Basic fibroblast growth factor increases allograft incorporation

Bone chamber study in rats

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We found increased penetration of new bone into a **frozen bone allograft which had been pretreated with basic fibroblast growth factor (bFGF). Pairs of grafts were placed in newly designed titanium bone chambers implanted bilaterally in rat tibiae. The ingrowing bone can enter the cylindrical interior of the chamber only at one end. It then penetrates the graft inside the chamber but, due to the length of the cylinder, it never reaches the other end. The distance which the ingrown bone has reached into the graft can then be measured on histological slides. With bFGF there was a 51 percent increase in the bone penetration distance at 6 weeks in this model. It also appeared that further penetration had almost ceased in the controls, whereas in the bFGF-treated specimens, membranous ossification was still going on.**

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bFGF is present in diverse tissues of the body, intracellularly or bound to heparin-like molecules of the extracellular matrix. It is thought to perform its physiological functions in tissue repair and neovascularization after being released from extracellular matrix molecules rather than from secreting cells (Baird and Walicke 1989, Gospodarowicz 1990, Vlodavsky et al. 1991). Free bFGF is highly angiogeneic, causing the formation of capillary sprouts by migration, proliferation, and organization of endothelial cells (Folkman and Klagsbrun 1987). bFGF causes mesenchyme formation and stimulates skin wound healing by increasing cell recruitment, mitosis, and collagenase production, the latter leading to faster reorganization of collagen (McGee et al. 1988, Buckley-Sturrock et al. 1989). bFGF can stimulate the proliferation of osteogenic cells and chondrocytes, while mostly reducing matrix production (Frenkel et al. 1990). We investigated whether bFGF is potentially useful for enhancing the incorporation of bone allografts.

Material and methods

The chamber

The bone conduction chamber (Aspenberg and Wang 1993) consists of a threaded titanium cylinder, formed from two half-cylinders held together by a hexagonal closed screw cap. The overall length is 13 mm, the screw cap is 7 mm, leaving 6 mm of the implant to be screwed into the bone. The bone ingrowth chamber has an inside diameter of 2 mm, and an inside length of 7 mm. The outside diameter is 3 mm. There are two bone ingrowth openings, 1 mm in diameter, at the bottom of the chamber (Figure 1).

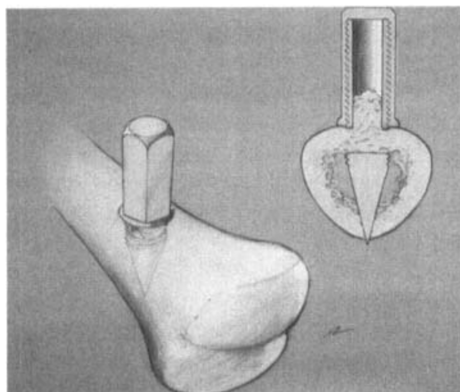

Figure 1. The bone conduction chamber is designed to allow bone ingrowth from one end of a long cylinder, through which the bone can be conducted. The implant is screwed into the bone so that the two bone ingrowth openings are located just below the periosteum, and the outer part extends into the subcutis.

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Graft preparation

We used 1% hyaluronate gel (Kabi Pharmacia, Uppsala, Sweden) as carrier for recombinant human bFGF (Synergen, Boulder, CO, U.S.A.) at a concentration of 2.5 μg bFGF/mL. This dose was chosen according to dose-response studies of bFGF-stimulation of bone induction (Aspenberg et al. 1991). Hyaluronate gel has been described as a useful carrier for growth factors (Prisell et al. 1992).

The allografts were taken from the proximal tibiae of donor rats. A 2 × 6-mm bone rod was resected in the axial direction from the knee joint with a hole-cutter. The epiphysis was excised. The proximal part of the graft had the densest cancellous bone and was later placed at the ingrowth end of the chamber. Each rat yielded two grafts. The pair was kept sterile and frozen at −70 °C. Before implantation, the pairs were lipid-extracted in chlorophorm/methanol overnight, rinsed 3 times in methanol and air dried. This extraction enhances the graft incorporation rate in another chamber model, by diminishing the immunologic response (Thorén et al. 1993).

One graft in each pair was placed in the gel with bFGF for 16 hours and the other graft in the gel without bFGF.

