Exogenous fibroblast growth factors-1 and -2 do not accelerate fracture healing in the rabbit

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Both fibroblast growth factors-1 (acidic FGF) and -2 (basic FGF) increase the proliferation of osteoblasts and chondrocytes in vitro and FGF-2 stimulates angiogenesis and bone formation in vivo. To test their effects on rabbit tibial fracture-healing under stable and unstable mechanical conditions, 3 µg of either FGF-1 or FGF-2 was injected around rabbit tibial fractures on day 4 after fracture. Neither growth factor had a significant effect on either the size of, or the amounts of bone and cartilage in, the 10-day callus irrespective of the mechanical conditions under which the fracture was healing. The 10-day FGF-2-treated calluses were, however, more mature than FGF-1-treated calluses because the cartilage was separated from the periosteum by bone and endochondral ossification had progressed further. In conclusion, the application of FGF-1 or FGF-2 to normally healing fractures of the rabbit tibia does not have a significant effect on the rate of healing.

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Acidic and basic fibroblast growth factors (aFGF = FGF-1 and bFGF = FGF-2) increase proliferation and the synthetic activity of osteoblasts and chondrocytes in vitro (Globus et al. 1988, 1989, McCarthy et al. 1989, Crabb et al. 1990, Suzuki 1992), but their effects on the synthesis of collagens are more equivocal (Horton et al. 1989, Shen et al. 1989, Kato and Iwamoto 1990, Nataf et al. 1990, Hill et al. 1992, Hurley et al. 1992, 1993, Suzuki 1992). In vivo, both raise the proliferative rate of osteoprogenitor cells in chick embryos (Frenkel et al. 1992) and subsequently bone formation, although no additional bone formation is observed on neonatal rat calvariae (Noda and Camilliere 1989). FGF-2 is also angiogenic in skeletal tissues (Eppley et al. 1988, 1991).

These observations led to experiments to determine whether FGFs induce additional bone formation and accelerate normal fracture healing. FGF-1 increases the amount of cartilage, but not bone, in 2-week rat femoral fractures (Jingushi et al. 1990), while the size of the callus of rat fibular fractures is increased after treatment with FGF-2 at the time of fracture (Kawaguchi et al. 1994). FGF-2 also increases bone yield in experimental implants in rats (Aspenberg et al. 1989, 1991, Wang and Aspenberg 1994).

The present experiments were designed to determine whether FGFs could be used to accelerate healing of rabbit tibial fractures healing under stable or unstable mechanical conditions (Ashhurst 1986). Under stable mechanical conditions, the callus consists almost entirely of bone, while under unstable conditions a large area of cartilage develops over the fracture site. To accelerate healing, the rate of callus formation and maturation must be increased. Therefore, the effects of FGFs-1 and -2 were investigated during the first 14 days after fracture.

Animals and methods

Experimental fractures

Fractures were made under sterile conditions and halothane anesthesia in the left tibiae of male New Zealand White rabbits (weight approximately 3.5 kg). A sawcut was made in the medial cortex to which the plate was attached and the remainder of the bone was fractured, using 3-point pressure (Ashhurst et al. 1982). The fractures were stabilized with a 6-hole plate. Two types of plate were used. The first was a stainless steel dynamic compression-plate (DC-plate) which stabilizes the fracture. The second was a plastic plate made to the same dimensions, but designed to leave a gap of 0.5 mm at the fracture site so that the fractures should heal under unstable conditions. Analgesics (Temgesic, 0.03 mg/kg) were administered twice daily on the first 3 postoperative days. Healing was monitored radiographically. Weightbearing was unrestricted.

Administration of FGF-1 and FGF-2

FGF-1 and FGF-2 (purified from bovine brain, R & D Systems Inc., Minneapolis, MN, U.S.A. and Oxford, UK) were dissolved in 0.9% saline containing 1 mg/mL bovine serum albumin (BSA–fatty acid-free). To ensure maximal exposure of the developing callus to the growth factor, 3 injections were given around the fracture site (at the midpoint of the plate), using a Hamilton syringe with a 25-gauge needle. Each injection was of 1 µg FGF-1 or FGF-2 in 20 µL carrier solution, so that the fractures received 3 µg of growth factor. Control animals were given 3 injections of 20 µL carrier solution only.

Experimental groups

For the quantitative analysis of the calluses, the fractures were divided into 2 groups. Group I fractures were stabilized with DC-plates and group II with plastic plates. In both groups I and II, 4 fractures were injected with 3 µg FGF-1 and 4 with 3 µg FGF-2 on day 4 and the rabbits were killed 10 days after fracture. 9 uninjected 10-day fractures (groups III and IV) were used as controls for the quantitative analysis. Thus, a total of 25 rabbits were used (Table 1).

