2 kg, purchased from Oriental Yeast Co. (Tokyo, Japan).

![Fig. 1. Experimental design: Radiographs showing the position of the needle at the time of operation (a) and at the end of distraction (b). The osmotic pump (c) was implanted subcutaneously on the back of a young rabbit (c). The pump was connected to a plastic catheter (d) that reached the needle (a, b) inserted into the lengthened segment. Local infusion of rhFGF-2 was achieved for 2 weeks at various stages of the experiment (f). The animals were sacrificed 8 weeks after operation.](image-url)
The protocol of animal experiment was approved by the local animal protection agency and the ethics committee of the University of Tokushima.

All rabbits were anesthetized by intravenous injection of ketamine hydrochloride and xylazine at doses of 20 mg/kg and 5 mg/kg body weight, respectively. After a unilateral external fixator (Orthofix M-100) was applied to the tibia with four self-tapping screws, subperiosteal osteotomy was achieved between the second and third screws using a fine wire saw. A small drill hole was made above the osteotomy and a 23-gauge needle (Fig. 1d) was inserted obliquely into the marrow cavity until it reached the distal bone fragment (Fig. 1a). The needle was connected with a polyvinyl catheter, which was embedded in the subcutaneous tissue until use.

There was a lag phase for 1 week before distraction was started at a rate of 0.35 mm every 12 h (0.7 mm/day). Distraction was continued for 2 weeks so that the actual lengthening of 10±0.1 mm was achieved. After completion of distraction, the animals were kept for 5 weeks until consolidation of the lengthened segment was obtained. At various stages of the experiment, the animals were anesthetized again for subcutaneous implantation of an osmotic pump [(Alzet 2ML4) purchased from Muromachi Kikai Co., Ltd., Osaka, Japan] on their back (Figs. 1c, e). The pump was connected to the polyvinyl catheter which was embedded subcutaneously at the time of primary operation so that the solution in the pump was gradually delivered into the center of the lengthened segment for 14 days (Fig. 1b).

Nine animals were used for preliminary experiment to determine the appropriate dose of the growth factor. Three different doses of rhFGF-2 (7.14 μg/60 μl/day, 14.28 μg/60 μl/day and 35.71 μg/60 μl/day) were infused into the lengthened segments for 14 days in the beginning of the consolidation phase. In the final experiment (43 animals), a constant dose of rhFGF-2 (14.28 μg/60 μl/day) was applied for 14 days at various stages of distraction.

Group 1 (3 animals) rhFGF-2 (14.28 μg/60 μl/day) was administered for 7 days in the lag phase and for a subsequent 7 days in the distraction phase.

Group 2 (4 animals) The same dose of rhFGF-2 as in Group 1 was administered for 14 days in the distraction phase.

Group 3 (16 animals) The same dose of rhFGF-2 as in Group 1 was administered for 14 days in the beginning of the consolidation phase.

Group 4 (15 animals) Normal saline (N/S) 60 μl/day was administered for 14 days in the beginning of consolidation phase.

Group 5 (5 animals) Sham operation; the needle was placed into the center of distraction gap and empty pump was implanted in the back.

Fig. 2. Radiological changes of the lengthened segment after infusion of rhFGF-2 (a–d, Group 3 animals) and N/S (e–h, Group 4 animals). (a, c) 4 weeks, (b, f) 5 weeks, (c, g) 6 weeks, and (d, h) 8 weeks after operation, respectively.
The animals were housed under standardized environmental condition with 12-h light/dark cycles, and fed standard rabbit chow (RC4, Oriental Yeast Co., Tokyo, Japan). The process of bone formation was monitored every week by soft X-ray (Model CMB-2T Softex Co., Kanagawa, Japan). All rabbits were sacrificed at 8 weeks after operation by injection of sodium pentobarbitone (60 mg/kg body weight) and the external fixator, the polyvinyl catheter and the osmotic pump were removed.

In a subset of the experiment (9 animals), distribution of the infused material was analyzed by using radio-opaque (Urografin) or Indian ink. These dyes were infused into the lengthened segment at various stages of the experiment using the same osmotic pump (60 μl/day).

**Densitometry**

At 4, 5, 6, and 8 weeks after operation, the animals were anesthetized and DXA analysis was performed using Hologic 2000W device (Waltham, MA, USA). Images of the lengthened tibiae were divided into three distinct regions, namely, the proximal tibia above the osteotomy, the lengthened segment, and the distal tibia below the osteotomy (Fig. 4). Each segment had 10 mm in length. BMC was measured for each region. BMC of the non-operated tibia was also measured as a control.

**pQCT studies**

At the end of experiment, the animals were sacrificed and the tibiae were prepared for pQCT study. Using the Stratec pQCT (XCT-960A; Norland/Stratec, Fort Atkinson Pforzheim, USA/Germany), a total of 18 slices were analyzed in each tibia. Six slices within the lengthened segment, 6 slices above and 6 slices below the osteotomy, each 1.67 mm in thickness, were analyzed. Six slices of the right tibia (non-operated bone) were used as controls.

