Basic fibroblast growth factor infused at different times during bone graft incorporation

Titanium chamber study in rats

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We investigated the effect of applying basic fibroblast growth factor (bFGF) to a bone graft during different stages of incorporation in an infusion bone chamber model. Bone chambers were implanted bilaterally into rat tibiae. Both chambers were connected to an implanted osmotic minipump. Ingrowing bone could enter the cylindrical interior of the chamber only at one end. The distance which ingrowing bone had reached into the bone graft was then measured on histological slides. Specimens were also analyzed by ^{99m}Tc-MDP scintimetry. The infusion of buffer during 2 weeks from implantation had no effects on tissue ingrowth distance or quality. bFGF was infused during 2 weeks from implantation in a dose of either 1.2 or 12 ng/day. Bone ingrowth was measured 6 weeks after implantation. The higher dose had a more marked effect and was used for studying the effect of application at different times.

The maximum stimulation of bFGF as measured at 6 weeks postimplantation was found after infusion during the first postimplantation week. Infusion during the third and fourth weeks had no effect at 6 weeks, but tended to increase the bone ingrowth distance at 8 weeks postimplantation. These findings suggest that bFGF infusion increases bone ingrowth into bone grafts when infused at both an early and a later stage, but the effect can be measured only several weeks later.

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The fibroblast growth factor (FGF) gene family comprises at least 9 members in mammals (Mason 1994). bFGF includes the two abundant prototypic members, FGF1 (aFGF) and FGF2 (bFGF), which are 55% identical in structure (Gospodarowicz et al. 1987, Baird and Böhlen 1991, Dionne et al. 1991). bFGF is a multifunctional polypeptide with mitogenic (Gospodarowicz et al. 1987), angiogenic (Folkman and Shing 1992) and embryogenic (Amaya et al. 1991, Cohn et al. 1995) effects. It is present in the extracellular matrix of bone and cultured bone cells (Globus et al. 1989). Under specific in-vitro cell culture conditions, bFGF is mitogenic for bone and bone-like cells and several studies have demonstrated an effect of bFGF on the expression of extracellular matrix and phenotypically characteristic osteoblast genes (Rodan et al. 1989, Hurley et al. 1994, Schedlich et al. 1994). bFGF promotes skin wound healing, it increases fibroblast proliferation and collagen accumulation, thus leading to faster reorganization of collagen (McGee et al. 1988, Buckley-Sturrock et al. 1989, Hom and Maisel 1992). bFGF increases cartilage and bone induction by demineralized bone matrix (Aspenberg et al. 1991, Wang and Aspenberg 1993), fracture-healing in normal and diabetic rats (Kawaguchi et al. 1993) and free bone graft reconstruction (Eppley et al. 1991).

We have previously demonstrated that bFGF in a hyaluronate gel increases the incorporation of allograft bone, as compared to untreated controls, in a bone ingrowth chamber in rats (Wang and Aspenberg 1994). By necessity, the bFGF was applied to the graft before implantation. The effect of a prolonged bFGF application, or application of the bFGF during a later phase of bone graft incorporation, is not known. No suitable in vivo model seemed to be at hand. In the present study, we modified the previously used titanium chamber (the bone conduction chamber (BCC)) to allow for the connection to an implantable minipump for continuous infusion. With this modification of the chamber, experiments could be designed to answer the question whether the FGF application at a later stage could also stimulate the incorporation of a bone graft.

Material and methods

The bone conduction chamber (Aspenberg and Wang 1993) is a titanium implant made of two half cylin-

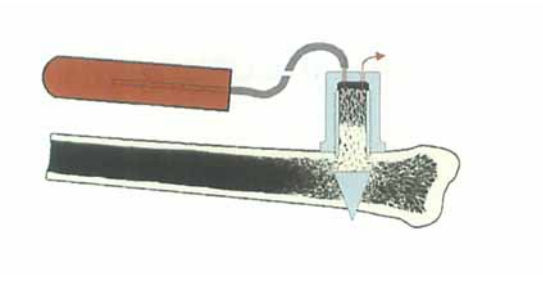


Figure 1. The bone conduction chamber was inserted into the proximal tibial metaphysis and connected to an implanted osmotic pump via a silicon tube. Bone enters the bottom of the chamber via two openings. The bone graft is grey and the new ingrown bone penetrating into the bone graft in the chamber is yellow.

