Fibroblast growth factor stimulates bone formation

Bone induction studied in rats

Per Aspenberg and L. Stefan Lohmander

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. It has been suggested that local growth factors may further regulate the process once it has been initiated. This investigation was designed to study the effect of adding a growth factor to the inductive implant. Pairs of demineralized rat femoral diaphyses were implanted intramuscularly in rats. The marrow canal of one implant in each pair was filled with a carboxymethyl cellulose gel containing 75 ng of recombinant human basic fibroblast growth factor (FGF). The other implant in each pair served as a control. It was either filled with the gel without FGF or left untreated. Bone formation was induced by all the implants after 3 weeks. The amount of mineralized tissue in the FGF-treated implants was 25 percent greater than in untreated controls. The carboxymethyl cellulose gel alone did not affect the bone yield.

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). Stromal tissue stem cells at the recipient site migrate into the implant and are induced to multiply and differentiate into chondroblasts. The cartilage thus formed will then be ossified by endochondral ossification. This process is believed to be initiated by an inductive matrix protein, which causes the attracted stem cells to determine their future differential pathway to cartilage- and bone-forming cells (Urist et al. 1983, Reddi et al. 1987, Aspenberg 1988, Triffitt 1987). Bone induction by this mechanism has similarities both with bone formation in the growth plate and with fracture healing.

Bone matrix contains large quantities of endogenous growth factors (Hauschka et al. 1986), and it has been suggested that these may participate in the regulation of the bone-induction process once it has has been initiated by the inductive protein.

Lund University Hospital Department of Orthopedics, S-221 85 Lund, Sweden

Fibroblast growth factor (FGF) is one of the endogenous factors found in bone matrix (Hauschka et al. 1986). It is known to stimulate colony formation by anchorage-independent chondroblasts in vitro (Kato et al. 1987). Further, FGF is a potent stimulant of capillary formation (Montesano et al. 1986). Both chondrocyte colony formation and capillary formation are important steps in the development of bone following an inductive stimulus. We therefore tested the effect of adding recombinant human basic FGF to inductive bone-matrix implants. The FGF was applied to rat bone matrix both in a solution and in a carboxymethyl cellulose gel intended for slow release. The matrix was then implanted intramuscularly in rats, and the resulting amount of bone was estimated after 3 weeks.

Material and methods

Animals

A total of 100 female Sprague-Dawley rats were obtained on different occasions from Møllegård avelslaboratorier (Copenhagen, Denmark) and kept in the animal facilities for 1 week before the experiments start-

Table 1. Rat weights (g) at implantation, and weight gain during the experiment. Mean *SD*

Group	n	Implantation		Gain	
1	7	209	*8*	51	*9*
2	7	203	*6*	34	*14*
3	10	212	*5*	42	*10*
4	10	217	*10*	26	*6*
5	10	227	*11*	27	*8*

ed. Five differentially treated groups were studied. For practical reasons, we performed the experiments with one group at a time over a 6-month period. In each group, half of the rats were killed to provide implants for the others. Body weights at the start of the experiment corresponded to an approximate age of 60 days (Table 1).

Matrix preparation

Femur 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 N HCl for 48 hours at room temperature with three changes, rinsed five times with sterile deionized water, lyophilized, and finally weighed. The implants appeared as 8-mm-long x 3-mm-diameter cylinders, which became soft after rehydration. Representative samples were checked for completeness of demineralization, and contained less than 1 µg calcium per milligram 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 experiment implant and the other as a control. We used recombinant human basic FGF (Synergen, Boulder, CO, U.S.A.). Solutions or gels with and without FGF were applied inside the former marrow cavity of the implants and allowed to moisten the lyophilized matrix for a few minutes before implantation.

Group 1. The experiment implant in each pair was treated with an FGF solution (5.5 µg/mL) in phosphate buffered saline (physiologic saline in sodium phosphate buffer, pH 7.4), produced by a 1:1000 dilution of a stock solution immediately before use. With a Hamilton syringe, 25 µL (140 ng) was deposited in the marrow canal under aseptic conditions. The solution was absorbed by the lyophilized matrix in a few minutes before implantation. The control implants were treated similarly with buffered saline only.

Group 2. The experiment implants were treated with FGF (2.5 µg/ml) in a carboxymethyl cellulose gel (3% carboxymethyl cellulose, 2.5% propylene glycol, 2.5% glycerol, 0.125% methyl parahydroxybenzoate, 0.0125% propyl parahydroxybenzoate in 10 mM sodium phosphate buffer pH 7). The marrow canal was filled with the gel using a Pasteur pipette. The mean gel volume per implant was about 30 µL, containing about 75 ng FGF per implant. The controls were treated similarly with gel without FGF.

Group 3. Experiment implants did not differ from experiment implants in Group 2 (gel with FGF). Control implants were implanted in their lyophilized state, without gel.

Group 4. This group was treated exactly as Group 3, so in reality it was an extension of that group. However, it was done 5–6 months later and due to possible seasonal differences in the animals, Groups 3 and 4 are reported separately.

Group 5. The experiment implants were treated with gel without FGF, and control implants were implanted in their lyophilized state.

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 stuck into the two pouches, which were closed with a suture. The skin was closed with wound clamps. There were 7 rats each in Groups 1 and 2 and 10 rats each in the other groups.

