Dose-dependent stimulation of bone induction by basic fibroblast growth factor in rats

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Implantation of demineralized bone matrix in rodents elicits a series of cellular events leading to the formation of new bone inside and adjacent to the implant. This process is believed to be initiated by an inductive protein present in bone matrix, and local growth factors may further regulate the process. We have previously shown that local application of recombinant human basic fibroblast growth factor (bFGF) in a carboxymethyl cellulose gel to demineralized bone matrix implants increases the bone yield as measured by calcium content 3 weeks after implantation in rats. We now report that this increase was seen at 3 and 4 weeks, but not earlier or later. Further, the stimulatory effect was seen with doses from 3 to 75 ng per implant. A dose of 0.6 or 380 ng did not increase the bone yield, and 1,900 ng had a marked inhibitory effect. This narrow dosage optimum may reflect the complex actions of the growth factor.

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The implantation of demineralized bone matrix in rodents elicits a series of cellular events leading to the formation of new bone inside and adjacent to the implant (Urist 1965). Demineralized bone matrix has been used for treatment of skeletal disorders in man, but its effects are uncertain (Aspenberg 1988). With the full characterization and cloning of inductive proteins (Reddi 1991), bone induction may become more clearly useful in medicine.

Bone matrix contains large quantities of endogenous growth factors, and it has been suggested that these may participate in the regulation of the bone induction process once it has been initiated by the inductive protein (Urist et al. 1983).

Basic fibroblast growth factor (bFGF) is one of the endogenous factors found in bone matrix (Hauschka et al. 1986). It stimulates the proliferation of differentiated chondroblasts in vitro (Kato et al. 1987) and in vivo (Cuevas et al. 1988). Further, bFGF is a potent stimulant of capillary formation in vitro (Montesano et al. 1986) and in bone grafts (Eppley et al. 1988).

Previously, we have reported increased bone yield from subcutaneously implanted demineralized rat femoral diaphyses after local application of 75 ng recombinant human basic FGF in a carboxymethyl cellulose gel (Aspenberg and Lohmander 1989). We now report results from the same experimental model with various bFGF doses and at various postoperative times.

Material and methods

Animals

A total of 500 female Sprague-Dawley rats were obtained on different occasions from Møllegaard (Copenhagen, Denmark) and kept in our animal facilities for 1 week before the experiments started. For practical reasons, we performed the experiments with one group of about 20 animals at a time over a 1-year period. In each group, half of the rats were killed to provide implants for the others. Most of the rats were 60 days old at the start of the experiment. The body weights of the others corresponded to the same age.

Matrix preparation

Femoral diaphyses were collected from donor rats and immediately cleansed of periosteum and marrow. They were kept as pairs from each donor in sterile glass tubes, defatted with 12 mL chloroform-methanol 1:1 for 2 h at room temperature, rinsed in methanol, demineralized in 12 mL 0.6 M HCl for 48 h at room temperature with three changes, rinsed five times with sterile deionized water, lyophilized, and finally weighed. The implants were shaped into about 8-mm long and 3-mm wide cylinders, which became soft after rehydration. Representative samples were checked for completeness of demineralization, and contained less than 1 μg calcium per mg dry matrix.

Matrix pretreatment

Each pair of implants from 1 donor were implanted in 1 recipient rat. One implant in each pair served as an experimental implant and the other as a control. We used recombinant human bFGF (Synergen, Boulder, CO, U.S.A.). The experimental implants were treated with FGF (0, 0.02, 0.1, 0.5, 2.5, 12.5, and 62.5 μg/mL) in a carboxymethyl cellulose gel (3 percent carboxymethyl cellulose, 2.5 percent propylene glycol, 2.5 percent glycerol, 0.125 percent methyl parahydroxybenzoate, 0.0125 percent propyl parahydroxybenzoate in 10 mM sodium phosphate buffer pH 7). The gel was applied inside the former marrow cavity of the implants and allowed to moisten the lyophilized matrix for a few minutes before implantation. The mean gel volume per implant was about 30 μL (27 SD ± 7). The control implants were moistened with saline.