For the qualitative assessment of the effects of FGFs on fracture healing under unstable mechanical conditions, 2 fractures were injected with 3 µg FGF-1 on day 4 and fixed on day 7, and 2 were injected with 3 µg FGF-2 on day 4 and fixed on day 14. Three control fractures were injected with vehicle on day 4 and fixed on days 7, 10 and 14 after operation. In addition, one mechanically stable fracture received injections of vehicle on day 4 and was fixed on day 10.

In preliminary experiments, 3 fractures that were healing under stable and 3 under unstable mechanical conditions were injected daily for 7 days starting on day 1 with 3 µg FGF-1, 3 µg FGF-2, or vehicle alone. At 10 days, the fractures showed no obvious effect of the multiple injections and the protocol was not pursued further.

Preparation of tissue for microscopy

The region of the tibia around the fracture site was removed and the tissue was fixed overnight at room temperature in 4% paraformaldehyde in 0.05 M Tris/HCl buffer, pH 7.3. After washing in buffer, the tissue was decalcified in 14.3% aqueous EDTA, pH 7.0, for 2–3 weeks at room temperature and the endpoint was determined radiographically. The tissue was then washed in buffer, dehydrated in graded ethanols and embedded in wax. Before fixation, the tissue was divided longitudinally into 3 pieces. One piece included the region under the plate and was not used. The other pieces were sectioned from both sides so that longitudinal sections were obtained from 4 parts of the callus. The sections were cut at 7 µm and stained with hematoxylin and eosin.

Quantification of tissues in the callus

One section was chosen at random from each of the 4 regions of the callus of fractures in groups Ia, Ib, IIa, IIb, III and IV (Table 1). Drawings were made of the region of each callus within a distance of 4 mm from the fracture site, using a drawing tube. The relative areas of bone, cartilage, fibrous tissue, including periosteum, and debris were determined using a point-counting method. The position of the regenerated periosteum was recognized by the bundles of longitudinally orientated collagen fibers. The actual area in mm^2 of the region of each callus drawn was obtained using an image analysis program. From these data, the actual areas of bone, cartilage, fibrous tissue and debris in each section were obtained. The means and standard deviations for the areas were calculated. The FGF-1 and FGF-2 injected fractures were compared with the uninjected control fractures, using the two-sample t-test (Tables 2 and 3).

Results

Fracture healing under stable mechanical conditions

Untreated fractures. Under stable mechanical conditions, the hematoma had disappeared 4 days after fracture. Bone was forming on the periosteal surface of the cortical bone which was covered with a thick layer of fibrous tissue. This developing bone crossed the fracture site. Over the next few days, bone formation continued and at 10 days the callus was complete (Figure 1) (group III, Table 1). In some sections, there were a few nodules of cartilage in the bone, but their position bore no relationship to the fracture site.

FGF-1-treated fractures. Treatment with 3 µg FGF-1 had little effect on the development of the callus

Table 1. Experimental groups

Mechanical conditions	Group	No. of animals	Type of FGF	Injection day
Stable	Ia	4	1 (acidic)	4
	Ib	4	2 (basic)	4
Unstable	IIa	4	1 (acidic)	4
	IIb	4	2 (basic)	4
Stable	III	4	none	none
Unstable	IV	5	none	none

Table 2. Callus data for fracture healing under stable mechanical conditions

	Total area (mm^2)			Bone (mm^2)			Cartilage (mm^2)			Fibrous tissue (mm^2)		
	Control	FGF-1	FGF-2	Control	FGF-1	FGF-2	Control	FGF-1	FGF-2	Control	FGF-1	FGF-2
Mean	8.21	7.41	7.98	6.06	6.23	6.44	0.61	0.18	0.41	1.54	1.01	1.13
SD	*2.66*	*2.11*	*3.44*	*2.51*	*2.05*	*2.71*	*0.35*	*0.18*	*0.55*	*0.52*	*0.23*	*0.54*
P-value[a]		0.66	0.92		0.92	0.84		0.07	0.58		0.11	0.32
CI[b]		-3.36–4.95	-5.10–5.55		-4.14–3.80	-4.90–4.13		-5.21–0.91	-0.60–0.99		-0.17–1.23	-0.51–1.34

[a] compared with that of the control using the two-sample t-test
[b] 95% confidence interval