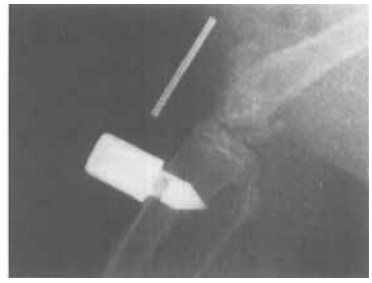


Figure 2. The position of the chamber in the rat proximal tibia. The metal rod is a part of the mini-osmotic pump.

ders which are held together by a hexagonal screw cap. The entire device is 13 mm long and the diameter of the threaded cylinder is 3.5 mm. This threaded cylinder is screwed into the bone. Bone tissue enters the bottom of the interior chamber (2 mm diameter, 7 mm long) via two openings. This original implant was then modified by drilling two 0.8 mm diameter holes through the cap at its upper end, into which was connected a 6 cm silicon tube (0.4 mm inner diameter, Silastic®, Medical Grade Tubing, Dow-Corning Corp., Midland, MI, USA). The other end of the tube was connected to an osmotic pump (ALZET, model 2002, ALZA Corp., Palo Alto, CA, USA). The other hole in the cap of the chamber was left to act as a drain into the subcutaneous tissue (Figure 1).

Graft preparation

72 female, 200 g Sprague-Dawley rats (Møllegaard, Copenhagen, Denmark) were killed with an overdose of mebumal, following which a 2 × 6 mm rod of trabecular bone was harvested from each proximal tibia under sterile conditions and kept as matched pairs. Using a specially designed hole-cutter, we resected each rod in the long axis of the tibia. After removal of any epiphyseal tissue, the grafts were stored sterile in a glass container at –70 °C. Before implantation, each pair of rods was lipid extracted (1:1 chloroform : methanol) in their glass containers overnight, then rinsed 3 times each in methanol and distilled water before air-drying. Each graft was positioned within the chambers so that the proximal and denser end of the graft was placed at the ingrowth end of the chamber.

Operative procedure

78 Sprague-Dawley rats (male, 320–350 g) were obtained from Møllegaard (Copenhagen, Denmark) and kept in the animal facilities (22 °C) for 1 week before the experiments started. 2 rats were kept in each cage, with free access to food pellets and water. Anesthesia was maintained with an intraperitoneal injection of 0.6–0.7 mL of a solution containing 15 mg/mL pentobarbital and 2.5 mg/mL diazepam. Using aseptic technique, the medial proximal tibial metaphysis was exposed with a longitudinal incision. The periosteum was elevated and cleared anterior to the insertion of the medial collateral ligament. The medial cortex was breached with a bone spike which was carried up to and through the opposite cortex. The medial hole was enlarged with a 3.2 mm drill. Each chamber was screwed into place, so that the pointed end engaged the opposite cortex and bone ingrowth holes were at the level of the cortical bone (Figure 2). A subfascial pocket was created with blunt dissection of the medial thigh and into this was inserted the osmotic pump and excess tubing. The wound was closed in layers with interrupted fascial, and continuous subcutaneous sutures.

Basic fibroblast growth factor

Basic FGF powder (CalBio, Scios Nova, Mountain View, CA, USA) was reconstituted in 10 mM sodium citrate, 1 mM sodium EDTA and 9% saccharose, pH 5, according to the manufacturer's instructions and stored at –70 °C. Stock solutions were then diluted in phosphate buffer saline (pH 7.0) containing rat serum albumin to the desired concentrations prior to use. Treatment group osmotic pumps were loaded with 200 µL of bFGF solution and had a delivery rate of 0.5 µL/h. Control osmotic pumps were loaded with similarly diluted buffer.

