Basic fibroblast growth factor and bone induction in rats

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Bone induction is initiated by bone morphogeneic proteins, but local growth factors present in demineralized bone matrix (DBM) 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 DBM implants increases the bone yield, as measured by calcium content. In the present study, similar experiments were evaluated with histomorphometry. The chondrocyte number at 2 weeks was increased by the application of 15 ng bFGF. This increase was due to an increased number of chondrocyte clusters, i.e., cartilage formation was initiated in more places within the implant. The size of the individual chondrocyte clusters was the same as in the controls. Thus, the bFGF had probably stimulated cellular events preceding chondrocyte proliferation. At 3 weeks, the chondrocytes were fewer than in controls, and instead there was more bone. Thus, cartilage formation was increased by bFGF, and its replacement by bone came earlier. However, 1900 ng of bFGF had a profound inhibitory effect on both cartilage and bone formation.

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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 has been initiated (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 femur diaphyses after exogenous application of recombinant human bFGF in a carboxymethyl cellulose gel at doses from 3 to 75 ng per implant. This effect was seen 3 and 4 weeks after the implantation, at which time the calcium content of retrieved implants was increased by 78 percent with an optimal bFGF dose of 15 ng per implant. In contrast, 1900 ng had a marked inhibitory effect (Aspenberg and Lohmander 1989, Aspenberg et al. 1991). However, it is not clear if the increased bone yield was caused by effects on one or more of the early cellular processes of bone induction—like inflammation, recruitment of stem cells, prechondroblast cell division, etc., leading to the formation of a bigger cartilaginous template. The increased amount of bone could also have been caused by a faster development of the capillary supply which is necessary for the replacement of cartilage by bone. In order to separate the effects on early cellular events from those on vascular supply, it was necessary to measure the amount of induced cartilage.

We have now repeated the experiments using optimal bFGF concentrations for stimulation and inhibition of bone formation, respectively. For evaluation, we counted cartilage cells and measured histologic bone areas to demonstrate a relationship between the previous calcium content values and histologic bone.

Animals and methods

Animals

54 female Sprague-Dawley rats weighing 199–229g were obtained from Møllegård (Copenhagen, Denmark) and kept in the animal facilities for 1 week before the experiments started. The animals were fed a standard laboratory diet. In each group, half of the rats were killed to provide implants for the others.

Matrix preparation

Femoral diaphyses were collected from donor rats and

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Table 1. Chondrocyte density index and ossification index. Mean *SEM*

bFGF	Pairs	Weeks	Chondrocyte index		Ossification index	
µg/mL	n		bFGF	Control	bFGF	Control
0.5	7	1	0	0	0	0
0.5	7	2	40 *6.4*[a]	26 *2.8*	8.1 *2.0*[c]	3.0 *1.2*
0.5	6	3	15 *3.3*[c]	35 *4.9*	38 *4.6*[b]	26 *3.9*
62.5	7	3	1.6 *0.9*[c]	29 *5.2*	5.4 *2.6*[c]	21 *0.9*

bFGF implants compared with controls. [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

immediately cleaned of periosteum and marrow. The specimens were kept as pairs from each donor, defatted and demineralized, as described previously (Aspenberg et al. 1991). The demineralized implants were shaped as 8 mm long and 3 mm wide tubes, which would soften after rehydration.

Matrix pretreatment

Each pair of implants from 1 donor was implanted in 2 separate muscle pouches in one recipient rat, as described previously (Aspenberg et al. 1991). One implant in each pair served as the experimental implant and the other as control. We used recombinant human basic FGF (Synergen, Boulder, Colorado, U.S.A.). The experimental implants were treated with bFGF (0.5 and 62.5 µg/mL) in a carboxymethyl cellulose gel (Aspenberg et al 1991). 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 32.9 ± 7.3 µL. In previous studies there was no difference between implants which were treated with the gel alone or moistened with saline (Aspenberg and Lohmander 1989). In this study, controls were only treated with saline.

Evaluation

The rats were killed after 7, 14, and 21 days, respectively (Table 1). No rat was lost or excluded from the study. The implants were dissected from the surrounding soft tissue, fixed in 4% buffered formalin, embedded in hydroxyethylmethacrylate (Cambridge Instruments GmbH, Heidelberg). Sections, 8 µm thick, were cut perpendicular to the long axis of the implant. The whole implant was sectioned throughout and sections at 1 mm intervals were taken for analysis. The sections were stained with hematoxylin and eosin (HE), Toluidine blue and von Kossa stain for different measurements.

