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Basic fibroblast growth factor for stimulation of bone formation in osteoinductive or conductive implants

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Abstract, abbreviations and definitions

Abstract

Basic Fibroblast Growth Factor (bFGF) is one of the endogenous factors found in bone matrix. bFGF is a mitogen for many cell types, including osteoblasts and chondrocytes. It can stimulate angiogenesis and osteoblast gene expression. The purpose of this study was to investigate whether exogenous bFGF can stimulate the formation of bone in bone grafts and in a bone graft substitute.

In a model using demineralized bone matrix implants for bone induction, a dose of 15 ng bFGF per implant increased the number of chondrocytes and the amount of bone, whereas 1900 ng greatly inhibited cartilage and bone formation. These results are consistent with previous studies with this model, showing that a lower dose of bFGF increased bone calcium content and a higher dose reduced it. Thus, exogenous bFGF can stimulate proliferation during early phases of bone induction.

A new device, the bone conduction chamber, was developed for the application of bFGF to bone conductive materials. This model made it possible to demonstrate a difference between the conductive properties of bone grafts and porous hydroxyapatite. bFGF increased bone ingrowth into bone graft inside the chamber and showed a biphasic dose-response curve, so that 8–200 ng per implant (0.4–10 ng/mm^3) increased bone ingrowth, but higher or lower doses had no effect. The same doses had the same effects in porous hydroxyapatite. In both bone grafts and porous hydroxyapatite, the highest dose still caused an increase in ingrowth of fibrous tissue. The effect on bone ingrowth was first detected after 6 weeks, regardless if administration of bFGF started at implantation or 2 weeks later, using an implanted minipump. Hyaluronate gel was effective as a slow-release carrier for bFGF.

In conclusion, bFGF stimulates bone formation in bone implants, depending on dose and method for administration.

Abbreviations and definitions

AAA-bone	Type of partly demineralized bone allograft (autolyzed, antigen-extracted allograft)
aFGF	Acidic fibroblast growth factor
Allografts	Grafts transferred between genetically different subjects of the same species
Autografts	Grafts transferred in a subject
BCC	Bone conduction chamber
BHC	Bone harvest chamber
bFGF	Basic fibroblast growth factor
BMP(s)	Bone morphogenetic protein (s)
DBM	Demineralized bone matrix
FGF(s)	Fibroblast growth factor (s)
HA	Hydroxyapatite
H&E	Hematoxylin and eosin
Osteoconduction	Process in which a material facilitates or stimulates bone formation in a skeletal defect
Osteoinduction	Stimulation of primitive mesenchymal cells to proliferate and differentiate to form cartilage and bone instead of scar tissue
^{99m}Tc-MDP	Radioactive ^{99m}technetium-labeled methylene diphosphonate
TGF-ß(s)	Transforming growth factor-beta (s)

Introduction

For more than a century, one goal of research has been to find a suitable material to repair or replace bony segments in the musculoskeletal system. MacEwen reported his clinical experience with a bone allograft in 1880. Between 1908 and 1925, Lexer performed hemijoint allograft transplants with 50% successful long-term results (Bolano and Kopta 1991). These studies contributed greatly to the beginning of modern bone transplantation and bone banking. Since then, the search for improved methods of preservation has led investigators to subject bone to many treatments, including refrigeration, freezing, freeze-drying, moderate heat application, boiling, irradiation, deproteinization, defatting and decalcification. Some of these methods reduce the immunogenicity and improve the incorporation of the graft.

Bone implants are routinely used to repair defects from injury, neoplasms, congenital malformations and infections. Allografts are frequently employed in revision hip replacement (Huo et al. 1992). Acetabular cavity defects can be filled with morselized allografts and peripheral defects by screw-fixed, structural, corticocancellous bone blocks (Einhorn and Grelsamer 1989). Both structural and morselized grafts give good clinical and radiographic results (Oakeshott et al. 1987). Impacted morselized frozen allografts seem to be incorporated with the formation of new structural bone (Kinzinger et al. 1991, Slooff et al. 1993). This technique has been successfully utilized on the femoral side in revision-cemented hip arthroplasty (Gie et al. 1993, Schreurs et al. 1994, Franzén et al. 1995). However, structural acetabular allografts seem to function well initially, but resorption, migration and cup-loosening may occur later (Jasty and Harris 1990, Mulory and Harris 1990).

The success of allografts depends on the union between host and donor bone and the ability of the graft to withstand physiological forces; non-union, delayed union and fracture are common. Factors which act by increasing the amount or strength of allograft incorporation would definitely be advantageous.

The incorporation of new bone in bone grafts is produced through the process of osteoconduction. During this process, a graft facilitates or stimulates the ingrowth of mesenchymal tissue into a skeletal defect. Ingrown cells differentiate into osteoblasts and form mineralized bone along the graft (Goldberg and Stevenson 1987). Recruitment and differentiation of mesenchymal cells may be modulated by bone morphogenic protein (BMP) (Urist 1965). This process is called osteoinduction. Allograft incorporation is distinguished from autograft incorporation by slower and less extensive vascular penetration and bone formation (Goldberg and Lance 1972, Burchardt et al. 1978). Many attempts have been made to improve the biological properties of bone allografts. Mechanical treatment of the graft (producing cancellous bone chips or milled bone) increases the contact area between the graft and host bone. Addition of fresh autografts, cancellous bone chips or marrow aspirate may increase the number of active osteoblasts. Another possibility would be to add specific growth factors, among of which much interest has focused on the BMPs, transforming growth factor betas (TGF-ßs) and fibroblast growth factors (FGFs).

Bone matrix contains large quantities of growth factors which may modulate bone formation by stimulating osteoblast proliferation and differentiation (Martin et al. 1989). In demineralized bone matrix (DBM) implants, these factors may participate in the regulation of bone induction once it has been initiated by the BMPs (Urist et al 1983, Hauschka 1986). Many bone-derived growth factors from bone matrix extracts and from media conditioned by bone cells and bone organs in culture have been isolated and characterized (Table 1, Canalis et al. 1989, Mohan and Baylink 1991, Joyce et al. 1991). These bone matrix-derived growth factors have various biologic activities, including mitogenesis, differentiation, chemotaxis and osteolysis. Growth factors act in an autocrine and/or paracrine manner to increase local osteoblast proliferation and bone matrix synthesis (Lundy et al. 1991). The production of growth factors by osteoblasts, their storage in bone matrix and modulatory effects on bone cells, suggest a role in bone growth. FGF, TGF-ßs, BMPs and insulin-like growth factors (IGFs) have all been shown to enhance the expression of differentiated osteoblast function (McCarthy et al. 1989, Wang et al. 1990, Centrella et al. 1992, Mayahara et al. 1993).

Basic fibroblast growth factor (bFGF) is one of the endogenous factors found in bone matrix (Hauschka et al. 1986). bFGF increases the bone calcium content in demineralized bone matrix implants (Aspenberg and Lohmander 1989, Aspenberg et al. 1991) and stimulates the proliferation of differentiated chondro-

Table 1. Bone growth factors

Origin of growth factors isolated from bone matrix		Actions on OPC	
		Prol.	Diff.
PDGF	Osteoblast? Endothelial cell? Platelets	+	+
FGFs	Osteoblast; Endothelial cell? Systemic circulation?	+	
IGF-I	Osteoblast, fibroblast, and systemic circulation	+	+
IGF-II	Osteoblast; Systemic circulation	+	+
TGF-ß	Osteoblast; platelets and inflammatory cells	(+)	+
BMP	Osteoblast	+	+

OPC osteoprogenetor cell; Prol. proliferation; Diff. differentiation; + increase.
(From Canalis et al. 1989)

blasts in vitro (Kato et al, 1987) and in vivo (Cuevas et al. 1988). Several studies have also demonstrated that bFGF increases fibroblast proliferation, collagen accumulation and angiogenesis, leading to faster reorganization of collagen during wound healing (Davidson et al. 1985, McGee et al. 1988, Buckley-Sturrock et al. 1989). An increased number of blood vessels is seen in and around autografts treated with bFGF in rabbits (Eppley et al 1988). In a similar study, the recipient site had been previously irradiated. The bone grafts then failed, unless the recipient site was pretreated with bFGF (Eppley et al 1991). Recently, Weiss et al. (1995) reported that bFGF stimulated the cells involved in osteogenesis and angiogenesis in a vascularized bone graft.

The function of bone allografts may be improved by growth factors, but the risk of HIV infection in banked bone exists. Bone allografts can be sterilized by exposure to chemicals such as ethene oxide or by ionizing radiation and heat application (Kühne et al. 1992), but the physical properties and the potential for osteoinduction and osteoconduction may be altered by the sterilization process (Friedlaender 1987, Aspenberg et al. 1990, Thorén and Aspenberg 1995). The use of bone substitutes will probably rise because they carry no risk of disease transmission, are easy to store and require no additional operation. A suitable artificial graft material reduces or, in some cases, eliminates the need for bone grafting. In the past two decades, several kinds of biomaterials such as porous ceramics, pyrolytic carbon or bioglass have been investigated for their use as bone graft substitutes. The biostability and biocompatibility of porous hydroxyapatite is well known and its use is becoming popular in reconstructive surgery and dentistry (Aoki at al. 1977, Rosen 1989, Salyer and Hall 1989). Porous hydroxyapatite or tricalcium phosphate combined with BMP and TGF-ß promotes bone formation (Ono et al. 1992, Ripamonti et al. 1992, Sumner et al. 1995, Lind et al. 1995). There is, to date, no study in the Medline register in which porous hydroxyapatite and bFGF have been combined.

We evaluated the effects of bFGF on stimulation of osteogenesis. Exogenous bFGF enhanced bone formation in DBM (Aspenberg and Lohmander 1989; Aspenberg et al.1991). After we performed another study on the relationship between DBM and bFGF, we wanted to determine whether the concept of stimulating bone induction by bFGF should be changed to a concept of facilitating the process of bone ingrowth in bone-conductive materials. In order to answer this question, the Bone Conduction Chamber (BCC) has been designed. This model is unique, since the chamber can be used to evaluate bone conductive materials in rats. The effects of growth factors and bone conductive materials in the chamber can be distinguished by determining the final amounts of formed bone, because the chamber is unlikely to become completely filled by ingrown bone.

Review of literature

Fibroblast growth factor

Structure and secretion of fibroblast growth factors (FGFs)

Structure. Fibroblast growth factors (FGFs) are a family of structurally related polypeptides characterized by a high affinity to heparin (Florkiewicz et al. 1991). The FGF gene family currently includes 9 members that occur in mammals (Mason 1994). The best known FGFs and the most abundant in normal adult tissues are acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), also named FGF-1 and FGF-2. aFGF comprises 140 amino acids with a molecular weight of 15.5 kDa. bFGF is a single-chain peptide of 146 amino acids with an apparent molecular weight of 16.5 kDa (Baird and Böhlen 1991). aFGF and bFGF have 55% sequence homology, suggesting that the two molecules have similar three-dimensional structures (Gospodarowicz 1990, Baird and Böhlen 1991, Dionner et al. 1991). In addition, FGFs show approximately 25% homology with interleukin-1, which is known to stimulate some FGF target cells (Eriksson et al. 1991). The other members of the FGF family are mainly expressed in the embryo and in certain tumors.

Secretion. Both aFGF and bFGF have been purified from many species and are found in most tissues in the body. aFGF exists mainly in the brain and retina. bFGF is found in blood vessels, macrophages, the brain, adrenal, liver and bone, i.e., in most tissues in the body. bFGF in bone is synthesized by osteoblasts (Globus et al. 1989). In general, bFGF has several properties that distinguish it from most other growth factors. One of them is the lack of a signal peptide which is normally required for the peptide to enter the endoplasmic reticulum and be secreted. Therefore, it has been difficult to describe how the FGFs are released (Logan and Hill 1992). Canalis et al. (1989) reported that aFGF and bFGF may be released from their cell of origin only after cell membrane disruption or cell death. Jackson et al. (1992) found that aFGF is released from NIH 3T3 cells in response to heat shock, and its release is interrupted by treatment with actinomycin D or cyclohexamide, thus suggesting that only molecules under going synthesis can enter the secretory pathway. Their findings are also consistent with previous data about these cells, reported by Rifkin et al. (1991), who showed that bFGF can be released from living cells and can act as an autocrine factor.