In 28 implants, 370 kBq of ^{125}I FGF (37 TBq/mmol bovine recombinant basic FGF, Amersham, specific activity 37 TBq/mmol) was added to the stock bFGF gel, which contained 0.5 μg nonlabeled bFGF per mL.

Implantation

The rats were anesthetized with intraperitoneal diazepam and pentobarbital. The skin was incised with an abdominal midline incision, and bilateral muscle pouches were created lateral to the rectus muscle by separating two oblique layers. The implants were inserted into the two pouches, which were closed with a suture. The skin was closed with wound clamps.

Treatment groups

Two doses of bFGF were used for studies of the duration of the effect. Rats given 15 ng of bFGF (0.5 μg/mL) were killed after 2, 3, 4, 5, and 6 weeks. Rats given 75 ng of bFGF (2.5 μg/mL) were killed after 2, 3, and 4 weeks (Table 1).

For the dose-response study, we used 0.6, 3, 15, 75, 380, and 1,900 ng. All the rats were killed at 3 weeks (Table 1). The ^{125}I bFGF-treated implants were retrieved after 0, 1, 6, 18, 24, 48, 96, and 216 hours (four implants for each time).

Evaluation

The rats were killed with an anesthetic overdose. The specimens were dissected, ashed in a muffle furnace (800 °C, 24 h), and dissolved in 1.5 mL of 6 M HCl.

Table 1. Number of implant pairs harvested (a few rats died before harvest). Exclusions were made on the basis of macroscopic appearance at harvest

Weeks/dose, ng	n	Excluded
Effect duration study, 15 ng		
2	9	0
3	9	0
4	9	0
5	10	0
6	9	0
Effect duration study, 75 ng		
2	20	1
3	30	1
4	30	3
Dose-response study		
0.6	27	3
3	35	3
15	28	2
75	30	3
380	37	0
1,900	13	1

The acid was evaporated in a vacuum centrifuge and the specimens redissolved. Calcium was measured in a DACOS machine using the thymol blue reaction. The activity of the entire ^{125}I bFGF-treated implants was measured in a gamma counter.

Specimens that appeared macroscopically infected were excluded, but no other exclusions were made. In each treatment group, the experiment-control difference in calcium content was tested with Wilcoxon's signed rank test and considered significant if $P < 0.01$.

Results

The absolute calcium yield varied between the different groups of animals. We therefore restricted the study to experiment-control differences within the groups.

The calcium content was increased by 15 ng of bFGF at 3 and 4 weeks, but not at 2, 5, or 6 weeks (Figure 1). The same was found with 75 ng, but less significant ($P < 0.03$).

Doses of 3, 15, and 75 ng of bFGF increased the calcium content at 3 weeks, with an optimum at 15 ng (78 percent increase). No effect was seen with 0.6 or 380 ng, whereas 1,900 ng had a powerful inhibitory effect (69 percent decrease; Figure 2).

The ^{125}I activity within ^{125}I FGF-treated implants declined exponentially by first order with a half-life of 114 hours (data not shown).

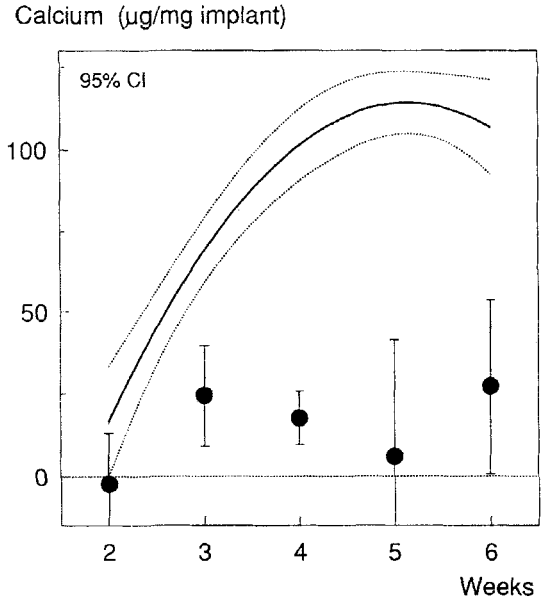

Figure 1. Implants treated with 15 ng bFGF for 2 to 6 weeks. Upper curve shows calcium content (2nd order regression and 95 percent confidence interval). Dots show the difference from control implants (mean and 95 percent confidence interval).