Operation

24 Sprague-Dawley rats were obtained from Møllegaard (Copenhagen, Denmark; 12 female donors 200–220 g; 12 male recipients 320–350 g). They were kept in our animal facilities for 1 week before experiments started (22 °C; 2 rats in each cage, free access to food pellets and water). The rats were anesthetized with peritoneal injections of 0.6–0.7 mL of a solution containing 1mL pentobarbital (60 mg/mL) and 2 mL diazepam (5 mg/mL) and 1 mL saline, and the rats were killed with an overdose of Mebumal.

Under aseptic conditions, longitudinal incisions were made bilaterally over the anteromedial aspect of the proximal tibial metaphyses. After incising and raising the periosteum, the medial and posterior lateral cortices were pierced with a 1.0-mm spike just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was manually enlarged with a 2.7-mm drill. The graft was placed in the chamber, which was then screwed into position so that the bone ingrowth holes were placed at the level of the cortical bone, and the pointed end of the implant was engaged through the opposite cortical bone. The wound was closed in layers with 5/0 Dexon interrupted fascial suture and a 4/0 monofilament nylon continuous, subcutaneous stitch.

There were no infections and no sample was excluded.

Evaluation

The rats were killed after 6 weeks. The harvested tissue was fixed in 4% formalin, decalcified and embedded in paraffin. The specimens were cut parallel to the long axis of the chamber with a microtome and stained with hematoxylin and eosin. Three sections from the middle of the specimens, each at 300 μm distance from the other, were used for histology and histomorphometry. This was done blindly, so that each specimen was given a code number, and all specimens were investigated in random order. The area of the new ingrown bone was measured with the Videoplan™ equipment at magnification 125 ×. This area includes marrow cavities and graft bone remnants which had been surrounded by new bone. Sometimes the border between the new bone and the graft was difficult to define, just as it is difficult to define the Swedish coastline due to all islands in the archipelagos. In those cases, straight lines were drawn to connect 7 most distant points, where new bone had reached (like connecting the most far out islands).

As we measured an area, but are more interested to know the distance which the new bone had reached into the graft, the mean ingrowth distance was calculated by dividing the new bone area with the distance between the walls of the chamber, i.e., the width of the specimen (Aspenberg and Wang 1993). This ingrowth distance was tested for significance, using 2-factor Anova.

The presence of osteoid just distal to the new bone, was scored as 0 (none), 1 (some) or 2 (abundant), and tested for significance with Wilcoxon's signed rank test.

Results

New vascularized tissue usually filled the entire chamber spaces. At the distal end, far away from the bone ingrowth openings, there was a loose connective tissue with small, mostly spindle-formed, nuclei surrounding the cancellous bone graft trabeculae. In the proximal end of the specimen there was an ossicle, with a large marrow cavity, containing some new woven bone trabeculae, surrounded by a thicker shell of new, partly lamellar bone. The interface between the proximal ossicle and the mostly non-resorbed graft located distally differed between controls and bFGF-treated specimens. In the controls, there was a clear-cut borderline: the new bone was covered by a mature fibrous tissue parallel to the borderline, and most of the graft bone was resorbed in that area (Figure 2). In the bFGF-treated specimens, there was no such borderline, but an interdigitation, with new bone sprouts protruding