Both aFGF and bFGF are tightly adsorbed to the extracellular matrix, presumably by their affinity for heparin-like glycosaminoglycans (Baird and Walicke 1989, Vlodavsky et al. 1991). The two peptides have also been found to be associated with heparan sulfate on cell surfaces, and with hyaluronic acid as well as with low molecular weight syndecan compounds in the matrix (Baird and Böhlen 1991). Hence, aFGF and bFGF can be regarded as extracellular matrix components required for supporting cell proliferation and differentiation (Vlodavsky et al. 1990). In the case of bFGF, peptide fragments 24–68 and 106–115 bind to heparin and interact with the bFGF receptor (Baird et al. 1988). Since both peptides display high affinity binding to heparin in the extracellular matrix, they can be released by various heparin- and heparan sulfate-degrading enzymes (Eriksson et al. 1991).

Mechanism of action of FGF. FGF family peptides transduce signals to the cytoplasm via a family of transmembrane receptors. These receptors have been demonstrated to contain intrinsic tyrosine kinase domains in their intracellular portions. Furthermore, 5 different genes encoding FGF receptors (FGFRs) have also been identified (Dionne et al. 1991, Givol and Yayon 1992). The products of these different genes display varying relative specificities for the various FGFs. bFGF is a potent mitogen for a wide variety of cell types that express FGF receptors on their surface. The activation of these receptors leads to mitosis.

In addition to the tyrosine kinase domain containing FGFRs, which is responsible for transducing the FGFs biological effects, FGFs may be bound by either secreted or cell surface-bound proteoglycans and, in particular, heparin-like structured proteoglycans (Moscatelli 1992, Xu et al. 1992). The free proteoglycan can serve as a depot of growth factor and /or compete with cell surface receptors, whereas the membrane-bound proteoglycan may "present" FGF to the signal-transducing FGFRs. Yayon et al. (1991) observed that, although the ability of proteoglycans to bind FGFs can compete with the "true" FGFRs, these cell surface FGF-binding proteoglycans facilitate presentation of the ligands to their receptors. Baird and Böhlen (1991) reported that FGFs bind more to proteoglycans with lower affinity than they do to their

transmembrane receptors, supporting the view that these proteoglycans serve as low-affinity, high-capacity "receptors". Thus, it is believed that a proteoglycan molecule may present multiple FGF molecules to receptors and facilitate receptor activation.

FGFs are internalized following binding to cell surface receptors and become translocated to the nucleus in some cells (Forough et al. 1993). This suggests that FGFs may possess properties which categorically distinguish them from other classes of peptide growth factors. Nuclear translocation seems necessary for mitogenic effects (Imamura et al. 1990).

Biological functions of FGF

General background. FGFs have a mitogenic and differentiation-promoting effect on many cell populations, including various epithelia (epithelium), myocytes, osteoblasts and chondrocytes (Baird and Walicke 1989, Gospodarowicz 1990). Under specific 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 (Noda 1991, Schedlich 1994). Hence, bFGF may be involved in the local regulation of bone formation. Its potent mitogenic, angiogenic and osteoblast gene regulatory effects suggest that bFGF may play an important role in fracture healing and bone formation. In this regard, several studies have demonstrated effects of bFGF on bone healing, bone graft and bone graft substitute incorporation in vivo (Table 2).

bFGF also stimulates the smooth muscle cell proliferation that occurs after injury to large blood vessels; modulates retinal cell differentiation, lens formation and photoreceptor development in the eye; plays a role in neuronal differentiation and survival in the brain; and influences oocyte differentiation and embryonic development. FGFs also have an important clinical potential for in vivo use such as preventing degenerative diseases of the retina, healing damaged cornea, promoting neuronal survival in stroke and Alzheimer's disease, inhibiting melanoma growth, promoting wound healing in diabetic patients, accelerating dermal wound healing and promoting the healing of duodenal ulcers (Baird and Klagsbrun 1991). Recent evidence suggests that bFGF also promotes hematopoietic cell growth (Bikfalvi and Han 1994).

FGF and the musculoskeletal system in early embryos. In the early 1970s, Searls and Janners (1971) suggested that FGF could initiate limb bud formation. Gospodarowicz et al.(1975) reported that administration of FGF promoted heterotrophic growth of amputated forelimbs of adult frog. Further studies indicated that FGFs act as signals in the developing limb and can promote proliferation of mesenchymal cells in the limb bud (Niswander and Martin 1993, Niswander et al. 1994). Beads soaked in aFGF, bFGF or FGF4 and implanted in the presumptive flank of a chick embryo induced the formation of ectopic limb buds, which developed into complete limbs (Cohn et al. 1995). These observations suggest that normal limb bud formation is initiated by a local source of FGF. Previous studies demonstrated that FGF stimulated the proliferation of myoblasts and delayed their differentiation (Seed et al. 1988). The period during which FGF-dependent myoblasts are found in the limb bud suggests that FGFs may play a role in early muscle morphogenesis and that FGFs have a differential effect on muscle development during embryogenesis (Seed et al. 1988, Olwin et al. 1994).

FGF and wound healing. Wound healing is characterized by three phases: the initial inflammatory phase, the proliferative phase accompanied by synthesis and deposition of extracellular matrix components and, finally, the remodeling phase with restructuring of the matrix and restoration of the function of the tissue. This is a complex, coordinated sequence of steps involving migration of cells into the wound, proliferation of different cell types and changes in the synthetic and secretory activities of the cells. Polypeptide growth factors regulate these processes.

Application of exogenous growth factors can modify the healing process, and growth factors can now be made in sufficient quantity to be used therapeutically. Endogenous growth factors, including epidermal growth factor (EGF), FGFs, platelet-derived growth factor (PDGF) and TGF-ß are related to the wound site and are presumed to be a necessary part of natural wound healing. Mechanically damaged endothelial cells and burn wound tissue release significant amounts of bFGF into the wound area. This observation suggests that damaged endothelial cells at the cut edges of blood vessels, as well as other damaged cells at the site of a wound, might provide an early source of FGFs (McNeil et al. 1989, Muthukrishnan et al. 1991, Gibran et al. 1994). A slightly later and more sustained supply of bFGF at the wound site is delivered by the invading activated macrophages (Baird et al. 1985, Rappolee et al. 1988, Fiddes et al. 1991). Moreover, all of the previously mentioned growth factors have been shown to enhance healing, if added exogenously to a wound site. It is now clear that they can act as angiogenic factors in vitro (Montesano et al. 1986) and in vivo (Folkman and Klagsbrun 1987) directing endothelial cell migration and proliferation. The effects of stimulating endothelial cell growth,

Table 2. The effect of recombinant human FGF on bone healing, bone graft and bone substitute incorporation in-vivo studies

Author	year	Animal	Model	FGF	Dose	Carrier+ delivery	Observation time	Effect
Wellmitz	1980	Rabbit	Joint cartilage lesion	bFGF*	1 or 2 mg in animal	Saline, local injection	12 days	Increased chondrocyte proliferation (12 d[b])
Cuevas	1988	Rabbit	Joint cartilage lesion	bFGF	1μg/ml, 0.5μl/h	Saline, pump infusion (2 w[a])	3 weeks	Healing of cartilage (3 w)
Eppley	1988	Rabbit	Autograft/ mandibular	bFGF	1μg/μl, 0.5μl/h	PBS, pump infusion	2–28 days	Increased angio-genesis (10 d)
Jingushi	1990a	Rat	Femur	aFGF	1μg/50μl once	PBS, injection	2–5 weeks	Increased amount of cartilage, not bone (2–4 w)
Eppley	1991	Rabbit	Autograft/ madibular irradiated site	bFGF	1μg/μl, 0.5μl/h	PBS, pump infusion	3 months	Increased angio-genesis and improved graft incorporation (3w)
Frankel	1992	Chick	Egg/embryo	bFGF	10μg/ml	Saline, egg injection	18 days	Increased osteo-progenitor replication (18 d)
Schnettler	1992	Pig	HA implant	bFGF	64 μg/cm^3	Implant soaked in solution	1.5–3 months	Comparable to autograft
Mayahara	1993	Rat	Endosteal bone formation	bFGF	0.03–0.3 mg/kg	Saline, i.v. injection (2 w)	1 week	Increased bone in endosteal bone, (Histol., Ca, hydroxy-proline content (1 w)
Baron	1994	Rabbit	Tibia growth plate	bFGF	0.1μg/h	PBS, pump infusion (6 d)	6 days	Accelerated vascular invasion and ossifica-tion of growth plate
Kawaguchi	1994	Rat	Fibular fracture	bFGF	0.4–50 μg/ rat	Fibrin gel, local injection	1–5 weeks	Increased bone callus size (3 w)
Thorén	1994	Rabbit	BHC+bone allograft	bFGF	0.5μg/ml	cmc gel, graft in gel	2 weeks	Increased preosteo-blastic tissue, not bone (2 w)
Wang	1994	Rat	BCC+graft	bFGF	1.8 μg/cm^3	Graft soaked in Healon gel	6 weeks	Increased bone ingrowth (6 w)
Weiner	1994	Rat	Femur cortical allograft	aFGF	2.75ng/graft	Graft in gelatin	1–4 months	No effects on graft incorporation (1–4w)
Wippermann	1994	Sheep	HA implant	bFGF	4.5 μg/cm^3	Implant soaked in solution	3 months	Improved bone ingrowth
Bland	1995	Rabbit	Tibia fracture	aFGF bFGF	3 μg 3 μg	Saline, site injection	4, 10 days	No effect on amount of bone and cartilage (4 and 10 d)
Nagai	1995	Rat	Endosteal bone formation	bFGF	0.1, 0.3 mg/kg	Citrate buffer, intravenous injection (2 w)	3 week	Increased osteoid, mineralized area (3w)
Schliphake	1995	Pig	Pyrolized bovine bone	bFGF	42 μg/cm^3	Implant soaked in PBS solution	5 months	No effects at 5 months (bone ingrowth throu-ghout the whole block)
Weiss	1995	Rat	Vascularized autograft	bFGF TGF-ß	0.5 μg/graft 0.25 μg/graft	Agarose beads with saline	2–4 weeks	Both growth factors increased osteoblasts and angiogenesis (2–4 w)

[a]w weeks; [b]d days

capillary differentiation and connective tissue cell growth by bFGF contribute to wound healing and tissue regeneration (Steenfos 1994, Gibran et al. 1994). An intravenous injection of aFGF stimulates endothelial regeneration of damaged arterial walls (Bjornsson et al. 1991).

In a variety of animal models, exogenous bFGF has been shown to accelerate wound healing by speeding granulation tissue formation, increasing fibroblast proliferation and collagen accumulation at the site of a subcutaneously implanted sponge in rats (Davidson 1985, Buckley-Sturrock et al. 1989). Fresh wound

tensile strength has also been increased (McGee et al. 1988). However, the greatest benefit from the application of exogenous bFGF can probably be obtained in cases where wound healing is impaired. Research on skin flaps indicates that FGF has the potential to increase viability by accelerating flap revascularization when administered in a sustained-release manner (Hom et al. 1988). This may have applications to open or non-union fractures with impaired wound healing.

Effect of FGF on cartilage and bone repair. In 1984, Kato and Gospodarowicz recognized the ability of bFGF to stimulate the proliferation and differentiated function of chondrocytes in vitro. bFGF has been detected by immunohistochemistry in the proliferative and upper hypertrophic zone chondrocytes of the growth plate (Jingushi et al. 1990b). In the fetal bovine rib, bFGF is present throughout the growth plate with the cartilage (Sasse et al. 1992). Exogenous bFGF, infused into the rabbit proximal tibial growth plate, stimulates vascular invasion from the adjacent metaphyseal bone and accelerates the invasion of bone cell precursors and the ossification of the cartilage at the metaphyseal border (Baron et al. 1994). It has also been reported that bFGF can act on injured articular cartilage to stimulate chondrocyte proliferation and cartilage healing in vivo (Cuevas et al. 1988).