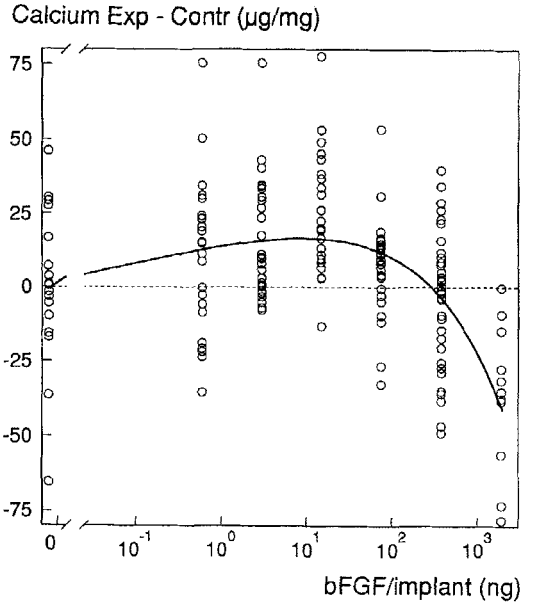

Figure 2. Implants treated with 0.6 to 1,900 ng bFGF for 3 weeks. Calcium content in µg per implanted mg bone matrix. To the left are controls treated with bFGF-free gel.

Routine histologic studies revealed cartilage and bone formation in both bFGF-treated and untreated implants.

Discussion

The present results confirm and extend our previous finding that the addition of exogenous bFGF to demineralized bone matrix can increase the amount of bone formed by bone induction. The long half-life of the ^{125}I activity within ^{125}I bFGF-treated implants does not necessarily imply a similar survival of active bFGF, but a prolongation of the bFGF activity by the carboxymethyl cellulose gel seems probable, because implant pretreatment with bFGF in a saline solution has no stimulatory effect (Aspenberg and Lohmander 1989). In vivo, bFGF is stored in the extracellular matrix, bound to glucosaminoglycans, which stabilize and protect it. The active bFGF concentration may be regulated by release from these glucosaminoglycans (Vlodavsky et al. 1991). The carboxymethyl cellulose gel of our model may act in a similar way.

The time until onset of ossification did not seem to be affected by the addition of 75 ng of bFGF to the implants, as no difference in calcium content was found at 2 weeks, when ossification had just started. The increased calcium deposition that followed during the third and fourth weeks in the bFGF-treated implants may have several explanations. Because the bFGF probably exerted most of its effects during the first few days, it seems possible that it has stimulated the formation of a larger cartilaginous template for ossification. Stimulation of cartilage formation by bFGF has also been shown in joint cartilage in vivo (Cuevas et al. 1988), and directly injected acidic FGF increased the size of cartilaginous fracture calluses in rats, but seemed to weaken the mechanical callus strength (Jingushi et al. 1990).

bFGF is a potent stimulator of vascularization (Montesano et al. 1986), and may also have stimulated the ossification of the nonvascularized cartilage inside the implants by increasing capillary ingrowth. This explanation would require that the FGF activity persisted in the implants for 2 to 3 weeks, which is uncertain due to the half-life of about 4 days for ^{125}I bFGF.

The maximum calcium content was reached after 5 weeks in both bFGF-treated (15 ng) and untreated implants. The bone resorption in this model is considerable already at early stages (Bauer et al. 1984). At 5 weeks, bone deposition and resorption are in balance and the implant is in a stage of remodeling, and no

stimulatory effect of bFGF was seen. Thus, we could not demonstrate a final net increase in bone mass, but these groups were small, with large variation.

The inhibitory effect of larger bFGF doses may have many explanations, because an enormous number of interactions with different cell types, other growth factors and their regulation are possible. The simplest explanation would be that bFGF-receptors are down regulated.

We conclude that bone formation in vivo may be both stimulated and inhibited by bFGF, depending on dosage. In future attempts to stimulate bone formation with bFGR, dose and time regimens must be well controlled.

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