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THESIS

Growth factor stimulation of bone healing

Effects on osteoblasts, osteomies, and implants fixation

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ACTA ORTHOPAEDICA SCANDINAVICA SUPPLEMENTUM NO. 283, VOL. 69, 1998

SCANDINAVIAN UNIVERSITY PRESS

Oslo - Copenhagen - Stockholm - Boston

Printed in Sweden
Wallin & Dalholm
1998

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Abstract

Bone tissue has been shown to contain numerous cell-to-cell signalling peptides called *growth factors*. These growth factors are thought to have important regulating effects for bone remodeling and bone healing, due to their potent effects on bone cell metabolism. In vivo studies over the last half decade have demonstrated that growth factors can stimulate bone formation and bone healing. These results have made bone growth factors candidates for future clinical use in orthopedic surgery. In numerous clinical situations enhanced bone formation and bone healing could lead to improved results of surgical procedures. This thesis describes the most important bone growth factors and their actions in vitro and in vivo. In vitro investigations of growth factor effects on osteoblast chemotaxis and metabolism are described as well as in vivo studies with growth factor stimulation of fracture healing and bone healing to prosthetic-like implants.

In vitro results: Several growth factors exhibited chemotactic effects towards human osteoblasts. TGF-ß1 and PDGF-BB had the strongest chemotactic effects, whereas PDGF-AA, IGF-1, and IGF-2 had less but significant chemotactic effects towards human osteoblasts. TGF-ß1 exhibited the highest chemotactic potency with maximal activity at 100 pg/mL, whereas the other growth factors had maximal effects at 10–100 ng/mL. BMP-2 was found to have chemotactic effects toward human osteoblasts, human bone marrow osteoprogenitor cells, and U2-OS osteosarcoma cells. BMP-4 and BMP-6 were without any chemotactic effects towards these celltypes. Human bone marrow osteoprogenitor cells were the most responsive celltype to BMP-2 stimulation. Growth factor combinations resulted in synergic stimulative effects on different metabolic functions on human osteoblasts. Combinations with TGF-ß1 and PDGF-BB strongly stimulated proliferation and chemotaxis. Combinations with TGF-ß1, PDGF-BB and BMP-2 strongly stimulated an osteoblast differentiation parameter (alkaline phosphatase activity). The different growth factor combinations had no effect on collagen synthesis in human osteoblasts.

In vivo results: Continuous application of 1 and 10 µg natural TGF-ß to a plated tibial osteotomy in rabbits increased mechanical bending strength and callus formation at 6 weeks observation. Diaphyseal cortical bone remodeling was not affected by the local growth factor application.

In a dog model with unloaded implants surrounded by a gap, 0.3 µg rhTGF-ß1 adsorbed to gritblasted tricalcium phosphate coated implants, was able to enhance mechanical fixation, bone ingrowth and gap bone formation. 3.0 µg rhTGF-ß1 had less but significant stimulative effect. In a weight-loaded model, 0.3 µg rhTGF-ß1, adsorbed to gritblasted tricalcium phosphate coated implants, was able to enhance bone ingrowth, without enhancement of mechanical fixation. In the unloaded model, 0.3 µg rhTGF-ß1, adsorbed to gritblasted hydroxyapatite coated implants, was able to enhance bone ingrowth, without enhancement of mechanical fixation. 3.0 µg rhTGF-ß1 had no stimulative effects.

The establishment of a biological implant fixation concept with growth factor absorbed to ceramic coatings of implants was successful. These data are promising for a possible future clinical usage of growth factors, especially for enhancement of bone healing to cementless prosthetic components.

Acknowledgements

The studies in this thesis were supported by grants from the Danish Rheumatism Association, the Danish Health Science Research Foundation, the Velux Foundation, the Foundation of 1870, the Foundation of 17.12.1981, the Novo Nordisk Foundation, the Institute of Experimental Clinical Research, Aarhus University, Kong Kristian Den Tiendes Foundation and the Danish Foundation for the Advancement of Medical Science.

Definitions and abbreviations

Definitions

Bioactivity The level of activity to biological processes by a peptide regulator molecule. Is modified by physical environment and other biochemical regulators.

Bioavailability The amount of peptide regulator molecules accessible to responsive cells.

Bone healing The cellular and biochemical events that initiate and maintain healing of bone lesions.

Bone induction The biochemical stimulation of new bone formation at an ectopical location.

Bone matrix The substance between cells in bone tissue. Consists of numerous different proteins and glycoproteins in which calcium phosphate mineral is deposited.

Bone remodeling Regulation of bone volume through bone resorption and bone formation at anatomically distinct foci.

Chemotaxis The directed cell migration controlled by a biochemical concentration gradient.

Cytokine Peptide regulator molecule that regulate cell to cell interactions mainly for the immunological system.

Growth factor Peptide regulator molecule that regulate local cell to cell metabolism and with generally stimulative effects.

Osteoblast The metabolic active bone forming cell. Derived from the mesenchymal cell liniage.

Osteoclast The cell capable of bone resorbtion. Derived from the hematopoetic monocytical cell liniage.

Osteogenesis The cellular and biochemical processes that takes place during bone formation and bone healing.

Osteoconduction Enhancement of bone formation due to a favourable physi cal environment and/or structure of the area where new bone formation will take place.