The histologic progression of fracture repair can be divided into four distinct stages, each characterized by different cellular and extracellular matrix features. Stage-1) Immediately after fracture, a hematoma forms at the fracture site and extends along the cortex above the periosteum and into the overlying soft tissue and muscle. Undifferentiated cells adjacent to the hematoma in the periosteum, muscle and the surrounding tissues proliferate. Stage-2) Bone matrix is synthesized by osteoblasts close to the fracture site and between the proliferating periosteal cells and the underlying cortex. Bone formation in the periosteum occurs by differentiation of osteoblasts from precursor cells without a cartilaginous intermediate. Stage-3) Undifferentiated mesenchymal cells are also seen in the granulation tissue overlying the fracture site. The region starts to develop the histological features of cartilage, and this continues until all fibrous tissue is replaced by cartilage. Stage-4) Bone forms from cartilage in the soft callus by a process that appears similar to bone formation in the growth plate. Capillaries invade the calcified cartilage. Osteoblasts appear following capillary ingrowth and synthesize osteoid on calcified cartilage. This process continues until all cartilage in the soft callus is replaced by bone.

While each of the four stages of fracture repair has distinct histological features, they share several underlying cellular events. These events include cell proliferation and differentiation, chemotaxis and the synthesis of extracellular matrix. The repair of fractures is believed to be regulated by both systemic and local factors. Systemic factors that affect fracture repair include endocrine and metabolic factors and drug treatment. Local factors are appreciated as important in fracture repair, but are less well characterized. Current investigations indicate that macrophages and other inflammatory cells secrete cytokines and growth factors to regulate the initial stages of fracture repair. Although many growth factors may play a role in regulating of fracture healing, only a few of these factors are presently understood. The effects caused by FGFs, TGF-ßs, PDGF and BMPs have been studied most extensively (Cornell and Lane 1992). These factors are synthesized by inflammatory cells, osteoblasts and chondrocytes throughout healing (Table 3, Bolander 1992). aFGF and bFGF genes are expressed from the early stage of fracture repair in the granulation tissue at the fracture site (Scully et al. 1990). aFGF stimulates cartilaginous callus enlargement in a rat fracture model (Jingushi et al. 1990a). bFGF has been shown to stimulate angiogenesis, volume and mineral content of callus in experimental fractures, in normal and diabetic rats (Inui et al. 1994, Cornell and Lane 1992, Kawaguchi et al. 1994) but, if instilled continuously, it will delay initiation of ossification (Cornell and Lane 1992). The data suggest that bFGF plays a role in stimulating bone formation during an early stage of the fracture healing process.

The effect of systemic administration of bFGF on bone has also been reported. Systemic daily injections of bFGF in rats stimulate endosteal bone formation in cortical and cancellous bones (Mayahara et al. 1993,

Table 3. Growth factor gene expression during fracture healing

	Fracture healing stage			
	I	II	III	IV
TGF-ß1	0	++	++++	+++
aFGF	+	+	++	+
bFGF	+	+	+	+
PDGF-B	0	++	++	++
BMP	+	+	+	+

Fracture healing stages defined as: I, initial injury response; II, intramembranous ossification; III, chondrogenesis; IV, endochondral ossification. 0 not detectable by Northern blotting; + detectable by reverse transcription followed by PCR amplification; ++ less than 50% maximal expression on Northern blotting; +++ greater than 50% maximal expression on Northern blotting; ++++ maximal expression on Northern blotting. Bolander et al. 1992. BMP from Bostrom et al. 1995.

Nagai et al. 1995, Nakamura et al. 1995). Systemic bFGF also increases the amount of intrinsic TGF-ß in the endosteal cells (Nakamura et al. 1995), as has previously been shown for TGF-ß expression in vitro (Noda and Vogel 1989). This implies that the stimulation of endosteal bone formation by bFGF may in part be mediated by TGF-ß.

Role of bFGF in bone implants

Demineralized bone matrix

Since Urist reported in 1965 that acid-extracted long-bone fragments stimulated endochondral osteogenesis, much work has been done to identify the factors responsible for osteoinduction. The use of molecular cloning revealed the existence of several BMP genes. The factors responsible for osteoinduction may play a physiologic role in endochondral bone formation and may be relevant to bone repair. They may accelerate fracture healing and the filling of bone defects; therefore, they may be of therapeutic value. bFGF has the ability to stimulate chondrocyte formation (Kato et al.1987) and capillary formation in vivo (Montesano et al. 1986). Exogenous bFGF increases the bone yield from subcutaneously-implanted demineralized rat femur diaphyses (Aspenberg and Lohmander 1989). This effect of bFGF was dose-dependent. Higher doses had an inhibitory effect (Aspenberg et al. 1991). However, it was not clear if the increased bone yield was caused by effects on one or more of the early cellular processes of bone induction i.e., was due to inflammation, recruitment of stem cells, prechondroblast cell division, or if it was caused by a faster development of the capillary supply. Clarification of this point may contribute to understanding the effect of bFGF on bone implants.

Bone graft

Incorporation of cancellous bone grafts. After the surgical procedure, bleeding and inflammation occur rapidly and the bone marrow and spongiosa in the nonvascularized autograft become necrotic. New osteoblast precursors and osteoclasts are brought to the grafts by revascularization. The ingrowth of host blood vessels is followed by graft resorption, which proceeds parallel to new bone formation. The parallel activity of resorption and bone accretion is seen throughout the interior of a cancellous graft within 4 weeks after surgery (Goldberg and Stevenson 1987).

During the first few weeks after transplantation, BMPs in the grafts are thought to induce host mesenchymal cells to migrate into the graft. These cells differentiate into osteoblasts and begin to produce new bone. However, it remains to be demonstrated whether the BMP content of undemineralized bone grafts has any practical importance. Differentiated osteoblasts line the edges of dead trabeculae during this early stage. The osteoid deposited eventually surrounds a core of dead bone. Increased bony density may be seen on roentgenograms during this phase because of the entrapment of necrotic bone by new host bone. During this phase, the mechanical strength of the cancellous bone may be greater than it was originally because of the admixture of live host bone and dead graft bone; with remodeling, however, the strength gradually returns to normal (Goldberg and Stevenson 1987).

Bone allografts are incorporated more slowly than autografts because of the immune response by the host, which delays and impairs new vessel ingrowth into the graft (Bolano and Kopta 1991, Kirkeby 1991). This results in a significant rate of infection, non-union and graft fracture. Methods of preservation and graft modification have improved graft incorporation. However, in clinical practice, the growth of new bone into cancellous allografts is shallow and incomplete (Enneking and Mindell 1991). The incorporation of the graft depends on angiogenesis. Exogenous bFGF increases angiogenesis in autografts (Eppley et al. 1988, Eppley et al. 1991, Weiss et al. 1995), However, it is not clear whether it increases osteogenesis in grafts.

Hydroxyapatite (HA)

If a bone substitute with some mechanical strength is needed, HA appears most appropriate, as it represents the natural mineral in human bone (Cooke 1992). There are some reports about successful integration of coral-derived HA in dental surgery (Pilliar et al. 1991, Block et al. 1992). Also, HA is used in bone defect (Loku et al. 1993). When HA with a pore size of 200 or 500 µ was implanted in rabbit femoral condyles, no ingrowth was found with the 200 µ pore size, but bone remodeling was observed with the 500 µ size at 12 and 26 weeks postoperatively (Kühne et al. 1994), thus suggesting that bone ingrowth is dependent on pore size in HA . On the other hand, priveous study hass also shown that a thin layer of a 200 µm pore size HA may be useful for coating a metal endoprosthesis to improve its attachment to the surrounding bone (Cook et al. 1985). These ovservations suggested that the ingrowth of bone was dependent not only on pore size but also on the interconnectivity of the pores. Later studies reported that porous HA combined with BMP induces bone formation under the periosteum of the rabbit skull (Miller et al. 1991, Ono et al. 1992) and in the intramuscular pockets and calvaria of baboons (Ripamonti et al. 1992 and 1993). TGF-ß

bound to tricalcium phosphate (TCP) stimulated bone formation in radial defects in rabbits (Beck et al. 1995), and recent studies have reported that TGF-ß enhanced bone ingrowth and mechanical fixation of (TCP)/HA ceramic-coated implants (Lind et al. 1995, Summer et al. 1995). bFGF may also increase bone ingrowth in porous HA (Schnettler et al.1992, Wipperman et al. 1994), but data on optimal dose and carrier require further clarification.

Bone chamber method

Titanium chambers have been used for almost two decades for studies of bone regeneration. The "Bone Growth Chamber" (BGC) and the "Bone Harvest Chamber" (BHC) are useful tools for quantifying bone generation under different experimental conditions (Albrektsson et al. 1989). Using the originally constructed BGC, bone generation was investigated after heat trauma (Eriksson et al. 1984), irradiation (Jacobsson 1985) and electrical stimulation (Buch et al. 1985). The major drawback using the BGC method is that the animal must be killed in order to remove the implant and harvest the ingrown bone. The BHC was developed by Albrektsson et al. in 1984. The advantage was that a single animal could be used for several consecutive experiments. The bone production is reproducible over a considerable time (Kälebo and Jacobsson 1988; Thorén et al. 1995). Bone formation in this model has been shown to be very sensitive to various forms of disturbance; small amounts of particulate biomaterials (bone cement, high density polyethylene, titanium alloy and chrome-cobolt, but not hydroxyapatite) diminish bone ingrowth (Goodman et al. 1993a, Wang et al. 1994, Goodman et al. 1995a, Goodman et al. 1995b). This chamber has been modified for continuous drug application to the bone ingrowth canal (Aspenberg et al. 1988, Aspenberg et al. 1989) and for micromotion (Aspenberg et al. 1992, Goodman et al. 1993b, Goodman 1994). The bone defect of the BHC is a canal measuring 1 mm in diameter and this defect is usually filled by new ingrown bone within 3 weeks.

In the evaluation of osteoconductive materials, the defect created should be large enough to challenge the adjacent bone with a space that it can hardly fill spontaneously. This is evident from an important mistake in orthopedic history. A standard model since the 1950s has been to drill a 5 mm hole in cancellous bone of animals and to place a plug of the test material in the hole (Maatz et al. 1954). In such experiments, Kiel bone and autografts gave the same results. The same experiment was performed in humans with similar findings (Hallén 1966). These findings led to the introduction of Kiel bone in clinical practice. Clinical results with a failure rate of 42% in 142 cases were reported (Haasch 1963). The disadvantage of the experimental model was that the spontaneous regenerative response caused healing, whatever material was tested, because the hole was not large enough (Schweiberer 1970). When the diameter of the burr hole was to be increased, Kiel bone was found by Schweiberer to prevent healing (1970). This observation caused Kiel bone to disappear almost totally from orthopedic practice.

The effect of growth factors on bone conductive materials are usually seen at an early stage during bone ingrowth. It is then difficult to find the right time to measure these effects, if new ingrown bone rapidly fills the chamber. This problem stimulated us to develop a chamber which is never completely filled with bone. This would be advantageous, since the final amount of bone in the chamber can be used to measure the effects of various attempts to increase or reduce bone formation.

AIMS OF THE STUDY

This thesis examines the effects of bFGF on osteoinduction and the incorporation of conductive implants. It summarises and discusses work performed at the Laboratory for Experimental Orthopedics in Lund (Aspenberg and Wang 1993, Wang and Aspenberg 1993, 1994, 1996a, 1996b and 1996c). The specific aims were to test the following hypotheses:

1. Increased calcium content in DBM with bFGF is due to increased number of chondrocytes, leading to more bone.

2. The newly developed bone conduction chamber makes it possible to demonstrate a difference between the conductive properties of different materials (defatted cancellous bone graft and porous hydroxyapatite).

3. bFGF can increase new bone ingrowth distances in cancellous bone graft and porous hydroxyapatite.

4. bFGF also increases the ingrowth of fibrous tissue in cancellous bone graft and porous hydroxyapatite.

5. The effect of bFGF on ingrowth is dose-dependent.

6. The effect of bFGF on ingrowth is different at different times after administration.

7. The effect of bFGF on ingrowth is dependent on the time-point for administration.

8. A slow-release carrier will contribute to the effect of bFGF on ingrowth.

Methods

Design of experiments

Effects of bFGF on bone induction (Wang and Aspenberg 1993). Demineralized bone matrix was implanted with or without bFGF. Each pair of implants from 1 donor was implanted in 2 separate muscle pouches in one recipient rat. One implant with bFGF in a carboxymethyl cellulose gel (cmc) in each pair served as the experimental implant and the other had saline as the control. Previous studies showed no difference between implants which were treated with the gel alone or moistened with saline (Aspenberg and Lohmander 1989). Histological changes were studied after 1, 2 and 3 weeks.