Evaluation

The rats were killed after 21 days. The specimens were dissected, ashed in a muffle furnace (800 °C, 24 h), and dissolved in 1.5 mL of 6 M HCl. The acid was evaporated in a vacuum centrifuge, and the specimens were redissolved. Calcium was measured in a DACOS machine using the thymol-blue reaction (Cossar and Fitzpatrick 1974). Parallel to Group 2, 4 animals were identically treated, but specimens were prepared for histology (Aspenberg et al. 1988). Two animals in Group 1 and 1 animal in Group 3 were excluded because of infections.

Table 2. Calcium yield from pairs of demineralized rat femur diaphyses implanted in rats. One implant was pretreated with FGF, the other serving as control. Mean *SD*

	Implanted matrix mg		Calcium/implant µg/mg		*P*-value [a]
Group 1 (n 5)					
FGF	13.1	*1.6*	75.2	*24.2*	
PBS	12.7	*1.4*	87.6	*18.8*	
FGF-PBS	+0.3	*2.2*	−12.4	*14.6*	NS
Group 2 (n 7)					
FGFcmc	17.8	*1.9*	64.9	*20.8*	
cmc	17.4	*1.0*	20.7	*20.2*	
FGFcmc-cmc	+0.4	*2.0*	+44.2	*10.8*	< 0.001
Group 3 (n 9)					
FGFcmc	17.2	*1.5*	73.1	*18.0*	
Untreated	17.3	*2.6*	63.8	*14.3*	
FGFcmc—untreated	−0.1	*2.8*	+9.3	*9.2*	< 0.02
Group 4 (n 10)					
FGFcmc	19.0	*2.1*	32.5	*12.0*	
Untreated	18.0	*1.3*	20.8	*10.4*	
FGFcmc—untreated	+1.0	*2.8*	+11.7	*14.7*	< 0.05
Group 3 and 4 combined (n19)					
FGFcmc	18.1	*2.0*	51.7	*25.5*	
Untreated	17.7	*2.0*	41.1	*25.2*	
FGFcmc—untreated	+0.4	*2.6*	+10.6	*12.1*	< 0.002
Group 5 (n10)					
cmc	19.3	*2.8*	32.1	*19.1*	
Untreated	18.0	*3.0*	31.0	*20.2*	
cmc—untreated	+1.3	*1.9*	+1.1	*12.2*	NS

[a] Student's *t*-test for paired observations.

Results

Simply adding FGF solution caused no difference in calcium yield compared with saline only (Table 2), whereas the FGF gel implants yielded three times more calcium than gel-treated controls ($P < 0.001$; Table 2) and 25 percent more calcium than untreated controls ($P < 0.002$; Table 2). There was no difference between gel-treated and untreated implants (Table 2).

Histologic examinations showed cartilage and bone formation in gel-treated implants with or without FGF. Calcium yield from the control implants containing neither FGF nor gel differed among the different groups of rats; it was positively correlated with weight gain during the experiment (n 36; $r = 0.6$; $P < 0.001$).

Discussion

Our results clearly show that the addition of exogenous FGF to demineralized bone matrix may increase the amount of bone formed by bone induction even in young rats.

In a similar system, Howes et al. (1988) demonstrated an increase of cartilage and bone formation by platelet-derived growth factor added to bone matrix powder in mature rats. They found no effect of 100 ng of FGF; and in young rats, they found no effects of platelet-derived growth factor either. Our experiment differs from theirs in that the FGF was delivered in a cellulose gel, and in that we used nonpulverized bone matrix. The role of the gel is not clear, but it may have provided a slow-release system and protection against the rapid inactivation of FGF under physiologic conditions. Because no effect of FGF was found without the gel, such functions seem necessary.

Our experiment was designed with untreated controls in each animal in order to eliminate the hazards of some animals or groups being more active bone formers than others. This was a fortunate measure, because the bone yield from control implants varied considerably between groups. This bone yield correlated with animal weight gain during the experiment. The groups with low weight gain may have been a few weeks older at the beginning. Further, these groups were delivered to us during the summer, so seasonal changes may have occurred.

We found no negative effect of the gel alone in comparison with untreated implants. Therefore, it is surprising that implants treated with FGF in the gel yielded three times more bone than gel-treated controls, but only 25 percent more than untreated controls. Due to the variation between groups, however, we prefer to

conclude merely that FGF has had a stimulatory effect and defer to further studies the determination of the quantitative aspects.

The cellular mechanisms influenced by FGF in this system are unknown. Cartilage proliferation may have been directly stimulated as demonstrated in vitro (Kato et al. 1987). Because FGF is a potent stimulator of capillary formation (Montesano et al. 1986), it may have facilitated endochondral ossification of the cartilage, which requires vascular supply.

Possibly, bone inductive implants will be available for clinical use in the future, consisting of a recombinant inductive protein on an appropriate carrier. The present result may suggest that one or several growth factors should be added to such an implant.

A considerable number of endogenous growth factors may be involved in fracture and soft-tissue healing. The functions of growth factors in vivo are, however, largely unknown and difficult to predict from the present in vitro knowledge. We have found exogenous FGF to enhance a process that probably occurs in the presence of a number of endogenous growth factors. Possibly, other related processes, such as bone healing, may also be influenced by exogenous growth factors.

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