Table 3. Callus data for fracture healing under unstable mechanical conditions

	Total area (mm^2)			Bone (mm^2)			Cartilage (mm^2)			Fibrous tissue (mm^2)		
	Control	FGF-1	FGF-2	Control	FGF-1	FGF-2	Control	FGF-1	FGF-2	Control	FGF-1	FGF-2
Mean	13.80	16.37	15.05	8.70	9.28	10.30	3.57	4.94	2.99	1.45	2.04	1.59
SD	1.12	3.12	1.30	0.99	1.60	2.40	1.70	2.21	1.17	0.51	0.60	1.24
P-value[a]		0.13	0.16		0.52	0.21		0.33	0.58		0.15	0.82
CI[b]		-6.07–0.94	-3.15–0.65		-2.64–1.46	-4.37–1.15		-4.43–1.70	-1.79–2.94		-1.46–0.28	-1.57–1.28

[a] compared with that of the control using the two-sample t-test
[b] 95% confidence interval

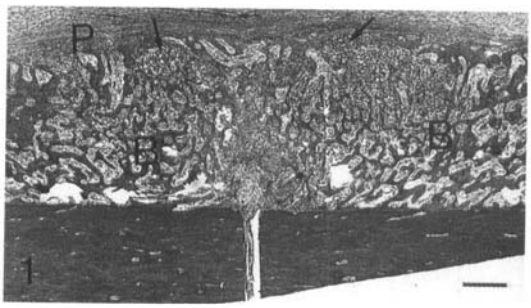

Figure 1. A 10-day untreated control fracture (group III) healing under stable mechanical conditions. The callus consists of bone (B) with very small areas of cartilage (arrows) just under the periosteum (P). Cells have moved into the fracture gap that has been created by the saw-cut used to "start" the fracture. Bar 0.5 mm.

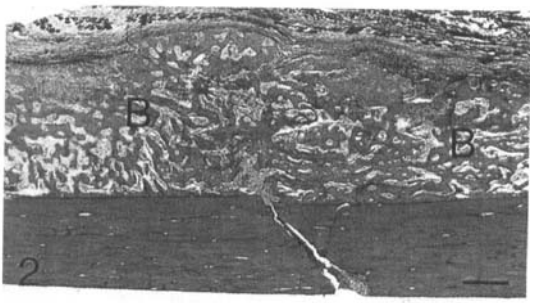

Figure 2. A 10-day mechanically stable fracture that was given 3 injections of 1 µg FGF-1 on day 4 (group Ia). The callus is similar to that shown in Figure 1 and consists in this region entirely of bone (B). Bar 0.5 mm.

(Figure 2). The quantitative analysis of the calluses of 10-day FGF-1-treated fractures (group Ia, Table 1) indicated that they did not differ significantly from those of control fractures (group III) (Table 2).

FGF-2-treated fractures. Injection of 3 µg FGF-2 on day 4 had no significant effect on the 10-day callus (group Ib, Table 1). This was confirmed by the quantitative analysis (Table 2).

Fracture healing under unstable mechanical conditions

Untreated fractures. Under unstable mechanical conditions, the first stages of callus development were similar to those under stable conditions, but the formation of new bone was restricted to regions away from the fracture site, leaving a central area of fibrous tissue. Within 7 days, the fibrous tissue was replaced in most regions by cartilage. The 10-day callus (Figure 3) (group IV, Table 1) was composed of a thick layer of bone that completely enclosed the cartilage and separated it from the periosteum.

Endochondral ossification had started. At 14 days, the area of cartilage was greatly reduced, but cells had not yet invaded the fracture gap.

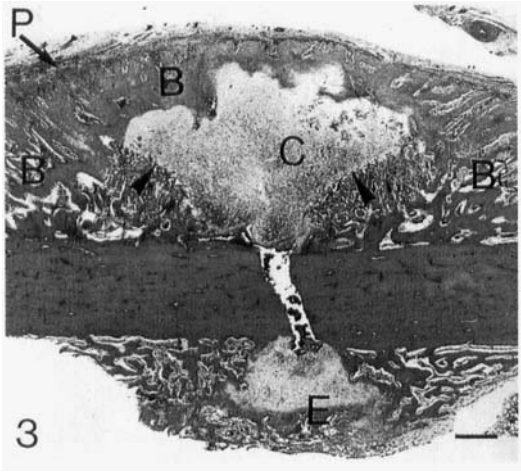

Figure 3. A 10-day fracture that is healing under unstable mechanical conditions. The region over the fracture is filled by cartilage (C), but it is completely surrounded by bone (B) which buttresses it from the sides and separates it from the periosteum (P). Endochondral ossification has started (arrowheads). There is some endosteal callus (E) that consists of both bone and cartilage. Bar 0.5 mm.