Evaluation of Bone Conduction Chamber (Aspenberg and Wang 1993). In order to test the BCC and the bone-conductive materials in the chamber, the empty BCCs were unilaterally implanted in rat tibiae in one group. The rats were killed after 3, 4 and 6 weeks. In another group, empty BCCs and BCCs containing porous hydroxyapatite rods and bone grafts were unilaterally implanted in rats. These rats were killed after 6 weeks.

Effects of bFGF on incorporation of bone graft and porous hydroxyapatite (Wang and Aspenberg 1994, 1996a and 1996c). In order to investigate whether bFGF is a stimulator of the incorporation of bone implants and clarify the effect of different doses of bFGF on bone implant incorporation and the onset of the effect of bFGF, the bone implants (bone grafts and hydroxyapatite rods) were soaked in the hyaluronate gel with or without bFGF. In dose studies, the BCCs containing bone grafts were unilaterally implanted in rats and harvested at 6 weeks. The doses per graft were 0.3, 8, 40, 200 and 1000 ng, respectively. In the time-effect study of the bone grafts and in the hydroxyapatite studies, the BCCs with bone grafts or hydroxyapatite rods were bilaterally implanted in rats, so that each rat had an internal control. The rats were killed after 1, 2, 4, 6 and 10 weeks, respectively.

The effect of the time-point for administration of bFGF (Wang and Aspenberg 1996b). In order to clarify whether bFGF application at a later stage could stimulate the incorporation of a bone graft, a modified BCC was used to infuse bFGF. Before implantation, each pair of grafts was moistened with saline and placed in two chambers connected with tubes to an osmotic minipump, one pump with bFGF, the other with buffer. The chambers were bilaterally implanted in rats. The bFGF was infused after implantation either immediately or 2 weeks later. The infusion time was 1 week or 2 weeks. The harvest time was 6 or 8 weeks after implantation.

Animals and material

Female Sprague-Dawley rats, weighing 200–220g (Møllegaard, Copenhagen, Denmark) were used to prepare demineralized bone matrix and bone graft and act as recipients of demineralized bone matrix. Four hundred and twenty-one Male Sprague-Dawley rats (320–350 g) were used as recipients of chambers. The animals were housed in a temperature-controlled room (22 °C) and fed a standard laboratory diet. Two rats were kept in each cage with free access to food pellets and water.

Recombinant human basic FGF delivered in a buffer (Synergen, Boulder, CO, USA) was used in some studies (Aspenberg and Wang 1993, Wang and Aspenberg 1994 and 1996a). In the others we used recombinant human basic FGF powder (CalBio, Scios Nova, Mountain View, CA, USA) reconstituted in 10 mM sodium citrate, 1 mM sodium EDTA and 9% saccharose, pH 5, stored at –70 °C (Wang and Aspenberg 1996b and 1996c). Stock solutions were then diluted in PBS (pH 7.0) containing rat serum albumin to the desired concentrations prior to use.

A carboxymethyl cellulose gel was used as a carrier for bFGF in one study (Wang and Aspenberg 1993). A 1% hyaluronate gel (Healon TM, Kabi Pharmacia, Uppsala, Sweden) was used as a carrier for bFGF in other studies (Wang and Aspenberg 1994, 1996a, 1996b and 1996c).

Osmotic pumps (ALZET, model 2002, ALZA Corp., Palo Alto, CA, USA) for the treatment group were loaded with 200 µL of bFGF solution and had a delivery rate of 0.5 µL/hr. Osmotic pumps for the control group were loaded with a similarly diluted buffer.

Bone conduction chamber

The bone conduction chamber consists of a threaded titanium cylinder, formed from two half cylinders held together by a hexagonal closed screw cap. The

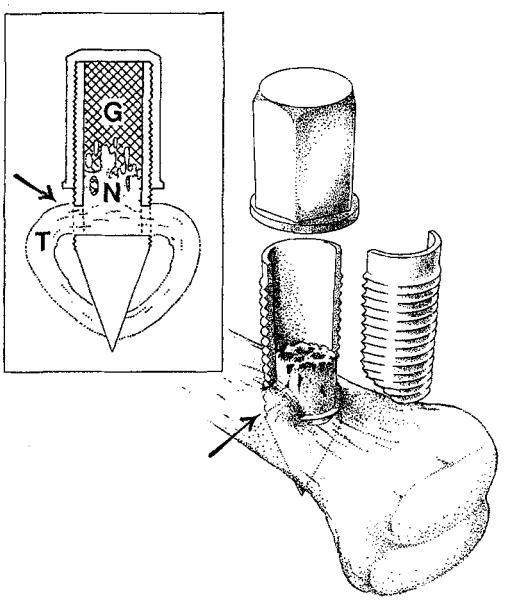

Figure 1. The Bone Conduction Chamber. Implant in position at the poximal tibial (T) metaphysis. The ingrowth entrances (arrow) are situated in bone. Inset shows schema with new bone ingrowth (N) and graft bone (G). (Reproduced with permission of European Journal of Experimental Musculoskeletal Research).

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 3.5 mm. There are two bone ingrowth openings at the bottom of the chamber (Figure 1). The bone conduction chamber was devised so that the bone enters one end of a cylindrical space at the cortical level. This space extends far out into the subcutaneous region, and the ingrown bone-derived tissue can fill the chamber without competition with other tissues.

In order to deliver the bFGF at different times, the original chamber was modified by drilling two 0.8 mm diameter holes through the cap at its upper end into which was inserted a 6 cm silicon tube (0.012 inch inner diameter, Silastic®, Medical Grade Tubing, Dow-Corning Corp., Midland, MI, USA), that was also connected to an osmotic pump. The other hole in the cap of the chamber was left open to act as a drain into the subcutaneous tissue (Figure 2).

Preparation of bone implants

Demineralized bone matrix was prepared by collecting femoral diaphyses, shaped as 8 mm long and 3 mm wide tubes, from donor rats. The femoral tubes were immediately cleaned of periosteum and marrow. The specimens were kept as pairs from each donor, 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, which was changed 3 times, rinsed 5 times with sterile deionized water, lyophilized, weighed and finally stored in sterile glass tubes.

Bone grafts were prepared by resecting a cylindrical 2 x 6 mm bone rod in the axial direction from the knee joint with a hole-cutter from the proximal tibiae. The epiphysis and the growth plate were excised. The proximal part of the graft had the densest cancellous bone and was later turned towards the ingrowth end of the chamber. The grafts were kept sterile and frozen at –70 °C. Before implantation, the pairs were lipid-extracted in a test tube containing a mixture of chloro-

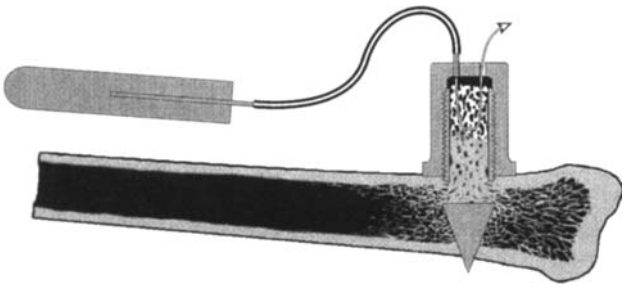

Figure 2. A. The bone Conduction Chamber was inserted into the proximal tibial metaphysis and connected to an implanted osmotic pump via a silicon tube. Bone enters the bottom of the chamber via two openings. The bone graft is shown white and the new ingrown bone penetrating into the bone graft in the chamber is shown grey.

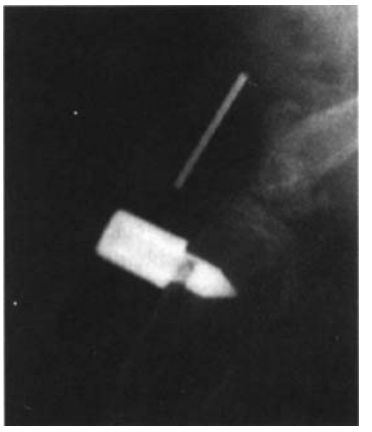

Figure 2. B. The position of the chamber in the rat proximal tibia. The metal rod is a part of the mini-osmtic pump.

form/methanol 1:1, oscillated overnight, then rinsed 3 times with methanol and air-dried.

Coralline hydroxyapatite (Interpore IP 200; Interpore International, Irvine, CA, USA) was delivered as cylindrical rods, manufactured to fit precisely into the chambers. This material has a pore size of about 200μ. The cylinder axis was oriented randomly to the growth axis of the coral and the coral rods were taken at random for bFGF or the control.

Surgical procedures

For DBM implants, an abdominal midline incision was made in the rat's skin and bilateral muscle pouches were created laterally to the rectus muscle by separating the two oblique layers. The implants were inserted in the two pouches, which were closed with a suture. The skin was closed with wound clamps.

For the chamber implantation, the medial proximal tibial metaphysis was exposed with a longitudinal incision under general anesthesia using an aseptic technique. The periosteum was elevated and cleaned 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. When using the infusion chamber, a subfascial pocket was created by blunt dissection of the medial thigh and into this was placed the osmotic pump and excess tubing. The wound was closed in layers with interrupted fascial and continuous subcutaneous sutures (Figure 2).

Evaluation of results

The implants of demineralized bone matrix were dissected from the surrounding soft tissue, fixed in 4% buffered formalin, embedded in hydroxyethylmethacrylate (Cambridge Instruments GmbH, Heidelberg, Germany). Sections, 6 μm thick, were cut perpendicularly to the long axis of the implant. The whole implant was sectioned throughout and sections at 1 mm intervals were taken for analysis. Three sequential sections for each 1 mm interval were stained with hematoxylin and eosin (H&E), toluidine blue and van Kossa stain, respectively.

The total area of the bone matrix implant and the calcified areas on the slides were blindly measured by using a microscope with a video camera connected to an image analysis system (Videoplan, Kontron Bildanalyse, Darmstadt, Germany). The total area of the bone matrix implant, not including the ingrown tissue in the marrow cavity, was measured in the H&E stained sections. The bone areas were delineated using van Kossa stained sections. The number of chondrocytes in the bone matrix was counted in the toluidine blue-stained sections. 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 sequential sections. The number of chondrocyte clusters and the area of each cluster per section were blindly measured with another morphometry computer (CAS, Inc. Becton Dickinson, Elmhurst, IL, USA). The cluster area was defined as the toluidine blue-stained area surrounding at least one chondrocyte. The analysis was done in the 3 most proximal sections of each implant as a cluster area measurement.

The tissue harvested from the bone conduction chamber, with or without bone graft or hydroxyapatite, was 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 were stained with hematoxylin and eosin. Three sections from the middle of the specimens, each 300 μm from the next slide, were employed for measurements, using a microscope with a computerized video digital table system (Videoplan™, Zeiss) at a magnification of 40 x. This was done blindly, so that each slide was given a code number, and all slides in each experiment were investigated in random order. In the specimens from chambers without grafts, the tissue and bone areas and their widths were measured. In the specimens containing osteoconductive implants, the bone area included implant areas and marrow cavities which had been surrounded by new bone. New bone close to the bone ingrowth frontier was always woven. It was defined as a dark pink, evenly stained matrix with dispersed, slightly rounded cells. In contrast, the fibrous tissue was pale with loosely arranged fibers and abundant spindle-shaped cells. The border between the new bone and fibrous tissue was easily identified. In some cases, the shape of the border line was very complex. In such cases, a straight line was drawn between about 7 points, where the new bone extended farthest into the graft. The total area of tissue ingrowth, including soft tissue, was measured in the same way as with bone ingrowth. The distal border was defined by a thin zone of inflammatory cells. The mean ingrowth distance of the total tissue and the

ingrown bone were calculated by dividing the area by the width of the specimen (Figure 3). The mean of the 3 sections from the same specimen was used for all statistical analyses.

When the bone implants were harvested, a red zone on the upper part of the implants was often seen under the operating microscope (x 5). Histological examination showed that the red part corresponded to extravasated erythrocytes. One group of specimens at 6 weeks in the time-response study and another group of specimens from hydroxyapatite rods treated with 0.04 µg bFGF with carrier were chosen for evaluating the effect of bFGF on the vascularity in the graft. The presence of a red zone was scored 0 (none), 1 (red patches), 2 (about 1 mm wide zone), 3 (about 2 mm) or 4 (about 3 mm or more).