The investigator did not know if the sections were derived from experiment or control specimens. Using a microscope with a video camera connected to an image analysis system (Videoplan, Kontron Bildanalyse GMBH, Germany), the total area of the bone matrix implant and the calcified areas on the slides were measured. The bone areas were delineated using von Kossa stain but were always compared to HE staining in adjacent sections. The number of chondrocytes in the bone matrix was counted manually by dividing Toluidine blue-stained sections in 1 × 1 mm areas and counting the chondrocytes in each of them. A "chondrocyte density index" was created by dividing the cell number by the total area of the bone matrix implant in that section, and an "ossification index" was calculated by dividing the induced bone area by the total area of implanted bone matrix in the same section.

The number of chondrocyte clusters and the area of each cluster were measured with another morphometry computer (CAS Inc., Becton Dickinson, Elmhurst, Illinois, U.S.A). Since the three most proximal sections of each implant turned out to contain the vast majority of the chondrocytes, the cluster measurements were restricted to these sections. The cluster area was defined as the Toluidine-stained area surrounding at least 1 chondrocyte.

All measurements were analyzed with 2 factor ANOVA.

Results

Chondrocytes in 0.5 µg/mL bFGF-treated implants

By week 1, numerous elongated fibroblast-like cells had appeared in close proximity to the implanted matrix, and the invasion of the implanted matrix had begun. Only a few chondrocyte-like cells but no cartilaginous matrix were found in the implanted matrix. There was no qualitative difference between bFGF-

Table 2. Chondrocyte clusters in bFGF 0.5 µg/mL-treated implants vs. controls at 2 weeks. Mean *SEM*

Cluster measurements	bFGF	Control	Ratio
Chondrocyte cluster number (per section)	100 *14*[a]	61 *4.1*	1.6
Chondrocyte cluster size ($mm^2 . 10^{-3}$)	1.1 *0.1*	1.1 *0.2*	1.0

bFGF implant compared with controls. [a] $P < 0.005$.

treated implants and controls and a quantitative comparison was not possible.

At 2 weeks, chondrocytes surrounded by a Toluidine staining matrix were extensively present within the bone matrix. In the bFGF-treated implants the chondrocyte density index was increased by 57 percent compared to that of the controls (Table 1; Figures 1 and 2). The chondrocyte cluster number was increased by 64 percent. The chondrocyte cluster size was unchanged (Table 2).

At 3 weeks, the chondrocyte density index in the bFGF implants was smaller than in the bFGF implants at 2 weeks. In the controls, the chondrocyte density index was higher than at 2 weeks (Table 1).

Ossified areas in 0.5 µg/mL bFGF-treated implants

At 1 week, there was no bone. At 2 weeks, the ossification index in bFGF-treated implants was higher than in controls (Table 1). At 3 weeks, the ossification index in the bFGF-treated implants was still higher than that of the controls (Table 1; Figures 3 and 4). As the osteogenesis continued, the cartilage mass was gradually replaced with bone. Numerous osteoblasts and multinucleated osteoclasts were seen surrounding the bone. The newly formed bone was associated with the dissolution of implanted matrix and the formation of an ossicle. Some ossified areas contained bone marrow elements, including fat cells and hematopoiesis.

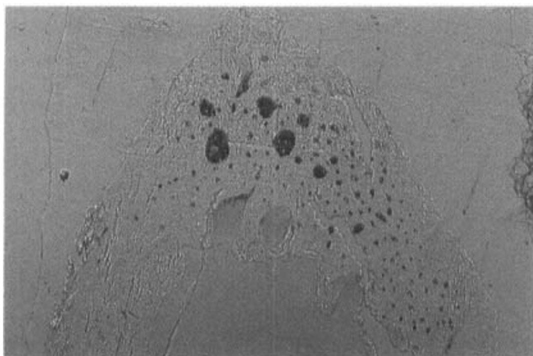

Figure 1. Induced cartilage in demineralized bone matrix treated with 0.5 µg/mL bFGF at 2 weeks (Toluidine blue, ×16).

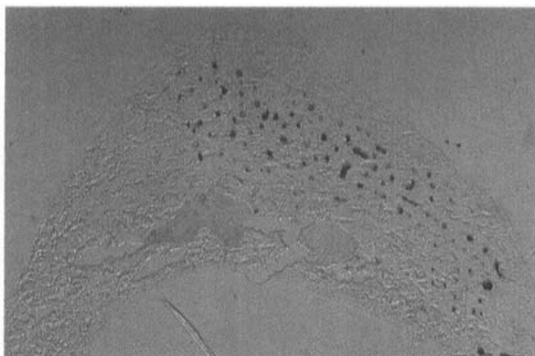

Figure 2. Induced cartilage in demineralized bone matrix without bFGF treatment at 2 weeks. Control implant from the same rat as Figure 1 (Toluidine blue, ×16).