FGF-1-treated fractures. 3 days after injections of 3 µg FGF-1 on day 4, bone was forming on each side of the fracture site, leaving a wide gap. Small areas of cartilage were developing next to the bone, but fibrous tissue remained over the the fracture site. 6 days after injections of 3 µg FGF-1 on day 4, i.e., 10 days after fracture (group IIa, Table 1), the area over the fracture was filled by cartilage buttressed laterally by bone (Figure 4). Bone was beginning to grow over the cartilage to separate it from the periosteum, but the layer was incomplete. Endochondral ossification had started. Occasionally, fibrous tissue remained in the region of the callus near the plate.

The quantitative analysis of the 10-day calluses suggested that there may be a small increase in the overall size of the callus and in the amounts of cartilage and fibrous tissue compared to those of the control 10-day fractures, but none of the increases was significant (Table 3).

FGF-2-treated fractures. The callus of 10-day fractures that received 3 µg FGF-2 on day 4 (group IIb, Table 1) consisted of bone with some cartilage over the fracture site (Figure 5). The cartilage was covered by a layer of bone. Endochondral ossification was underway. In some regions near the plate, fibrous tissue was still present over the fracture site. At 14 days, the area of cartilage was greatly reduced, but cells had not yet entered the fracture gap.

The means of the size of the callus at 10 days and the amount of bone were slightly greater, but the amount of cartilage was lower than in the control fractures (group IV). All the differences were small and not significant (Table 3).

Discussion

Fracture healing occurs in 2 stages. The healing stage starts with the development of the callus and ends when cortical union is achieved. The remodeling stage then starts; the callus is resorbed and the cortical bone is remodeled to regain its original structure. Growth factors have been given continuously for approximately 6 weeks after fracture, that is, into the remodeling stage of healing (Lind et al. 1993, Nielsen et al. 1994); this may, however, hinder the restoration of normal structure because the addition of bone at the periphery of the callus will retard remodeling.

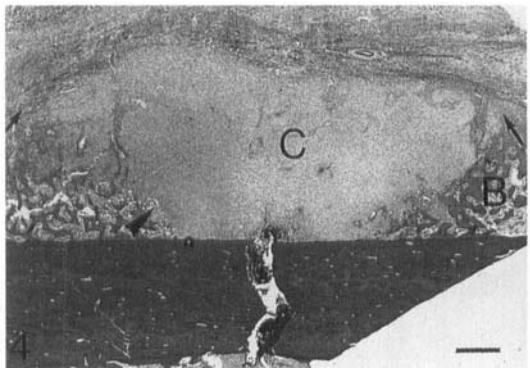

Figure 4. A 10-day mechanically unstable fracture that was given 3 injections of 1 µg FGF-1 on day 4. The area of cartilage (C) over the fracture is large. It is buttressed by bone (B) laterally, but it is only beginning to grow over the cartilage to separate it from the periosteum (arrows). Endochondral ossification has started in only one small region (arrowheads). Bar 0.5 mm.

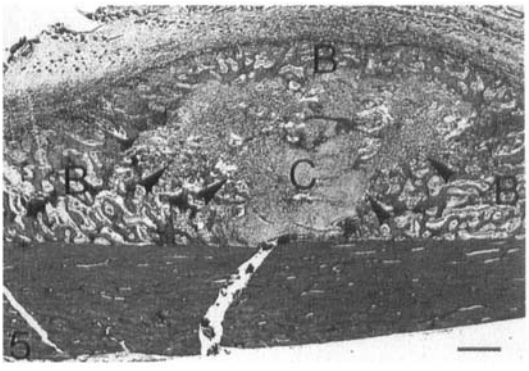

Figure 5. A 10-day fracture that was given 3 injections of 1 µg FGF-2 on day 4. The cartilage (C) over the fracture site is completely surrounded by bone (B) and endochondral ossification is underway (arrowheads). Bar 0.5 mm.

Indeed, this occurred after prolonged treatment of rat tibial fractures with growth hormone (Mosekilde and Bak 1993). To accelerate fracture repair, the rate of callus formation and maturation must be increased in the early part of the healing stage. In our study, the growth factors were, therefore, given on day 4 and the calluses were examined during the next 10 days.