At the bone ingrowth front, active osteoblasts were visible laying down osteoid. The osteoid was defined as spindle cells and round cells surrounded by a light pink matrix. In mineralized specimens of bone grafts, this tissue was easily distinguished by using the Goldner stain. The presence of similar tissue stained with H&E (osteoid or primitive bone) was scored blindly in decalcified sections as 0 (none), 1 (interrupted appearance) or 2 (almost continuous along the border between the bone and fibrous tissue).

In some studies, ^{99m}Tc-MDP was given in the tail vein 3 hours before the rats were killed. The scintimetric activity of the tissue harvested from the chambers was measured in a well counter. The values were corrected for time-dependent decay.

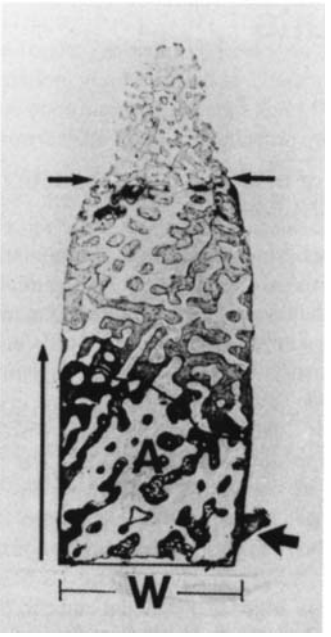

Figure 3. Control specimen at 6 weeks. Fat arrow points at bone ingrowth opening. Vertical arrow shows growth direction. The horizontal arrows point at the distal border of tissue ingrowth where a dark band of inflammatory cells is seen. Above this border, the specimen has shrunken during histologic preparation, due to absent tissue support. The bone ingrowth frontier is marked with a solid line. The area (A) consists of new bone, marrow and dissolved hydroxyapatite (white). Specimen width is marked as W. The mean ingrowth distance is calculated as A/W (H&E; original magnification, x5; Published with permission from Clin Orthop).

Results

Effects of bFGF on bone induction

(Wang and Aspenberg 1993)

At one week, 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. At 2 weeks, in the 15 ng bFGF-treated implants, the chondrocyte density index was increased by 57% compared to the controls (Table 4). The number of chondrocyte clusters was increased by 64%. The chondrocyte cluster size was unchanged. The ossification index was also higher than that of the control (Table 4). At 3 weeks, the chondrocyte density index in the bFGF implant was lower and the ossification index was higher than in the controls. In the latter, the chondrocyte density index was higher at 3 weeks than at 2 weeks. As osteogenesis continued, the cartilage was gradually replaced by bone. Numerous osteoblasts and multinucleated osteoclasts were seen surrounding the bone. The newly formed bone was associated with the dissolution of the implanted matrix and the formation of an ossicle. With the higher dose of bFGF (1900 ng), the chondrocyte density index and the ossification index were lower than in the controls and the implants with 15 ng per graft at 3 weeks. There was no sign of an inflammatory reaction or increased vascularity. Only fibroblast-like cells were seen in the cavities in the center of the bone matrix.

Evaluation of bone conduction chamber

(Aspenberg and Wang 1993, Wang and Aspenberg 1994, 1996a, 1996b, and 1996c)

The total mass of tissue in the empty chamber was unchanged between the 3rd and 4th weeks, but there was more tissue in the 6th week. The mean ingrowth distance of the bone gradually increased and the thickness of the fibrous tissue diminished with time (Figure 4).

The distal end of the specimen (the greatest distance from the bone ingrowth openings), consisted of a zone with vascularized fibrous tissue. A zone with woven bone was present in the middle portion. The proximal end contained a zone with more mature, re-

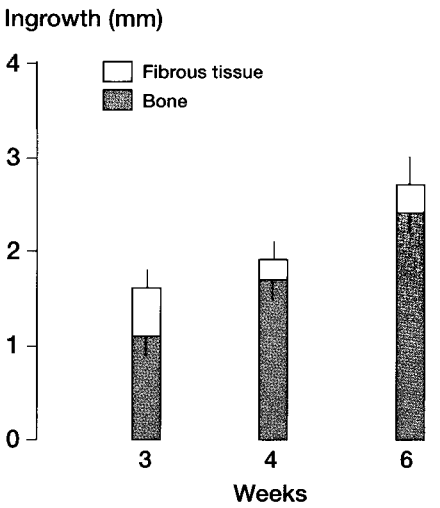

Figure 4. Total tissue and bone ingrowth distance within non-grafted chamber at 3, 4 and 6 weeks.

Table 4. Bone induction by DBM with bFGF

Weeks after implantation	Histologic finding	Control	bFGF	bFGF (higher dose)
1	Cartilage	+	+	ND
	Bone	0	0	ND
2	Cartilage	++	+++	ND
	Bone	+	++	ND
3	Cartilage	+++	+	0
	Bone	++	+++	0

ND not determined.

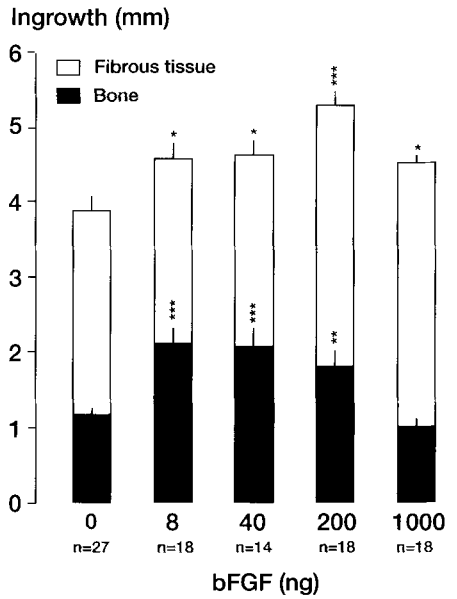

Figure 5. Dose-response curves for the effects of bFGF on bone and total tissue ingrowth distances in rat bone grafts (mean±SEM). Bone and total tissue ingrowth distances were increased by bFGF. (Published with permission from J Orthop Res).

modeled cancellous bone surrounding marrow cavities. The transition from mesenchymal cells to woven bone was gradual, apparently through membranous ossification. The cancellous zone gradually expanded and the fibrous zone gradually disappeared with time. Thus at 6 weeks, most of the tissues consisted of remodeled cancellous bone. Since a small amount of woven-fibered bone remained at 6 weeks, membranous bone formation appeared to be continuing.

In the experiment that was specificly designed to compare conductive materials (Aspenberg and Wang 1993), empty chambers and the chambers containing hydroxyapatite or bone grafts were harvested at 6 weeks. The mean ingrowths of bone (mean ± SD) were 0.8±0.2, 1.4±0.5 and 2.4±0.2 mm, respectively (Anova, $p<0.0001$; all intergroup post hoc comparisons, $p<0.01$). Also, when the untreated control implants from all later 6 weeks experiments were pooled, bone grafts showed more bone ingrowth than porous hydroxyapatite ($p<0.05$).

In a comparison of grafts with or without the hyaluronate gel in BCC at 6 weeks, we found no significant difference in ^{99m}Tc-MDP activity, histology or histomorphometry. Two way ANOVA showed that most in the variation of the bone ingrowth distance was due to differences between animals, and only a minor part was due to left-right differences in the animals (variance ratio 4.3). Thus, the bone ingrowths on the two sides correlate ($r^2=0.6$, $p<0.01$). The methodological error in the single specimen at 6 weeks, including biological variation, was estimated at 27% for scintimetry, 20% for bone ingrowth distance and 14% for total tissue ingrowth. The histomorphometric measurment error for this method is 6% (Thorén 1994).

Fluid flow from the osmotic pumps into the ungrafted chambers caused no difference in the amount or quality of ingrown bone, and also in chambers with graft there was no difference in the quality of bone or its penetration distance into the graft at 2 weeks.

Effects of bFGF on incorporation of bone graft *(Wang and Aspenberg 1994 and 1996a)*

Quantitative evaluation

Specimens treated with 40 ng bFGF had a higher score for the presence of a red color at the distal end of the graft than did the controls at 6 weeks. On microscopic examination, almost all bFGF-treated specimens showed more erythrocytes in this region, which explains the red color on macroscopic inspection. The bFGF-treated specimens also had a higher score for the presence of osteoid.

In the grafted chambers, the total ingrowth distance was increased by all doses except 0.3 ng per implant. Bone ingrowth was increased by 8, 40 and 200 ng (by 88%, 80% and 54%, respectively), but was unchanged by 0.3 and 1000 ng. The thickness of the fibrous zone (i.e., total ingrowth minus bone ingrowth) was increased by 200 and 1000 ng (Figure 5).

In the time-effect study, the bone grafts were treated with or without 40 ng bFGF. At 3 and 4 weeks, no effect of bFGF on bone ingrowth could be shown. At 6 weeks, the bone ingrowth was increased by 51% with bFGF, compared to the controls. At 10 weeks, both the total tissue ingrowth and bone ingrowth distances in the bFGF-treated specimens were significantly increased, compared to the controls (for bone 67% more than the controls; Figure 6).

The uptake of ^{99m} Tc-MDP increased with time. At 2 and 4 weeks after implantation, the uptake of ^{99m}Tc-MDP was higher in the bFGF-treated grafts than in the controls. There was a correlation between new bone ingrowth distance and scinitimetric activity ($r=0.81$; $p=0.001$).

In the infusion chamber with a graft, a lower concentration of bFGF (0.1 µg/mL) caused an increase in 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%. The ^{99m}Tc-MDP uptake was also increased.

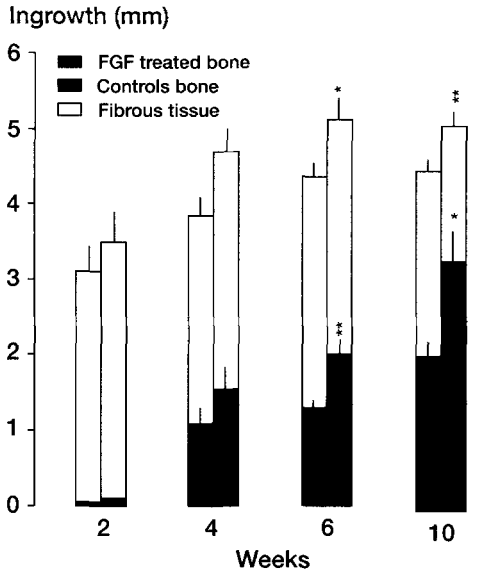

Figure 6. Time-effect study. Total tissue and bone ingrowth distances (mean±SD) in the grafts. *p<0.05, **p<0.01. Data from Wang and Aspenberg 1994 and 1996a. (Published with permission from J Orthop Res).

Following the infusion of 1.0 μg/mL bFGF for 1 week, the ^{99m}Tc-MDP uptake and the bone ingrowth distance were increased at 6 weeks. With a delayed infusion when buffer was infused first for 2 weeks, and then the bFGF for 2 weeks (2 + 2 + 2), no effects could be demonstrated at 6 weeks. However, when the same treatment was given, but the harvest was delayed to 8 weeks (2 + 2 + 4), we found a trend towards increased bone ingrowth distance ($p < 0.07$; Table 5).

One-way ANOVA showed significant differences in the effect of bFGF between these groups ($p < 0.05$). By post hoc analysis, bFGF had a larger effect in the animals that received an immediate infusion (1+5 and 2+4) than in those with a delayed infusion (2+2+2), but not delayed harvest ($p < 0.05$). When both infusion and harvesting were delayed (2+2+4), the bFGF effect was larger than in the animals with a delayed infusion only ($p < 0.05$). These data may indicate that bFGF has a stimulatory effect, regardless of the infusion time, but this effect was seen only 6 weeks after the infusion started.

Qualitative evaluation

The new bone in the bone grafts was very similar to that in the non-grafted chambers, but the soft tissue in front of the ingrown bone behaved differently. At 6 weeks, new vascularized tissue usually entirely filled the grafts. At the distal end, far from the bone ingrowth openings, there was loose connective tissue with small, mostly spindle-shaped nuclei, surrounding the cancellous bone graft trabeculae. In the proximal end of the specimen we found an ossicle, i.e., a large marrow cavity containing some new woven bone trabeculae, and often surrounded by a thicker shell of new, partly lamellar bone. The interface between the proximal ossicle and the mostly unresorbed graft located distally, usually had a clear-cut borderline: the new bone was covered with mature fibrous tissue parallel to the borderline, and most of the graft bone was resorbed in that area (Figure 7).