The effect of infusion of saline on bone ingrowth

6 rats received empty chambers in both legs, with an osmotic pump containing saline connected to one side. 6 other rats received bilateral chambers containing grafts with an osmotic pump containing buffer on

Table 1. Number of rats in different groups and the reason for exclusion of specimens

Group	No. of rats	Implant infection	Tube disconnection	Chamber loosening	Valid no. of rats studied
Empty chamber-test	6	–	–	–	6
Graft+buffer-test	6	–	–	–	6
0.1 µg/mL–2+4 wk	13	–	–	–	13
1.0 µg/mL–2+4 wk	21	6	1	1	13
1.0 µg/mL–1+5 wk	13	1	–	–	12
1.0 µg/mL–2+2+2 wk	6	–	–	–	6
1.0 µg/mL–2+2+4 wk	13	–	2	1	10
Total	78	7	3	2	66

one side. The infusion time was 2 weeks and the rats were killed at 2 weeks after implantation (Table 1).

Effect of different doses of bFGF on bone ingrowth

34 rats received bilateral chambers containing grafts. An osmotic pump containing buffer was connected to one side and another pump, containing bFGF 0.1µg/mL or 1.0 µg/mL (corresponding to 1.2 ng/day or 12 ng/day) was connected to the opposite side. The bFGF was infused for 2 weeks (thereafter the pumps were empty) and the rats were killed 6 weeks after implantation (Table 1).

Effect of early versus delayed infusion of bFGF

32 rats received bilateral chambers containing bone grafts. An osmotic pump containing buffer was connected to one side, and another pump, containing bFGF (1.0 µg/mL), was connected to the opposite side. In 13 rats, the pumps were taken out operatively after one week, and the animals were killed after 6 weeks (1 + 5 weeks). In 19 rats, both chambers were infused with buffer for 2 weeks, thereafter the pumps were changed so that bFGF was delivered to one side for another 2 weeks and the other side still received buffer. 6 of these rats were killed 6 weeks after postimplantation (2 + 2 + 2 weeks) and 13 rats were killed 8 weeks after implantation (2 + 2 + 4 weeks, Table 1).

Evaluation

12 recipient rats were excluded. 7 rats had an infection when harvested. 3 rats had disconnected tubes and 2 rats had loosened chambers (Table 1).

An intravenous injection of 2.3 MBq ^{99m}Tc-MDP was given 3 hours before the rats were killed. Immediately following harvest, the uptake of ^{99m}Tc-MDP was measured in a well counter. The harvested tissue was then fixed in 4% formalin, decalcified and embedded in paraffin. The specimens were cut with a microtome parallel to the long axis of the chamber and stained with hematoxylin and eosin.

3 sections from the middle of the specimens, each at 300 µm distance from the other, were used for histology and histomorphometry. Specimens were coded and assessed in a blind and randomized fashion. The area of the new ingrown bone was measured using the Videoplan™ equipment (Zeiss, Stockholm, Sweden) at a screen magnification of 40 ×. This area included marrow cavities and remnants of graft bone which were surrounded by new bone. A straight line was drawn between approximately 7 points, where new bone had reached the farthest distance into the graft. The mean ingrowth distance of the new bone was calculated by dividing the area by the width of the specimen (Aspenberg and Wang 1993). The mean of the 3 sections from the same specimen was used for the statistical analysis. The paired grafts were analyzed by Student's paired t-test. Comparisons between groups (i.e., different infusion times) were performed with one-way ANOVA of the bFGF-control difference for each animal, followed by Fischer's PLSD test.

Results

Effect of infusion on bone ingrowth

Fluid flow from the osmotic pumps into the ungrafted chambers did not cause a difference in the amount or quality of ingrown bone (Table 2). Ingrown tissue consisted of an osseous component adjacent to the ingrowth portals extending up into the body of the chamber and blending into a fibrous component at the most distal point from the portals. The osseous tissue was immature and disorganized and appeared to form without a cartilage precursor. The osseous tissue was invaded by fine-walled, single-layered capillaries which were most concentrated at the portal end. Numerous spindle-shaped cells with prominent nuclei surrounded the clusters of osteoid-producing osteoblasts and extended beyond the area of bone to occu-

Table 2. ^{99m}Tc-MDP and bone ingrowth in grafted and ungrafted bone conduction chambers (BCC) at 2 weeks. Mean *SD*

Graft[a]	Infusion[b]	^{99m}Tc-MDP (cpm)		Bone distance (mm)	
–	–	558	*228*	0.5	*0.3*
–	+	698	*384*	0.5	*0.3*
+	–	598	*76*	0.2	*0.2*
+	+	452	*208*	0.1	*0.1*

[a] Graft: – no graft, + with graft
[b] Infusion: – no infusion, + with infusion

py the fibrous component of the ingrown tissue.