In our rabbit model, the callus is fully developed by 10 days after fracture under stable mechanical conditions and bony union occurs by 3 weeks. Under unstable mechanical conditions, the callus at 10 days consists of a central region of cartilage with bone on either side. Endochondral ossification has started and replaces the cartilage in 3–4 weeks. Cortical union occurs in 4–5 weeks.

Neither FGF-1, nor FGF-2, has an appreciable effect on the size or composition of the callus formed under stable mechanical conditions.

Under unstable mechanical conditions, FGF-1 does not enhance the rate of cartilage formation because little is present 7 days after fracture, but by 10 days, an area of cartilage, slightly, but not significantly, larger than that of untreated controls has formed. It is not covered by a layer of bone. The callus is, therefore, not as mature as that of the uninjected control fractures and healing is retarded. A similar observation was made on healing rat fractures injected with FGF-1 (Jingushi et al. 1990).

FGF-2 has little effect on unstable fractures, but in contrast to FGF-1-treated fractures, the cartilage is completely covered with bone at 10 days, which indicates that the callus is more mature. Hence, FGF-2 may slightly accelerate the formation of the callus and, therefore, healing. At 14 days, the callus is qualitatively similar to that of uninjected fractures, which is a further indication that healing is not accelerated by FGF-2. Recently, Kawaguchi et al. (1994) found that FGF-2 implanted in a fibrin gel at the time of fracture of the rat fibula stimulates the development of a callus of significantly larger volume at 3 weeks if doses of 10 or 50 µg are given; the effect of 2 µg is minimal. It was pointed out above that a larger callus does not necessarily mean that healing is accelerated.

The efficacy of exogenous growth factors in vivo has been tested by injecting the factor of interest onto neonatal calvariae; FGF-2 does not induce extra bone formation at this site (Noda and Camilliere 1989). When cylinders of demineralized bone treated with FGF-2 were implanted intramuscularly in rats, Aspenberg et al. (1989, 1991) found that only amounts of 3–75 ng FGF-2 per implant induced a significant increase in bone mass (measured as Ca content) at 3 weeks, whereas doses of 1,900 ng caused a decrease in bone mass. In later experiments, a bone allograft treated with FGF-2 in hyaluronan was placed in a chamber implanted in the rat tibia and the ingrowth of bone from the tibia, determined 6 weeks later, was 51% higher than in the controls (Wang and Aspenberg 1994). Systemic treatment with FGF-1 and -2 (100–300 µg/kg/day) in young and old rats promoted endosteal, but not periosteal, bone formation (Mayahara et al. 1993, Nagai et al. 1995). The later experiments revealed, however, that whereas the low dose of FGF-2 increased the growth rate of 8-week-old rats, the high dose retarded it. It was suggested that the high dose reduced chondrocyte proliferation and bone formation at the growth plate (Nagai et al. 1995). It should be noted that Mazué et al. (1992) reported significant side-effects after a single intravenous dose of 100 µg/kg FGF-2 in both monkeys and rats (see below). Although these results show that increased bone formation was induced by FGFs, the amount of induced bone is relatively small, especially when compared to that produced in response to fracture.

The majority of the in vitro investigations of the actions of exogenous FGFs on osteoblasts and chondrocytes have used fetal or neonatal cells or animals. Both FGF-1 and -2 increase proliferation of calvarial cells, osteoblastic-cell lines and growth plate chondrocytes in vitro (Globus et al. 1989, McCarthy et al. 1989, Shen et al. 1989, Hiraki et al. 1990, Frenkel et al. 1992). Treatment with either FGF has no significant effect on the size of the fracture callus, which suggests that their mitogenic effects on the osteoprogenitor cells of young adult rabbits are minimal. A similar observation was made about the mitogenic effects of TGF-ß2 on osteoprogenitor cells in rabbit fracture callus (Critchlow et al. 1995). Further evidence is provided by the small amounts of bone formed in the rat experiments described previously (Aspenberg et al. 1989, 1991, Mayahara et al. 1993, Wang and Aspenberg 1994).

Our findings suggest that, at the dose levels we used, neither FGF-1 nor FGF-2 accelerates fracture healing. It could be argued that a higher dosage might produce better results, but Mayahara et al. (1993) and Mazué et al. (1992) found that while daily intravenous injections of FGF-2 in monkeys and rats produced increased bone formation, the side-effects were significant. High doses lead to hypertrophy of glomerular epithelium in the kidney and alveolar epithelium in the lung, while lower doses induce anemia. The side-effects of the administration of exogenous growth factors require more attention than they have so far received before their clinical use, at the dosages required, can be considered.

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