In the bFGF specimens treated with doses between 8 and 200 ng per graft, there was no clear borderline between the proximal ossicle and the unresorbed graft. Instead, there was an interdigitation, with new bone sprouts protruding into the graft. Distal to these sprouts, there was usually a dense mesenchymal-like tissue with larger nuclei (Figure 8). A gradual transition to osteoid and bone appeared to be continuing. The woven bone area in the bFGF-treated specimens was wider than in the controls. At bFGF concentrations of 1000 ng, dense fibrous tissue was sometimes present on the distal surface of the new-formed bone, isolating it from the graft (Figure 9).

Table 5. ^{99m} Tc-MDP and tissue ingrowth distances in bone grafts with 1.0 μg/ml bFGF infusion at different times

Infusion time (weeks)	^{99m}TcMDP (cpm)		Ingrowth distance (mm)			
			Total tissue		Bone	
	bFGF	Control	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 = first 2 weeks buffer, then bFGF infusion 2 weeks, after that no infusion for 2 weeks; 2+2+4 = first 2 weeks buffer, then bFGF infusion 2 weeks, after that no infusion for 4 weeks.

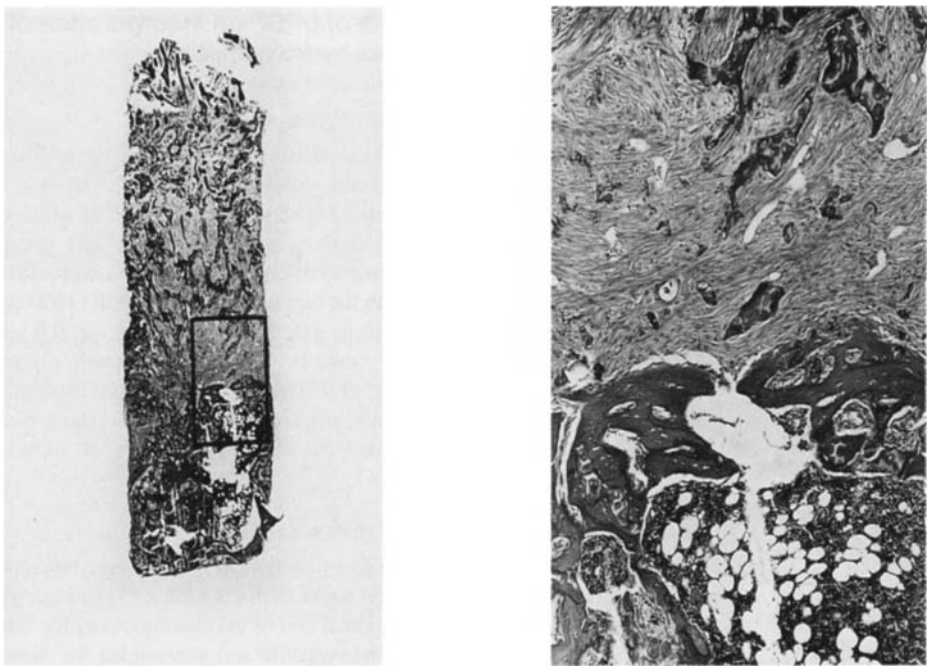

Figure 7. Control specimen of bone graft treated with hyaluronate gel. There is a clear-cut borderline between new bone and fibrous tissue. The partly lamellar new bone was covered by a mature fibrous tissue parallel to the borderline, and most of the graft bone was resorbed in that area. (Stain, H&E; original magnification, x 5, x 25).

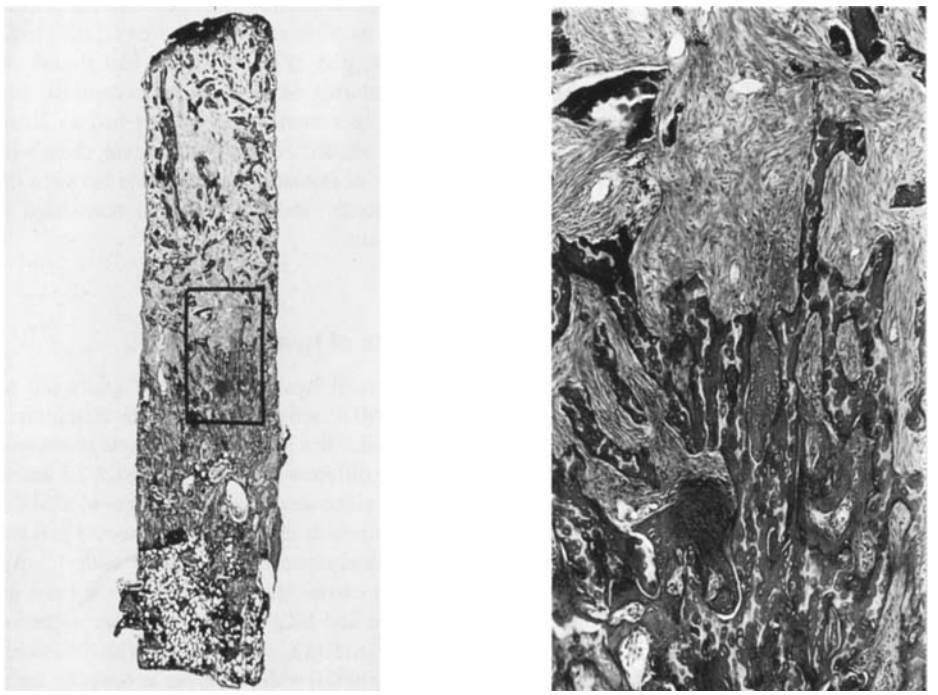

Figure 8. Specimen from the same animal as Figure 7, treated with 40 ng bFGF in a hyaluronate gel. There was no clear borderline between new bone and fibrous tissue, but an interdigitation, with new bone sprouts protruding into the graft. Distal to these sprouts, there was osteoid. Note that the bone has penetrated a longer distance into the graft, as compared to Figure 7. (g graft bone, n new bone, o osteoid; Stain, H&E; original magnification, x 5, x 25).

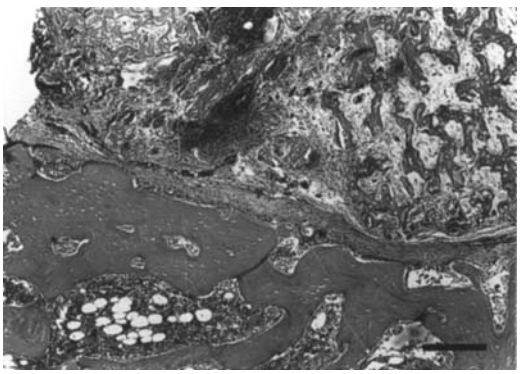

Figure 9. Similar specimens as in figure 7, but treated with a higher dose of bFGF (1000 ng). A dense fibrous tissue covers the mature ingrown bone (Stain, H&E; original magnification, x5. Published with permission from J Orthop Res).

The following section describes the findings that are seen at at different times after implantation of a bone graft.

Week 1. Polymorphonuclear leukocytes, lymphocytes and histocytes were present in all specimens. No difference in cell counts was detected between bFGF-treated grafts and controls. Organized tissue ingrowth had scarcely begun.

Week 2. The formation of new bone in the grafts had started in both bFGF-treated specimens and controls, and no differences were found between the paired specimens. A few islands of bone formation could be seen in some sections, but most bone was near the bone ingrowth holes or at the bottom of the chamber. Only woven bone was observed. Soft tissue, forming a loose reticular stroma with capillaries, had penetrated the graft-half way to the top of the chamber.

Week 4. The amount of new bone in the grafts had gradually increased. The woven bone was remodeled to a more trabecular shape. No differences were seen between bFGF-treated and controls.

Week 6. The zone of woven bone and remodeled trabeculae in bFGF-treated specimens were larger than in the controls. There were more mesenchymal cells along the distal aspect the woven bone in bFGF-treated specimens.

Week 10. At the proximal end of the specimens, a large marrow cavity was surrounded by a thicker shell of new bone. The new bone was more mature. In some bFGF-treated grafts, the new bone had almost reached the top of the graft.

Effects of bFGF on incorporation of porous hydroxyapatite

(Wang and Aspenberg 1996c)

Quantitative evaluation

The mean redness score was 1.7 in the hydroxyapatite rods treated with 40 ng bFGF with carrier at 6 weeks versus 0.3 for controls ($p<0.01$). The mean bone ingrowth distance was increased by 70% and the total tissue ingrowth distance was increased by 58% (Table 6). With the higher amount of bFGF (1000 ng) in the hyaluronate gel, the redness score was 0.6 versus 0.2 in the controls. The bone ingrowth distance was slightly decreased. In contrast, the total tissue distance was increased by this dose, which means that there was an increased amount of fibrous tissue (Table 6).

Qualitative evaluation

Qualitative histological examination revealed essentially the same findings as those with bone grafts. In the proximal end of the chamber, lamellar bone lined the hydroxyapatite and surrounded the marrow elements. No signs of resorption were found. More distally, woven bone mostly filled the available space. In some places, membraneous ossification was taking place at the interface to a more distal zone of fibrous tissue. In no control specimens did the fibrous tissue fill the distal third of the hydroxyapatite rod. Therefore this part of the specimen had shrunk or disappeared during decalcification. In contrast, the bFGF-treated specimens of all groups had an intact shape during decalcification. Furthermore, there was a wider zone of immature woven bone between the distal bone border and the proximal remodeled bone in specimens.

Effects of hyaluronate gel

Addition of hyaluronate gel to grafts did not alter ^{99m}Tc-MDP activity, histology or histomorphometry at 6 weeks. In a pilot study, the bone grafts were treated with different doses of bFGF (0.5, 2.5 and 12.5 µg/mL) in saline solutions. No changes in total tissue and bone ingrowth distance were detected at 6 weeks. In the hydroxyapatite rods treated with 1.5 µg bFGF without carrier, at 6 weeks, both the bone ingrowth distance and total ingrowth distance were increased by 41% and 33%, respectively. With the lower dose of 0.15 µg bFGF without carrier at 6 weeks, only the total ingrowth distance was increased (Table 7). The controls treated with carrier or without carrier at 6 weeks did not differ regarding bone or total tissue ingrowth.

Table 6. The dose-effects of bFGF with carrier on coralline hydroxyapatite incorporation at 6 weeks

Dose (μg)	Rats (n)	Redness Score bFGF/Con	Total tissue ingrowth			Bone ingrowth		
			Control (mm)	bFGF (mm)	Effect (%)	Control (mm)	bFGF (mm)	Effect (%)
0.04	9	1.7 / 0.3	3.6±0.8	5.7±0.6	+58 ***	1.7±0.8	2.9±1.2	+70 *
1.0	8	0.6 / 0.2	3.3±0.4	5.1±0.3	+55 ***	1.3±0.5	1.2±0.8	−7.7

* p<0.05; ** p<0.005; *** p<0.001.

Table 7. The effects of bFGF with or without carrier on coralline hydroxyapatite incorporation at 6 weeks

Dose (μg)	Rats (n)	Hyaluronate carrier	Total tissue ingrowth			Bone ingrowth		
			Control (mm)	bFGF (mm)	Effect (%)	Control (mm)	bFGF (mm)	Effect (%)
0.04	9	+	3.6±0.8	5.7±0.6	+58 ***	1.7±0.8	2.9±1.2	+70 *
0.15	9	–	3.5±0.6	4.7±0.5	+34 **	1.5±0.8	1.9±1.2	+24
1.5	10	–	4.0±0.7	5.3±0.6	+33 ***	1.2±0.5	1.7±0.7	+41 *

* p<0.05; ** p<0.005; *** p<0.001.

Discussion

bFGF-stimulated bone induction

The addition of exogenous bFGF to demineralized bone matrix may increase the amount of bone formed by bone induction (Aspenberg et al 1991). This observation was also found in a similar system in which platelet-derived growth factor was added to the bone matrix powder in mature rats (Howes et al. 1988). Bone induction by demineralized bone matrix is a multistep cascade (Reddi et al. 1987). The major phases of osteoinduction are chemotaxis, mitosis and differentiation. Chemotaxis may be defined as the directed migration of cells in response to a chemical gradient. Implantation of demineralized bone matrix promotes chemotaxis of cells from the vicinity. Proliferation of mesenchymal cells indicates that the bone matrix is also a local mitogen (Rath and Reddi 1979, Reddi 1989). Compared to DBM powder, bone induction by DBM femurs was slower, but exogenous bFGF accelerated the process (Table 4).