Likewise in grafted chambers, there was no difference in the quality of bone or its penetration distance into the graft between chambers that did or did not have osmotic pumps attached (Table 2). The graft was clearly visible as an intricate network of trabeculae with empty lacunae. The area closer to the ingrowth portals was invaded by a cellular mass of spindle-shaped cells insinuating themselves between the trabeculae and extending some distance into the graft (Figure 3).

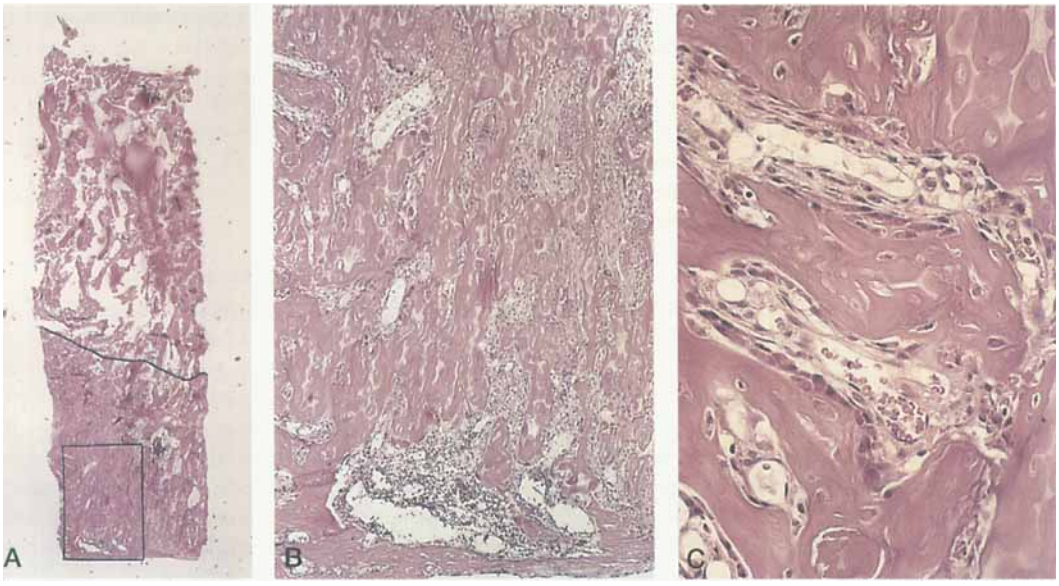
There were no differences in the amount of incorporated ^{99m}Tc-MDP between tissue from chambers that did or did not have osmotic pumps attached in both groups (Table 2).

Effect of different doses on bone ingrowth

A lower concentration of bFGF (0.1 μg/mL) caused an increase of total tissue ingrowth and ^{99m}Tc-MDP uptake, but there was no effect on the bone ingrowth distance. The higher concentration of bFGF (1.0 μg/mL) increased the bone ingrowth distance by 18%. ^{99m}Tc-MDP was also increased (Table 3).

Qualitative histology generally revealed that, in the proximal end of the chamber, ingrown bone lined the bone graft and surrounded the marrow elements. The trabecular appearance of the graft differed from the woven character of the ingrowing bone front, the matrix of the grafts stained differently, and the grafts contained very few osteocyte nuclei. Most of the chamber space was filled with new vascularized tissue; at the distal end, far away from the bone ingrowth openings, there was a loose connective tissue surrounding the bone graft trabeculae. At the border of the non-invaded part of the graft, we found an accumulation of inflammatory cells and extravasated erythrocytes. The interface between the proximal new bone and the non-resorbed graft sometimes differed between controls and bFGF-treated specimens. In the controls, there was a tendency to form a borderline at which the cortical-like new bone was covered by fibrous tissue (Figure 4). In the bFGF-treated specimens, there was no such borderline, but an interdigi-

Figure 3. Specimen 2 weeks after implantation. Buffer has been infused.