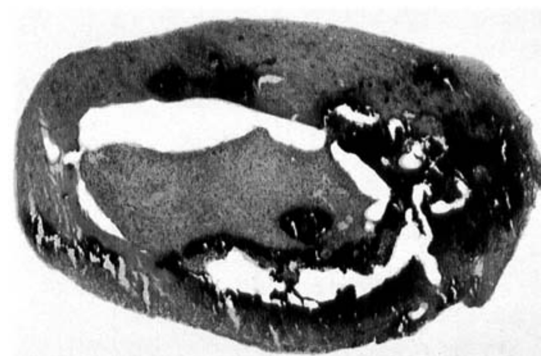

Figure 3. Induced bone (black) in demineralized bone matrix treated with 0.5 µg/mL bFGF at 3 weeks (von Kossa and HE, ×10).

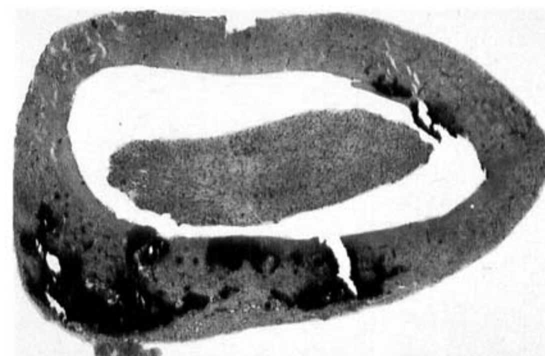

Figure 4. Control implant from the same rat as Figure 3 (von Kossa and HE, ×10)

Chondrocytes and ossified areas in 62.5 μg/mL bFGF-treated implants

At 3 weeks, the chondrocyte density index and the ossification index were lower than in the controls and the implants with 0.5 μg/mL bFGF (Table 1). In all implants (controls as well as bFGF-treated with both 62.5 and 0.5 μg/mL doses), there were areas without any new cartilage or bone present. In these areas, cavities within the bone matrix contained mostly fibroblast-like cells. The implants treated with bFGF 62.5 μg/mL appeared similar to those inert areas of the other implants. There was no sign of inflammatory reaction or increased vascularity. In the controls of the bFGF 62.5 μg/mL series, the number of chondrocytes and the ossified area did not differ from the controls of the series with bFGF 0.5 μg/mL at 3 weeks (Table 1).

Discussion

If the bFGF had stimulated bone formation in this model only by increasing capillary ingrowth into the implant, the number of chondrocytes would probably not have been affected. The increased number of chondrocytes indicates that the bFGF has acted on one or more of the early events during bone induction, starting with inflammation and stem cell recruitment, and ending with cartilage formation.

There were no signs of increased chondrocyte proliferation, i.e., chondrocyte clusters had not grown more than controls, but cartilage formation had started in more places within the implants. Thus, either the bFGF made more places in the implant suitable for cartilage formation or it had exerted its effect earlier in the process.

One step in the bone induction cascade is the proliferation of undifferentiated mesenchymal cells that later will transform to chondrocytes. As bFGF is a known potent mitogen for mesenchymal cells, this step is likely to be influenced by bFGF in our model. This would be consistent with the findings of Jingushi et al. (1990) that repeated injections of acidic FGF into fracture calluses made them grow bigger, but less mature. However, in our model, there are many other early events which may have been modified by the bFGF as, for example, the initial inflammation or the expression of BMP-receptors.

The ossification came earlier with bFGF treatment. This might indicate that the cartilage had matured more quickly. However, bFGF has been shown to inhibit the terminal differentiation of chondrocytes in vitro (Kato and Iwamoto 1990). Since capillaries are necessary for bone formation, the earlier ossification may also reflect a stimulated capillary ingrowth into the interior of the implant, as has been shown in bone grafts (Eppley et al. 1988). Although bFGF is a potent stimulator of capillary formation and penetration into collagenous materials (Montesano et al. 1986), a stimulated capillary formation would require a remaining bFGF effect at 2–3 weeks after the implantation, which seems unlikely with respect to the half-life of bFGF in this model (Aspenberg et al. 1991).

The way of applying the bFGF is important: dropping a bFGF-saline solution (140 ng) onto the dry implant was not effective (Aspenberg and Lohmander 1989). Risto et al. (1991) found that continuous application of a PDGF solution with a tube into similar implants seemed to cause resorption, and this was our experience with bFGF also.

In addition to the BMPs, bone matrix contains large quantities of a number of growth factors. It is possible that these growth factors contribute to the effects of bone matrix implants. However, they are not indispensable, since pure BMP in a synthetic carrier can induce bone, if large enough amounts are applied (Yasko et al. 1992). Synthetic implants containing BMP may solve some of the problems associated with bank bone. The addition of growth factors, like bFGF, to such implants may further increase their efficiency.

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