Our finding indicates that 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. These results confirm and extend the previous finding that exogenous bFGF can increase the amount of bone (Aspenberg and Lohmander 1989, Aspenberg et al. 1991). In those studies, the calcium content was increased by bFGF, after 3 and 4 weeks, but not earlier. This delay seemed to indicate that bFGF primarily increased vascular ingrowth, which must occur before bone formation can take place. However, the present results showed that the chondrocyte density and chondrocyte cluster number were increased at 2 weeks. The amount of bone was increased at 3 weeks. These observations suggest that bFGF produced most of its effects during the first few days of the bone induction cascade, i.e., on the proliferation of undifferentiated mesenchymal cells that later transform into chondrocytes. As bFGF is a known potent mitogen for mesenchymal cells, this step is likely to be influenced by bFGF in our model. The increased amount of cartilage led to more bone formation at a later stage. This would be consistent with the findings of Jingushi et al. (1990a), that repeated injections of acidic FGF into fracture calluses made them grow bigger although not stronger.

The influence of bFGF on bone induction is dose-dependent. An optimal bFGF dose of 15 ng per implant increased the content of bone calcium by 78 %. In contrast, 1900 ng had a marked inhibitory effect (Aspenberg and Lohmander 1989, Aspenberg et al. 1991). In the present results, the same dose (1900 ng) reduced both the chondrocyte density index and the ossification index.

In order to increase new bone formation, various materials have been combined with DBM to form functional composites. By far the most popular composite appears to be DBM + autogenous bone marrow (Lindholm and Urist 1980, Wittbjer et al. 1983, Aspenberg et al. 1986, Green et al. 1986, Sakata and Takagi 1987). Other natural composites have also been investigated. Kohler et al. (1990) used a combination of autoclaved autogenous bone with allogeneic demineralized bone matrix. They concluded that DBM improved the incorporation of autoclaved bone, while unaugmented autoclaved graft generally failed to be incorporated into the new bone. DBM did not increase the amount of bone in the bone ingrowth canal in the bone harvest chamber, although bone induction was demonstrated in a muscular site (Aspenberg et al. 1988). Comparing DBM powder with bone powder in the BCC, there was no difference in total tissue and bone ingrowth distances (data not published). Fully demineralized bone cannot be used to replace extensive bone defects because of its lack of mechanical strength (Ma et al. 1991). Some experimental studies have used inductive bone matrix together with porous hydroxyapatite, which increased bone formation (Kawamura et al. 1987, Ripamonti 1990). DBM is of very little practical use, but serves as a model for bone formation. The experience gained from our DBM studies encouraged us to try a model for bone grafting that resembles the clinical use of bone grafts, but is still as highly standardized as possible. BMP and some growth factors have been reported to enhance osteogenesis or osteoinduction in bone graft or bone graft substitutes. In order to investigate the incorporation of these bone implants in bone sites and the effect of bFGF on bone formation in bone conductive materials, a highly standardized model was required.

Validity of chamber model

The methodological error for bone ingrowth in the BCC model, including biologic variation (20%), was considerably larger than the previously reported error of the histomorphometric measurement (6%; Thorén 1994). Thus, there seems to be no indication for refining the bone ingrowth measurement method. Rather, if smaller effects are to be detected, the number of rats must be increased. The number of sections per specimen also seems sufficient, as the variance between animals was considerably larger than the variance between sections.

The BCC made it possible to demonstrate differences in bone ingrowth distances between different osteoconductive materials. Although the interior space in the BCC is 5 times larger than in the BHC (Albrektsson et al. 1984), it can be used in rats, which makes it possible to use more animals than with the previous rabbit chamber models and thus perform more experiments each time. Further, the fast ingrowth of new bone BHCs made it difficult to measure the long-term effects (Thorén et al. 1995). In contrast, the impaired ingrowth in the BCC also made it possible to investigate the ingrowth distance into grafts over a longer period. This model permitted measurement with histomorphometry of ingrowth distances at 6 and 10 weeks after grafting. The BCC has been used for evaluating the effect of growth hormone on bone graft incorporation (Aspenberg et al. 1994) and assessing sensory nerve ingrowth in bone grafts (Madsen et al. 1996). Aufdemorte et al. (1992) used a larger version of the bone harvest chamber to find that the application of TGF-ß1 promoted osteoblastic activity and proliferation in the tibiae of baboons, although the harvested amount of bone remained unchanged.

The BCC has several applications. It can be viewed as a "bone culture" in situ, since the BHC has been used as a tool to explore molecular processes occurring during the process of bone formation (Zhou et al. 1995). The BCC is so quick and easy to handle, that one can undertake large series. Osteoconductive materials can be tested inside the chamber, and the local conditions for bone ingrowth may be manipulated by adding growth factors and drugs. Studies are in progress to observe the effect of growth factors, such as BMPs and TGF-ß, on bone formation within bone replacement materials in the BCC.

Bone conductive properties of bone implants

A graft should be able to support a load, when necessary. This requires that the structure be maintained while the newly formed bone becomes remodeled to the same quality and dimensions as the original it is replacing. Osteoconductive materials allow ingrowth of vessels and osteoprogenitor cells from the recipient bed into the graft (Urist 1980). In this case, the graft acts as a trellis along which new bone forms. Alternatively, a material may exhibit creeping substitution, whereby graft resorbs as new bone forms in its place.

Words like "scaffold" are descriptive metaphors which imply a simple mechanical function by which a porous material can protect a preosteoblastic tissue from repeated deformation, which would disturb its differentiation into bone and produce a fibrous scar. This mechanical function includes the effects of pore size, elasticity and the ability to isolate a bone defect from soft tissue.

On the other hand, bone conduction is sometimes thought of as a surface phenomenon, which should indicate that the surfaces of an osteoconductive material would selectively favor the proliferation of bone-forming cells. This would probably require a selective anchorage of bone cell precursors. Cell adhesion is dependent on the protein film which forms on any implanted material which, in turn, may be dependent on material surface characteristics. A selective affinity for osteoblasts is theoretically possible, for example, if an implant is artificially covered with a protein having affinity for cell surface attachment molecules (integrins) on osteoblastic cells. If an implant has not been manipulated for selective osteoblast affinity, it is probably wise to expect "mesenchymal tissue conduction" rather than bone conduction. In comparing the empty bone conduction chambers with chambers containing a cancellous bone graft and porous hydroxyapatite, the most striking effect of the implants has been that on fibrous tissue ingrowth. In a chamber without a graft, the ingrown bone is covered by a thin layer of fibrous tissue. In a chamber with a graft, the bone ingrowth distance was increased by about 30%, whereas the fibrous tissue layer, in front of the bone, had increased greatly in thickness, sometimes reaching the upper portion of the implants. This indicates, again, that a so-called osteoconductive material is often really "mesenchymal tissue conductive". The extent to which the ingrown mesenchymal tissue is metaplastically ossified seems to depend more on other factors, like mechanical load or distance from loaded bone although, once formed, osteoblasts tend to lay down their matrix on bone graft trabeculae and hydroxyapatite surfaces.

Incorporation of bFGF-pretreated bone implants

Vascular penetration and bone formation in allograft incorporation are slower and less extensive than in autografts (Burchardt 1987). Successful incorporation includes the formation of callus around the graft-host junction, as well as the internal repair of the graft. Although the significance of the naturally occurring endogenous growth factors in a bone graft is not known, we have shown that an addition of exogenous growth factor-bFGF has stimulatory effects on the bone graft incorporation. Some bFGF effects on autograft incorporation have also been seen by others (Eppley et al. 1988 and 1991, Weiss et al. 1995).

Effects of bFGF dose on bone ingrowth

bFGF showed a biphasic dose-response curve for the ingrowth distance of new bone into bone grafts. The optimal dose range for bone ingrowth was between about 8 to 200 ng of bFGF per implant with the carrier. Lower or higher doses did not stimulate bone ingrowth (Table 8). These doses also had the same effects in porous hydroxyapatite and showed similarity to the dose-response curve in demineralized bone matrix. In the DBM model, the highest dose inhibited bone induction. In bone graft and porous hydroxyapatite, the highest dose had no effect on bone ingrowth, but instead caused an increased penetration of fibrous tissue (Table 8). It is possible that the highest concentration of bFGF influenced cell differentiation in favor of fibrous tissue formation or that this dose had a stronger influence on fibroblasts than on osteoblast precursors. The effect may have been the same in both the DBM and the chamber models, but no increase in fibrous tissue formation could be detected in DBM. Local application of bFGF to a fracture site stimulates callus formation and facilitates the repair of a fracture in a dose-dependent manner in normal rats and diabetic rats (Kawaguchi et al. 1994). In this model, the effect of bFGF increased with the increase in doses (0.4-50 μg in one injection). Nagai et al (1995), using a low dose (0.1 mg/kg per day) of bFGF administered intravenously for 7 days to growing rats, increased the longitudinal growth rate, the production rate of cartilage cells, and the area of metaphyseal bone. In the tibial shaft, the endocortical bone formation rates, total bone area, total osteoid, and medullary bone areas were increased, but the rate of formation of periosteal bone was depressed. Two weeks after the cessation of treatment, the total mineralized area in the tibial shaft was found to be increased. However, with a higher dose (0.3 mg/kg per day) of bFGF, the width of the growth plate was increased and the rates of longitudinal growth and endosteal bone formation were decreased in rat tibia. Several experiments showed that a higher dose resulted in an inhibition of bone formation (Aspenberg et al. 1991, Nagai et al. 1995). This effect was probably due to the inhibitory effect of excess bFGF on type I collagen gene expression in osteoblasts (Hurley et al. 1993). Noff et al. (1989) showed that a high dose of bFGF (3 ng/mL) maximally increased the activity of alkaline phosphates and of mineralization in a bone marrow culture system, whereas one-tenth of that dose (0.3 ng/mL) caused maximal proliferation. These findings suggest that bFGF may induce both proliferation and differentiation of bone cells by different concentrations.

Effects of time of administration of bFGF on bone ingrowth

The effect of bFGF first presented as an increased ^{99m}Tc-MDP uptake at 2 and 4 weeks, while the bone ingrowth difference on histomophometry developed later. These observations indicated that the late increase in bone ingrowth is a consequence of earlier events, stimulated by bFGF. The increased bone ingrowth into bone implants became evident as a histomophometric difference 6 weeks after the administration of bFGF. This also appears to be true of the delayed use of bFGF. Two weeks after implantation, when delayed use of bFGF was started, the chamber contained a considerable amount of soft tissue, mostly seen as an undifferentiated blastema. Bone formation had hardly begun. The stimulatory effect of bFGF initiated at this time may indicate that bFGF has an effect on preosteoblast proliferation and possibly also on differentiation. Furthermore, the effect of initial bFGF persisted even at 10 weeks. In allograft incorporation, a second phase of osteogenesis may be initiated by the host tissue, approximately 4 weeks after grafting (Burchardt 1987). In the BHC model, bFGF treatment of allografts caused only a difference of the preosteoblastic tissue, but not osteoid and bone, at 2 weeks (Thorén and Aspenberg 1993). Therefore, when histomorphometry is performed, it is important to ensure that sufficient time is available for observing the stimulation effect of bFGF. Schnettler et al. (1992) reported that bone ingrowth in HA with bFGF was quicker than in controls and comparable to an autograft at 6 weeks, but there was no difference from controls after 3 months. This result could suggest that a bFGF supplement to implants accelerates the incorporation of bone.