**Histological analysis**

Harvested tibiae were fixed with 10% neutral formalin for 5 days, decalcified in 20% aqueous EDTA for 6 weeks and then embedded in paraffin. Coronal sections of 3 μm in thickness were stained with hematoxylin and eosin.

**Mechanical analysis**

The mechanical properties of the tibiae were examined at the end of experiment (8 weeks after operation). The lengthened tibiae were cleaned of soft tissue and three-point bending strength was measured until failure with a support span of 40 mm between the second and third pin holes, using a servohydraulic.

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**Fig. 3.** Longitudinal sections of the lengthened segment prepared at 8 weeks after operation (hematoxylin–eosin staining). (a, b) rhFGF-2 infused tibia in Group 3 animals. (c, d) N/S infused tibia in Group 4 animals.
materials testing system (S-100; Shimazu, Kyoto, Japan) with a 1-kN load cell under displacement control (5 mm/min). The ultimate force, stiffness, and work to failure were determined as described previously [10].

**Statistical analysis**

Statistical analysis was performed using unpaired Student’s t test. Each data show the mean±the standard deviation (SD). Values of \( p < 0.05 \) were considered to be significant.

**Results**

**Radiological findings**

In all groups of the animals, lengthening of 10±0.1 mm was successfully achieved and bone consolidation was obtained by the end of experiment. In the control animals, the lengthened segment showed a characteristic zone structure consisting of central radiolucent zone and two sclerotic zones (Fig. 1b) as described previously [6]. After completion of distraction, the proximal and distal sclerotic zones became fused, shrank and were eventually absorbed. By the end of experiment, the lengthened segment showed tubular bone structure with a new cortex.

Three different dose of rhFGF-2 were tested in the preliminary experiment. The radiological findings suggested that bone formation was significantly enhanced when rhFGF-2 was infused at the dose of 14.28 μg/60 μl/day or 35.71 μg/60 μl/day for 14 days in the beginning of consolidation phase. Very small effect was detected when rhFGF-2 was applied at the dose of 7.14 μg/60 μl/day (data not shown). Subsequently, a constant dose of rhFGF-2 (14.28 μg/60 μl/day) was used in the final experiment.

Continuous local infusion of rhFGF-2 was achieved at various stages of distraction. There was no significant effect on bone formation when rhFGF-2 was applied in the lag phase (Group 1) or in the distraction phase (Group 2) (data not shown). When the same dose of rhFGF-2 (14.28 μg/60 μl/day) was applied for 2 weeks in the beginning of the consolidation phase (Group 3), however, the periosteal callus dramatically expanded and the outer diameter of the lengthened segment was significantly increased (Figs. 2a–d). In N/S-infused tibiae in Group 4 animals, the periosteal callus was gradually resorbed and replaced by a thin cortical bone by the end of experiment (Figs. 2e–h).

**Histological findings**

Consistent with the radiological findings, continuous local infusion of rhFGF-2 in the lag phase (Group 1) or in the distraction phase (Group 2) produced no significant effect on

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**Fig. 4.** The time course of bone mineral content (BMC) in the proximal, lengthened, and distal parts of the tibia measured by DXA (4, 5, 6 and 8 weeks post-operation).  
*\( p < 0.05 \), **\( p < 0.001 \).
histological structures of the lengthened segments (data not shown). Infusion of rhFGF-2 in the beginning of consolidation phase, however, produced dramatic increases in cortical bone thickness and overall diameter of the lengthened segment (Fig. 3).

**DXA studies**

BMC and BMD were measured exclusively in Group 3 and Group 4 animals. Three distinct areas of the lengthened tibia were analyzed by DXA (Fig. 4). The proximal and distal segments contained the original tibia above and below osteotomy, each 10 mm in length, respectively. The middle segment was the lengthened segment consisting of new bone regenerate.

In control animals (Group 4), consistent with radiological findings, BMC of the lengthened segment declined gradually during consolidation phase (Fig. 4). Not only the lengthened segment but also the proximal and distal segment gradually lost BMC. Continuous local infusion of rhFGF-2 into the lengthened segment in the beginning of the consolidation phase (Group 3) perfectly prevented the declination of BMC (Fig. 4). Consequently, by the end of experiment, BMC of the new bone regenerate in Group 3 animals showed seven times higher than that in Group 4 animals ($p<0.001$).

The BMD measured by DXA showed similar patterns as BMC (data not shown).

**pQCT studies**

Fig. 5 shows cross sections of the tibia made by pQCT at the end of experiment. Interestingly, the lengthened segment in Group 3 animals had double-layer bone cortices, whereas those in Group 4 animals had a single layer bone cortex. Consequently, CBT of the lengthened segment in Group 3 animals was two times larger than that in Group 4 animals ($p<0.01$) (Figs. 5 and 6b). There was no significant difference in cross-sectional morphology of the proximal and distal segment among Group 3 and 4 animals. There was no significant change in CBT of the contralateral tibia in both groups.