Tissue has grown in at the ingrowth end (bottom) and penetrated the graft to below the line. The rest of the specimen is necrotic bone graft. (HE, ×5).

Higher magnification of inset area in (A) showing new tissue between the graft trabeculae (HE, ×25).

Higher magnification of (B) showing osteoblasts and new bone matrix lining the graft trabeculae (HE, ×100).

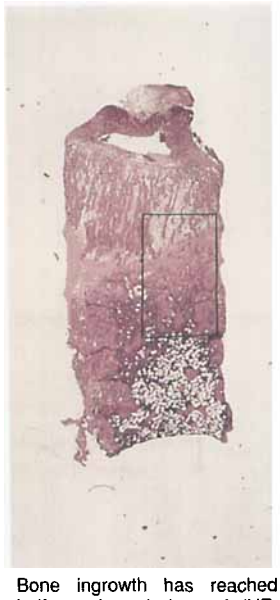
tation, with new bone sprouts protruding into the graft (Figure 5). Distal to these sprouts, there was usually a dense mesenchymal-like tissue with larger nuclei. A gradual transition to osteoid and bone appeared to be in progress.

Effect of bFGF infusion at different times

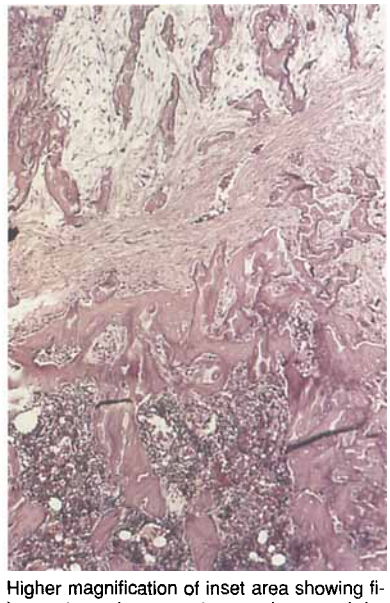
bFGF infusion for 1 week (1 + 5) increased the ^{99m}Tc-MDP uptake and the bone ingrowth distance at 6 weeks postoperatively (Table 4). With delayed infusion (2 + 2 + 2), no effects could be demonstrated at 6 weeks. When infusion was delayed, and the harvest was likewise delayed by 2 weeks (2 + 2 + 4), there was a trend towards an increased bone ingrowth distance (p 0.07; Table 4).

When comparing the effects of various infusion times, 3 groups were analyzed. The first group consisted of all animals which received an immediate infusion postoperatively with 1.0 µg/mL bFGF (1 + 5 and 2 + 4). The second group consisted of the animals with delayed infusion, but not delayed harvest (2 + 2 + 2), and the third group had both delayed infusion and delayed harvest (2 + 2 + 4). One-way ANOVA showed significant differences in the effect of bFGF between these groups ($p < 0.05$). In a post hoc analysis, bFGF had a larger effect in the animals that received an immediate infusion than in those with delayed infusion, but not delayed harvest ($p < 0.05$). Moreover, when both infusion and harvest were delayed, the bFGF effect was larger than in the animals with delayed infusion alone ($p < 0.05$). These data indicate that, with bFGF application, there was a stimulatory effect regardless of the infusion time, but this effect was seen not 4 but 6 weeks after the infusion started.

Figure 4. Control specimen at 6 weeks (1 + 5 weeks).



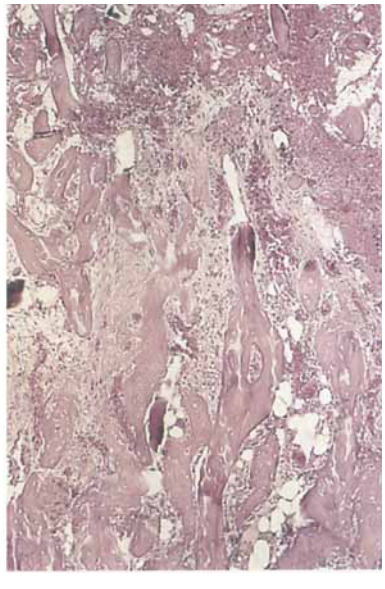
Bone ingrowth has reached half-way through the graft (HE, ×5).