Possible mechanism of bFGF effects on bone ingrowth

We found large amounts of mesenchymal cells and

Table 8. Ingrowth distance and thickness of fibrous zone in front of ingrown bone, expressed as percentage of controls

		bFGF dose (ng/implant)			
Implant	Ingrown tissue	8	40	200	1000 (ng)
Graft	Bone	188***	180***	154**	92
Graft	Fibrous	88	92	128*	128*
HA	Bone		170*		92
HA	Fibrous		147**		195**

* p<0.05; ** p<0.005; *** p<0.001.

osteoid-like tissue in the bFGF-treated implants, indicating increased proliferation of osteogenic cells. Several in vitro studies have shown that bFGF has a mitogenic effect on undifferentiated mesenchymal cells and on chondrocytes and osteoblasts (Linkhart et al. 1986, Froger-Gaillard et al. 1989). In the BCC, we found an increased width of the immature bone in bFGF-treated specimens. This resembles the finding in a study which showed that aFGF increased the size of cartilage callus, but inhibited cartilage gene expression (Type II procollagen and proteoglycan core protein mRNA) in aFGF-injected calluses (Jingushi et al. 1990a). This indicates a proliferation of osteogenic or cartilaginous cells, while their production of matrix is inhibited. The inhibitory effects of bFGF on synthesis of matrix products are probably due to the ability of FGF to keep cell populations in active proliferation, thereby delaying their mature synthetic function (Lathrop et al. 1985). Another observation is that bFGF increased the DNA content in a dose-response manner, but reduced the synthesis of type I collagen, a major bone matrix protein and alkaline phosphate activity which affected bone remodeling (Hurley et al. 1993, Iwasaki et al. 1995).

It is also possible that bFGF stimulates angiogenesis in the implants. We regularly observed that the bFGF-treated bone implants were more red than the controls at harvest, probably indicating that bone implants were more vascular. Administration of bFGF has proved to be angiogenic in other in vivo models (Folkman 1985, Shing et al. 1985, Folkman and Klagsbrun 1987). In autografts, bFGF significantly favored the formation of new vessels and the extent of their penetration (Eppley et al. 1988 and 1991, Weiss et al. 1995). Despite accelerated vascular ingrowth in the bFGF-treated grafts, some studies revealed no concurrent increase in osteogenesis. This probably indicated an insufficient period of bFGF stimulation or observation and an inadequate dosage of bFGF.

In our model, bone marrow stromal cells can enter the bone implants with the blood flow. Isolated bone marrow cells from adult animals have been shown to have osteogenic properties when cultured in vivo in diffusion chambers (Ashton et al. 1980). In a study on bone marrow cultures, bFGF played a major role in the proliferation and differentiation of bone marrow-derived cells, leading to the production of a bone-like tissue (Noff et al. 1989, Pitaru et al. 1993). It is possible that bFGF may stimulate the differentiation of proliferated osteoblasts through the response of bone marrow stromal cells in the in vivo environment.

In the present study using optimal doses, new bone sprouts protruded into the grafts. These sprouts consisted of a dense mesenchymal-like tissue with large nuclei, showing a continuous transition to osteoid and bone. This suggests an active membraneous ossification. Intravenous administration of bFGF to rats stimulated osteoblast proliferation and new endosteal bone formation in various bones in the skeleton (Mayahara et al. 1993, Nagai et al. 1995, Nakamura et al. 1995). Previous studies have shown that bFGF is a more potent mitogen for fibroblasts and preosteoblasts than for differentiated osteoblasts (McCarthy et al. 1989). Further studies demonstrated that bFGF enhanced the osteocalcin gene expression of extracellular matrix (Noda et al. 1991, Schedlich et al. 1994). In addition, bFGF was shown to inhibit bone-cell differentiation or matrix synthesis in vitro (Canalis et al. 1988, Rodan et al. 1989, Hurley et al. 1993). Nevertheless, when administered in vivo, bFGF markedly stimulated not only the proliferation but also the differentiation of osteogenic cells and accelerated bone formation. Thus, bFGF may stimulate the secretion or the actions of other growth factor(s) to cause such effects. bFGF has been reported to increase the expression of TGF-ß in osteoblastic cells (Noda and Vogel 1989). TGF-ß stimulates synthesis of collagen and other matrix proteins by osteoblastic cells in vitro and enhances bone formation in vivo (Robey et al. 1987, Noda and Rodan 1987, Pfeilschifter et al. 1987, Centrella et al. 1992). A recent study showed that bFGF stimulated endosteal bone formation and the expression of TGF-ß in the endosteal cells. The effect of bFGF may at least in part be mediated by TGF-ß (Nakamura et al. 1995). The present study demonstrates that the local application of bFGF in rats stimulates bone ingrowth in bone grafts and hydroxyapatite. The stimulatory effect of bFGF might be divided into two phases: an initial phase with an increase in mesenchymal cell numbers and possibly a second phase with stimulation of new bone formation, due perhaps to the release of additional factors, such as TGF-ß from these cells. However, the mechanisms and the sequence of cellular events that lead to the stimulation of bone formation by bFGF remain to be clarified.

Carrier effects

The experiments with bone grafts showed an effect of bFGF pretreatment when using a hyaluronate carrier, whereas bone grafts treated with bFGF in saline solution showed no effect. The study of bFGF effects on porous hydroxyapatite was therefore initiated, using the same carrier and bFGF concentrations. The effects of 40 ng per implant were similar to those found for the bone graft. However, bFGF without a carrier gel required about 40 times higher doses in order to increase bone ingrowth. This dose (1.5 µg bFGF per implant in saline) is the same as that previously reported by Schnettler et al. (1992). In the bone induction experiments, neither bFGF alone nor the carboxymethyl cellulose gel had any effect on these implants, but bFGF combined with the gel increased the bone calcium content (Aspenberg et al. 1991) and chondrocyte number. In the same bone induction model, a hyaluronate gel has shown about the same effects as the cmc gel (data not shown). The carriers seem to play a slow-release role for bFGF. The absence of the carrier probably cannot be fully compensated by an increased dose of bFGF, because a prolonged concentration of bFGF within an effective dose range may enable it to work on cells that were not present during the initial phase. Thus, both qualitative and quantitative differences can be expected between the effective doses, with or without a carrier. Differences in the temporal pattern of the bFGF effects may explain why an increase in the doses of bFGF increased the total tissue, but not bone, when a carrier was used, whereas the same response was found when the dose without a carrier was reduced. Regardless of the explanation, the promotion of bone ingrowth requires better control of the local concentrations than the stimulation of tissue ingrowth in general.

Several studies using slow-release carriers or osmotic pumps have shown that bFGF increased the bone callus in bone fracture and angiogenesis or bone formation in bone graft (Table 2). This indicates that bFGF may require a consistent optimal concentration to play a stimulating role at a bone site. In clinical work, sodium hyaluronate, which has been used in our studies, is of value in the treatment of osteoarthritis (Strachan et al. 1990), wound healing (Casadel et al. 1989, King et al. 1991) and for local delivery of drugs (Camber and Edman 1989, Ludwig and Van Ooteghem 1989). As a carrier, it retards the release of peptide growth factors (Prisell et al. 1992). Other growth factors than bFGF also need certain carriers to protect and slowly release them, in which the growth factors exhibit significant effects in stimulating bone formation, such as BMP or rhOP-1 in collagen or inactive DBM increases bone healing (Cook et al. 1994 and 1995b, Kirker-Head 1995). TGF-ß in methylcellulose had a significant effect in terms of osteoblast proliferative activity (Aufdemorte et al. 1992). This suggests that carriers probably play a role in the storage and protection of growth factors and can prolong their biological activity. In clinical practice, this may become important, especially as hyaluronate is well tolerated. Hopefully, the present data can provide information about how much bFGF should be used in other models, which have more relevant clinical applications.

Possible clinical applications

BMPs, TGF-ßs and IGFs have been studied extensively in models of clinical relevance in orthopedic and dental surgery (Table 9). These studies indicate that large segmental osteoperiosteal defects can be healed in rabbits, dogs and monkeys and these effects can reach the autograft level. Moreover, it is claimed that BMP present in AAA-bone stimulates the formation of bone in bone defects of patients (Johnson et al. 1990). In vivo studies have shown that BMPs with collagen or a porous biodegradable polymer are an effective bone graft substitute for obtaining a stable posterior spinal fusion (Cook et al. 1994). The use of such materials may provide an efficacious alternative to autografts (Muschler et al. 1994). Recent studies have indicated that TGF-ß increased the mechanical anchorage in ceramic-coated implants inserted into trabecular bone of dogs (Lind et al. 1995, Sumner et al. 1995). A combination of IGF-1 and PDGF increased bone ingrowth and bone formation in dental implants in dogs (Lynch et al. 1991). Application of nerve growth factor (NGF) maintained bone graft volume by inhibiting graft resorption in dogs (Eppley et al. 1992). A synergistic effect between bFGF and TGF-ß initiated and propagated the cellular events involved in osteogenesis and angiogenesis in bone grafts and endosteal bone formation (Nakamura et al. 1995, Weiss et al. 1995). As mentioned above, growth factors may play an important role in the autoinductive capacity for bone formation and the stimulation of bone ingrowth in bone implants. Autografts and allografts have been used in orthopedic surgery, such as hip replacement (Gie et al. 1993, Slooff et al. 1993, Schreurs et al. 1994) and spinal surgery (Meril 1994, Cook et al. 1995a, An et al. 1995). With research on growth factors, bone replacement materials may improve.

Table 9. Application of growth factors other than bFGF in bone defects and bone grafts

Author	Year	Animal	GH	Carrier	Model	Time	Effect
Heckman	1991	Dog	BMP	Polylactic acid	Radial defect	3 months	Increased bone formation
Lynch	1991	Dog	IGF-1 PDGF	Dental implant	Root canal	1, 3 weeks	Increased bone ingrowth and bone formation in implant
Eppley	1992	Rabbit	NGF	Solution+pump	Iliac to mandibular autograft	2 months	Maintained bone graft volume
Johnson	1992	Human	hBMP	AAA	Nonunion and segmental defect	Average 30 months	Promotion of union
Ono	1992	Rabbit	BMP	HA pellets	Skull defect	3, 6, 9 wks	Increased bone mineral density
Gerhart	1993	Sheep	rhBMP2	Inactive bone matrix	Femur defect	3 months	Increased bone healing comparable with autograft
Ripamonti	1993	Baboon	rhBMP3	HA discs	Intramuscular	1 month	Induced bone differentiation
Cook	1994	Dog	rhBMP7	Collagen	Posterior spinal fusion	6, 12, 26 weeks	Stable fusion, more rapid than autograft
Muschler	1994	Dog	rhBMP2	Porous biodegradable polymer	Posterior spinal fusion	3 months	Comparable to autograft in union score and mechanical testing
Stevenson	1994	Rat	BMP3	HA+TCP	Femur defect	1, 2, 4 mo	Increased bone area and amount
Cook	1995b	Monkey	rhBMP7	Collagen	Ulnar, tibial defect	3 months	Comparable to autograft; new cortical formation
Beck	1995	Rabbit	TGF-ß	TCP+amylopectin	Radial defect	2 months	Increased bone formation
Kirker-Head	1995	Sheep	rhBMP2	Inactive DBM	Femur defect	3 months	Bone mineral content equal to intact bone, not greater than autograft
Lind	1995	Dog	TGF-ß	Ceramic+TCP	Ceramic coated implant	6 weeks	Increased bone ongrowth and gap healing of TCP coated implant
Summer	1995	Dog	TGF-ß	HA-TCP	HA-TCP coated implant	1 month	Increased bone ingrowth

BMP-2 and 7 will probably be the first growth factors used in clinical orthopedic surgery (Johnson et al. 1990). Several preclinical experiments demonstrated good results regarding bone formation in bone defects. Application of bFGF may stimulate the proliferation of differentiated cells and enhance or accelerate the healing of bone defects. bFGF has already proved effective in increasing wound healing and angiogenesis in the healing of fractures and the incorporation of bone grafts. In total hip revision, the bone defect in the bone marrow cavity can be filled by a compacted bone graft. In this situation, bFGF would probably accelerate bone graft incorporation. It may also facilitate fracture and non-union healing when the circulation is impaired. TGF-ß is used to coat prostheses in order to increase bone ingrowth and mechanical integration. The addition of bFGF may play a synergistic role. Before these substances are used in clinical work, more experiments must be performed in order to understand the regulation of bone formation and bone remodeling, and develop new ways of treating difficult clinical conditions. Our findings hopefully provide data for planning future studies.

CONCLUSIONS

From these experiments, it may be concluded that exogenous bFGF in a slow-release carrier stimulates early proliferative events during bone induction and increases bone ingrowth into bone grafts and porous hydroxyapatite depending on dose. Effects on bone ingrowth could be observed first after several weeks. Without a carrier, increased bone ingrowth could still be achieved, using higher bFGF doses. These studies were enabled by the use of the BCC which could also be used to demonstrate a difference in conductive effect between bone grafts and porous hydroxyapatite. Thus, all the hypotheses on page 9 were verified except that the time-point for administration did not clearly influence the results.

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