Fig. 6 shows volumetric BMD calculated by pQCT data. Consistent with DXA studies, continuous local infusion of rhFGF-2 into the lengthened segment (Group 3) had a significant effect to prevent decrease in BMD of all three distinct areas. Consequently, BMD of the lengthened segment in Group 3 animals was 162% of those in Group 4 animals ($p<0.001$).

**Mechanical analysis**

Three-point bending test demonstrated that the ultimate force of the lengthened tibia in Group 3 animals was 72% larger than that in Group 4 animals ($p<0.008$, Fig. 6c). There were no significant differences of the ultimate force in contralateral tibia between two groups.
Distribution of infused materials

In order to examine the distribution of locally infused materials, osmotic pump was filled with radio-opaque marker (Urografin) or Indian ink. Figs. 7a and b demonstrate the accumulation of Urografin in the center of the lengthened segment after 24 h infusion in the beginning of consolidation phase. Fig. 7c demonstrates the accumulation of Indian ink (blue) within the lengthened segment in the same condition. When these marker dyes were infused either in the lag phase or in the distraction phase, they rapidly leaked into the surrounding tissue and only a limited accumulation of the dye in the lengthened segment was detected (data not shown).

Discussion

FGF-2 is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor being expressed at low levels in many tissues and cell types. The molecule consists of 154 amino acids and has a relatively short half life [20].

The effect of FGF-2 on bone formation has been examined in animal experiments. Systemic administration of FGF-2 stimulated endosteal bone formation, resulting in an increase in the cortical bone thickness of the rat tibia [18,21,22]. Systemic administration of high-dose FGF-2, however, is not recommended for clinical use because it may produce undesirable side effects. Cooper et al. [23] reported that intravenous injection of FGF-2 was associated with a high rate of proteinuria in humans.

The effect of local administration of FGF on bone formation has also been examined in animal experiments. Kawaguchi et al. [24] applied the recombinant human FGF-2 on fracture healing model of non-human primates. Bone union was obtained by 6 weeks in all 10 animals treated with FGF-2, while 4 of 10 animals treated with the vehicle alone remained in a nonunion state even after 10 weeks. They proposed rhFGF-2 as a potent bone anabolic agent for clinical use, since it had...
improved the mechanical properties of the bone. Nakamura T. et al. [25] reported in a dog experiment that local administration of rhFGF-2 accelerates fracture healing and remodeling of the callus.

Nakamura K. et al. [26] examined the effect of intraosseous injection of rhFGF-2 at various dose (4–1600 μg). A relatively high dose (>400 μg) of rhFGF-2 was required to induce new bone formation around the injection site. Okazaki et al. [16] demonstrated that a single-shot injection of FGF-2 (200 μg in 150 μl N/S) into the distraction osteogenesis stimulated bone formation in rabbits.

In the present study, we established a novel method to deliver the growth factors directly into the regenerating bone in limb lengthening. An overall dose of rhFGF-2 used in the present study was 10 times less than those in the previous studies with systemic or local injection [18]. In addition, application of rhFGF-2 into the regenerating bone segment resulted in expansion of the periosteal callus. By the end of the experiment, CBT of the lengthened segment reached the same level as that of the normal bone. In the control animals, severe bone atrophy was observed during the consolidation phase not only in the lengthened segment but also in the proximal and distal segments. Continuous infusion of rhFGF-2 in the beginning of the consolidation phase dramatically prevented those bone atrophy, resulting in an improvement of mechanical properties of the lengthened tibiae at the end of the experiment. There was no significant effect on the contralateral tibia, suggesting that systemic effect is negligibly small in this system.

It is generally known that significant osteopenia occurs during limb lengthening especially in the distal segment below osteotomy [27]. Indian ink injection in the present study suggested that locally infused rhFGF-2 should mostly have stayed in the regenerated part of the lengthened segment. Some of them, however, may infiltrate into the periosteum and also into the proximal and distal parts of the regenerated part and stimulate the local bone formation. This phenomenon should explain the increases in BMD not only in the lengthened segment but also in the proximal and distal segments.

It is not known why infusion of rhFGF-2 in the lag phase or in the distraction phase resulted in a small effect on bone healing. Indian ink study suggested that a low-dose of rhFGF-2 infused at this stage might have leaked into the surrounding tissue.

Various growth factors besides FGF-2 have been used to stimulate bone healing during distraction osteogenesis. Li et al. [17] reported that rhBMP-2 had positive effects when it was injected subcutaneously or applied with absorbable collagen sponge. The BMP-2 may stimulate osteogenic differentiation of the periosteum-derived mesenchymal cells, while FGF-2 may stimulate proliferation of those mesenchymal cells [28,29].

In conclusion, the present study has demonstrated that continuous local injection of FGF-2 into the lengthened segment during the consolidation phase definitely stimulates bone healing during distraction osteogenesis. Increased BMC, BMD and CBT of the lengthened segment as well as the proximal and distal segments should produce increased mechanical properties.
of the whole tibia. The potential risk of the present method is infection of the implanted pump. By using an extracorporeal pump, instead of an osmotic pump, this method is applicable to clinical treatment and will definitely contribute to shorten the treatment time of limb lengthening by distraction osteogenesis.

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