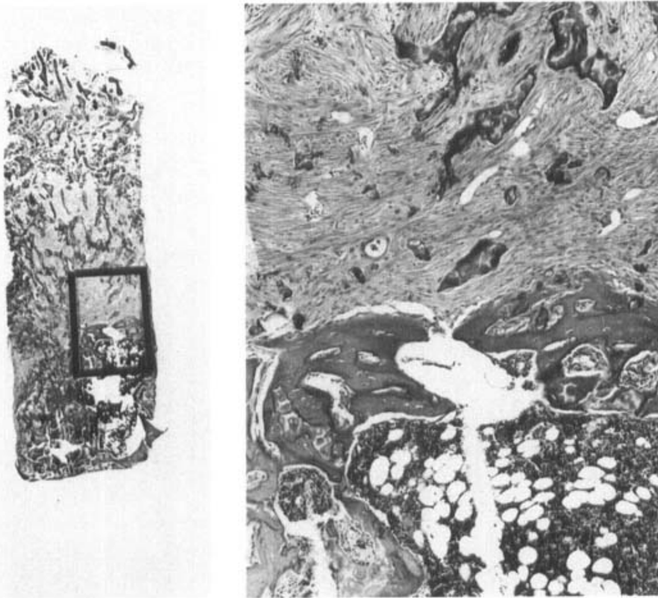

Figure 2. Control specimen treated with hyaluronate gel. There is a clear-cut borderline between new bone and fibrous tissue. The partly lamellar new bone was covered by a mature fibrous tissue parallel to the borderline, and most of the graft bone was resorbed in that area. (HE × 7.5, × 37.5).

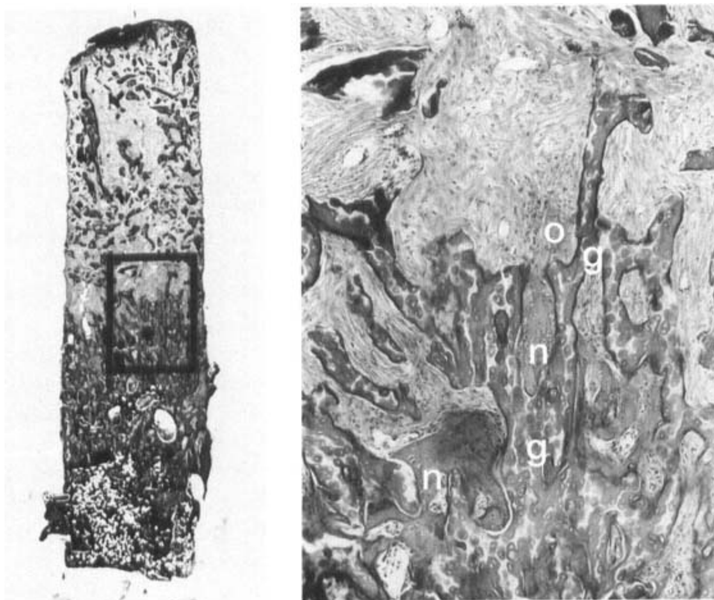

Figure 3. Specimen from the same animal as in Figure 2, treated with bFGF in a hyaluronate gel. There was no clear borderline between new bone and fibrous tissue, but an interdigitation, with new bone sprouts protruding into the graft. Distal to these sprouts, there was osteoid. Note that in this specimen the bone has penetrated a longer distance into the graft, as compared to Figure 2. (g graft bone, n new bone, o osteoid; HE × 7.5, × 37.5).

into the graft (Figure 3). The new bone often formed without obvious contact with graft trabeculae. Distal to the new bone sprouts, there was osteoid. A gradual transition to bone seemed to be occurring (metaplastic bone formation). The fibrous tissue in the distal part of the bFGF-treated specimens was more dense than in controls, but capillaries and sinusoids were seen within this tissue, both in bFGF-treated specimens and controls.

The bone ingrowth distance into the allografts in the rat chamber was increased by 51 percent with bFGF compared to hyaluronate vehicle controls (*P* 0.0001; Table 1). The bFGF-treated specimen had a higher score for the presence of osteoid (*P* 0.001; Table 2).

Discussion

Increased penetration of new bone into a bone graft due to local treatment with a growth factor has not been previously described. The mechanism for this effect of bFGF is unclear. As an increased vascular formation due to bFGF has been observed in other models with autologous grafts, the angiogeneic effect of bFGF may be responsible (Eppley et al. 1988, 1991). However, when our specimens were harvested, even the controls were revascularized all the way to the top of the chamber, though the penetration of new bone had only reached half that distance. It is possible that revascularization is not a limiting factor in this model, and that other than angiogeneic effects of bFGF, like the stimulation of mesenchyme or preosteoblast proliferation, are important (Folkman and Klagsbrun 1987, Joyce et al. 1991). We have tested various concentrations of bFGF in the hyaluronate gel in the same chambers without grafts, and found no effects (data not shown). As we only see

Table 1. The distance (mm) which new bone ingrowth had penetrated into defatted bone allografts in bilateral bone conduction chambers in rats. Mean value of 3 sections