Higher magnification of inset area showing fibrous tissue between the new bone and the unresorbed graft. Bone marrow has extended almost to the distal border of the new bone (HE, × 25).

Figure 5. Paired specimen from the same animal as in Figure 4, treated with bFGF 12 ng/day.

Bone ingrowth has reached three-fourths of the way through the graft (HE, × 5).



Higher magnification of inset area showing an interdigitation with new bone sprouts protruding into the graft. Distal to these sprouts, a mesenchymal-like tissue with large nuclei was connected with the osteoid (HE, × 25).

Table 3. ^{99m}Tc-MDP and tissue ingrowth distance in bone grafts with different concentrations of bFGF infused for 2 weeks and harvested at 6 weeks. Mean *SD*

Dose (µg/mL)	^{99m}Tc-MDP (cpm)				Ingrowth distance (mm)					
	bFGF		Control		Total tissue				Bone	
					bFGF		Control		bFGF	Control
0.1	4176	*1235**	3387	*803*	4.5	*0.3**	4.1	*0.5*	2.3 *0.7*	2.1 *0.4*
1.0	5164	*2055**	4471	*2101*	4.4	*0.6*	4.7	*0.6*	2.0 *0.5**	1.7 *0.6*

* $p < 0.05$ as compared to control

Table 4. ^{99m} Tc-MDP and tissue ingrowth distances in bone grafts with 1.0 µg/ml bFGF infusion at different times. Mean *SD*

Infusion time (wk)	^{99m}Tc-MDP (cpm)				Ingrowth distance (mm)					
	bFGF		Control		Total tissue				Bone	
					bFGF		Control		bFGF	Control
1+5	6464	*2078***	5077	*1517*	4.2	*0.4*	3.8	*0.6*	2.2 *0.5***	1.7 *0.5*
2+4	5164	*2055**	4471	*2101*	4.4	*0.6*	4.7	*0.6*	2.0 *0.5**	1.7 *0.5*
2+2+2	6352	*906*	7433	*2168*	4.7	*0.4*	4.9	*0.5*	1.7 *0.4*	1.9 *0.4*
2+2+4	5599	*1366*	5324	*1179*	4.2	*0.5*	4.2	*0.4*	2.3 *0.9*	1.6 *0.5*

* $p < 0.05$; ** $p < 0.01$. 1+5 = 1 week bFGF infusion, no infusion for 5 weeks; 2+4 = 2 weeks bFGF infusion, no infusion for 4 weeks; 2+2+2 = buffer first 2 weeks, then bFGF infusion 2 weeks, after that no infusion for 2 weeks; 2+2+4 = buffer first 2 weeks, then bFGF infusion 2 weeks, after that no infusion for 4 weeks.

Discussion

Despite significant advances in biomaterials, allograft bone remains an important resource for regaining continuity or structural support in reconstructive surgery. The success of allografting techniques is measured by union at the interface between host and donor bone and its ability to tolerate physiological forces without failure. Problems such as nonunion, delayed union and fracture are not infrequent and still compromise the complete success of this procedure. Factors which act by increasing the amount or strength of allograft incorporation could therefore be advantageous.

bFGF, transforming growth factor beta, bone morphogenetic proteins and the insulin-like growth factors have all been shown to enhance the expression of differentiated osteoblast function (McCarthy et al. 1989, Wang et al. 1990, Mayahara et al. 1993, Centrella et al. 1994). bFGF has been found in the extracellular matrix of bone and cultured osteoblasts, and its osteotrophic action includes the replication of cells of the osteoblastic lineage and augmentation of osteocalcin synthesis (Canalis et al. 1989, Gospodarowicz 1990). Thus, bone appears to be an important target for bFGF function. Together with its angiogenic and fibroblast-stimulating actions (Eppley et al. 1991, Folkman and Shing 1992), it seems likely that bFGF plays a specific role in fracture healing.