Rat	bFGF	Control
1	2.9	1.3
2	1.3	1.4
3	1.9	1.1
4	1.1	0.8
5	1.3	1.4
6	1.7	1.4
7	2.1	1.7
8	2.9	1.2
9	1.9	1.2
10	2.2	0.9
11	2.5	1.5
12	1.7	1.8
Mean, *SE*	2.0 *0.2*	1.3 *0.1*

Table 2. Osteoid in specimens with bFGF and without (control). Blinded examination. Score 0–2 represents increasing amounts

Rat	bFGF	Control
1	2	1
2	0	0
3	2	0
4	2	0
5	1	0
6	1	0
7	2	1
8	2	0
9	2	0
10	2	0
11	2	0
12	1	1
Mean	1.6	0.3

an effect of bFGF if a graft is placed in the chamber, this might indicate that the effects of bFGF are somehow coupled to the penetration of collagenous or osseous tissues by the new cells, rather than to mitogenic effects. Such increased penetration may have a relation to the reported stimulation of collagenase production by bFGF (McGee et al. 1988, Buckley-Sturrock et al. 1989).

We do not know during which time-period the bFGF was active. Probably we have observed only late effects of a stimulation that occurred during the early phases of tissue ingrowth. However, regardless of the persistence of the hyaluronate, the bFGF may have become stored and protected within the implanted bone graft matrix, thus producing a prolonged effect. Judged by histological appearance, bone penetration of the graft had ceased in the controls, but in the bFGF-treated implants, the large amount of osteoid indicates that bone ingrowth was still going on.

Quantitative differences between various bone-grafting procedures are difficult to measure, especially in small animals. In the past, clinical mistakes, like the wide use of Kiel bone, were preceded by seemingly ambitious experimental studies where quantitative differences between different types of grafts were not found (Hallén 1966). Some of the problems that are encountered are the standardization of the shape and quality of the graft and the recipient site, but also to define the graft after it has become more or less incorporated. The titanium bone chamber techniques (Albrektsson et al. 1984) can minimize these problems, and we thought that they could demonstrate a possible stimulation of bone graft incorporation by bFGF. However, in preliminary experiments it turned out that the commonly used Bone Harvest Chamber (Albrektsson et al. 1984) was unsuitable for our experiment, because the grafts were too easily remodeled. A major change in the principle and design of the chamber was required in order to perform the present study. The Bone Conduction Chamber was designed so that bone ingrowth is challenged by such a large graft volume, that new bone formation may cease before the chamber is filled. This is rare in animal models of bone-grafting and is a great advantage, because we become less dependent on timing: we can study how much of the graft was replaced in the end. In other models, comparisons have to be performed at several time-points before all of the graft is eventually replaced. Further, incomplete incorporation resembles more the clinical situation, where bone allografts are usually only replaced to a small extent (Enneking and Mindell 1991).

The potential uses of bFGF in orthopedic surgery include the repair of flexor tendons, cartilage, and bone. An increased vascular penetration into repaired avascular flexor tendon segments has been suggested. The healing of incisional wounds in joint cartilage has been reported (Wellmitz et al. 1980, Cuevas et al. 1988), but was based on qualitative histology, and so far no quantitative measurements have been published. The injection of acidic FGF into fracture callus has caused a large increase in the dimensions of the cartilaginous callus. However, there was a lower production of cartilage-specific molecules (Jingushi et al. 1990).

We know of 2 studies regarding bFGF and the incorporation of bone grafts. In the first, fresh autologous iliac bone was transplanted into a mandible

defect in the rabbit, and bFGF was continuously applied with a minipump. An increased number of blood vessels in and around the grafts was found, but no signs of increased bone turnover (Eppley et al. 1988). In the second study, the same model was used, but the recipient site had been previously irradiated. The bone grafts failed, unless the recipient site was pretreated with bFGF (Eppley et al. 1991).

It could be questioned to what extent bone ingrowth into a graft is desirable, because remodeling of a dead bone may jeopardize its mechanical integrity. If, however, increased ingrowth is desired—which may be the case especially with synthetic bone replacement materials—the use of growth factors like bFGF for this purpose deserves extended studies. The bone conduction chamber may then prove a useful tool.

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