We have previously reported that bFGF increases the ingrowth of rat bone into allograft bone (Wang and Aspenberg 1994). It is unclear whether this is primarily due to an effect on mesenchymal cell proliferation or whether bFGF-induced angiogenesis is responsible. When large pieces of demineralized bone matrix were implanted intramuscularly in rats with bFGF, we found an increased number of sites of cartilage induction in this matrix. Later, this was followed by an increased amount of bone (Wang and Aspenberg 1993). Since no vascular formation had reached into the cartilagenous areas of the implant, this finding indicates an effect of bFGF directly on the chondrocyte precursors. As the tissue in the present experiment was vascularized far ahead of the bone ingrowth front, we find likely that also in this experiment the effect of bFGF was due to a direct stimulatory effect on preosteoblasts rather than to an indirect effect via increased capillary formation.

The biological half-life of bFGF in this buffer at 37 °C is about 4 days (Berthold Nies, Darmstadt, Germany, personal communication). The half-life of an ^{125}I marker of the bFGF close to the bone ingrowth

openings was approximately 20 hours (Wang and Aspenberg 1996a). Nevertheless, the biological half-life of bFGF in vivo is probably short, and when bFGF was applied in a hyaluronate gel, as in our previous studies, it probably exerted its effects during the first few days. Considering the half-life of the biological activity in buffer, the second week of infusion supplied only small amounts of bFGF compared to the first week. Therefore, the pooling of the groups (1 + 5) and (2 + 4) seems justified and, indeed, there was no statistical difference between these 2 groups.

In previous experiments with a hyaluronate carrier, we have found a stimulatory effect on bone ingrowth at 6 and 10 weeks postoperatively, but there was no significant difference at 4 weeks. This is consistent with the present finding that one has to wait more than 4 weeks after bFGF application before an increased bone ingrowth distance can be demonstrated. This appears to be true also for delayed application of bFGF. At 2 weeks postimplantation, when delayed bFGF application was started, the chamber contains a considerable amount of soft tissue, mostly appearing as an undifferentiated blastema. Bone formation has hardly begun (Wang and Aspenberg 1996b). The stimulatory effect of bFGF applied at this time indicates that bFGF affects pre-osteoblast proliferation, and possibly also differentiation.

2 weeks after implantation, tissue ingrowth was not affected by the fluid flow from the minipump. We have not specifically investigated whether this holds true also after 4 weeks of infusion. However, total tissue ingrowth at 6 weeks in the group with 4 weeks of infusion was not different from other 6-week groups, indicating that also 4 weeks of fluid flow may be harmless. On the other hand, the total tissue ingrowth was not stimulated by bFGF infusion. This finding differs from other studies using a hyaluronate gel as a slow-release carrier for bFGF, instead of a minipump (Wang and Aspenberg 1996a, b). Whether this difference is due to fluid flow, concentration gradients or a different time course for the local bioactive bFGF concentration is not known. It is possible that fluid flow disturbed the advancing front of fibrous tissue when it came close to the tube openings.

A similar model for use in larger animals has been described (Aspenberg et al. 1988a), but that model was considerably more complicated to handle. Several other intraosseous chambers without infusion have been reported. They are all developments from the bone harvest chamber (Albrektsson et al. 1984), which has been successfully used to study the effects of particulate materials on tissue differentiation, bone graft preparations, bone induction and conduction (Aspenberg et al. 1988b, Goodman 1994, Thorén et al. 1995). Aufdemorte et al. (1992) has mentioned another development of the bone harvest chamber, the analytic bone implant, which was used to examine the effects of transforming growth factor beta in baboons. Common to all these models is the need to wait 6 or more weeks after the initial insertion for stabilization of the implant to occur before any experiments can be started. On the other hand, with the larger animals required for those chambers, the possibility of repeated harvests is a great advantage. In the bone conduction chamber, immediate use is possible. Since the amount of bone ingrowth was not impeded by the use of osmotic pumps, we believe that it could be a good model for delivering and testing the effects of potentially stimulating agents.

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