Abbreviations

AP	Alkaline phosphatase
BMP	Bone Morphogenetic Protein
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked ImmunoSorbent Assay
FGF	Fibroblast growth factor
HA	Hydroxyapatite
IGF	Insulin Like Growth Factor
PDGF	Platelet Derived Growth Factor
RIA	Radio Immuno Assay
TCP	Tricalcium phosphate
TGF-ß	Transforming Growth Factor Beta

List of papers

This thesis is based on the following papers

I **Lind M**, Schumacher B, Søballe K, Keller J, Melsen F, Bünger C. Transforming growth factor-ß enhances fracture healing in rabbit tibia. Acta Orthop Scand 1993; 64(5): 553–556.

II **Lind M**, Deleuran B, Thestrup-Pedersen K, Søballe K, Eriksen EF, Bünger C. Chemotaxis of human osteoblasts. Effects of osteotropic growth factors. APMIS 1995; 103: 140–146.

III **Lind M**, Eriksen EF, Bünger C. Bone morphogenetic protein-2 but not bone morphogenetic protein-4 and 6 stimulate chemotactic migration of human osteoblasts, marrow osteoblasts and U2-OS cells. Bone 1996; 18(1): 53–57.

IV **Lind M**, Eriksen EF, Bünger C. Effect of growth factor combinations on human osteoblast metabolism and chemotaxis. Eur J Exp Musculoskel Res 1995; 4(3-4): 164–170.

V **Lind M**, Søren Overgaard, Søballe K, Nguyen T, Ongpipattanakul B, Bünger C. Transforming growth factor beta enhances fixation and bone ongrowth of ceramic coated implants. J Orthop Res 1996; 14(3): 343–350.

VI **Lind M**, Søren Overgaard, Nguyen T, Ongpipattanakul B, Bünger C, Søballe K. Transforming growth factor beta stimulates bone ongrowth to weightloaded tricalcium phosphate coated implants. J Bone Joint Surg [Br] 1996; 78-B: 377–382.

VII **Lind M**, Søren Overgaard,, Nguyen T, Ongpipattannakul B, Bünger C, Søballe K. Transforming growth factor beta stimulates bone ongrowth to hydroxyapatite coated implants in dogs. Acta Orthop Scand 1996; 67: 611–616.

Introduction

Principles for enhancement of bone healing and bone formation

Three basic principles exist for enhancement of bone healing and bone formation. When investigating the possibilities for experimental enhancement of bone healing, these principles must be considered: *bone induction* is new bone formation from determined osteogenic precursor cells; *bone conduction* is enhanced bone formation due to a favorable structural environment where bone is formed; and stimulation of *bone genesis* effectuated by modulations of the natural biochemical processes that initiate and maintain bone formation during a healing response (Table 1).

Table 1. Principles for enhancement of bone healing and bone formation

Osteoinduction	Osteogenesis	Osteoconduction
Bone precursor cells	Growth factors	Porous coatings
TGF-ß superfamily	Ca/P ceramics	Ca/P ceramics
Bone autograft	Bone autograft	Bone allograft

Bone induction can be obtained by two approaches. 1) *Cell mediated*: Bone precursor cells can be harvested from bone marrow and placed in, for example, bone defects that need bone induction to heal sufficiently. Bone precursor cells can also be cultured from crude bone marrow, and by this method large numbers of bone precursor cells can be obtained for bone induction. During osteoinduction, osteogenic precursor cells placed in both a osseous and a non-osseous biological environment can proliferate and differentiate to form mature osteoblasts, which form bone matrix that subsequently mineralize to mature bone tissue. *2) Growth factor mediated:* A special family of growth factors called bone morphogenetic proteins (BMP's) have a unique property of stimulating mesenchymal stemcells to differentiate towards a chondro- and osteoblastic lineage. Local application of BMP's, produces extensive bone induction and this principle can be used for creating bone formation in bone defects and as substitute for bone grafts. After implantation of autologous bone graft, bone formation is probably accomplished by both the cell and growth factor mediated bone induction, which occurs with viable precursor cells from the bone marrow stroma and BMP growth factors released from the bone matrix.

By ***bone conduction,*** normal bone formation is helped to extent into an area due to a favorable structural environment where the bone conductive material serves as a scaffold for new bone formation. An example is porous coatings with the optimal pore size of 100–400 µm, which are able to favor bone ingrowth into endoprosthetic components. The use of allogenic bone grafts and other processed bone graft materials most likely serves mainly as bone conductive material. Ceramics, like calcium phosphate ceramics and glass ceramics, have osteoconductive properties, but are also hypothesized to stimulate ***osteogenesis*** by releasing non-organic mineral-ions, which activates cellular processes during bone formation and healing.[1] Bone matrix is a storage for growth factors that participate in activation and maintenance of cellular processes during bone formation and healing. Some of these growth factors have been shown to be able to accelerate bone formation and bone healing when applied locally to both intact and healing bone tissue. This principle is pure stimulation of osteogenesis.

The main topic of this thesis is to describe in review and from experimental studies the possibilities for biochemical stimulation of osteogenesis.

The clinical problem of impaired bone healing

Inadequate bone stock and impaired bone healing are potential problems in various clinical situations in orthopedic surgery and new approaches for enhancement of bone healing could improve clinical outcome in these situations. Type III complicated fractures often heal insufficiently resulting in pseudarthrosis formation due to extensive soft tissue damage and subsequent bone loss. Surgical treatment of bone tumors often leaves large defects which heal inadequately and require extensive bone grafting for acceptable clinical results. In spine surgery, large amounts of trabecular bone grafts are used for several procedures where spondylodesis is required. In endoprosthetic surgery, late loosening of the prosthetic components is a problem, both in primary and especially in revision joint replacements.[2,3] Loosening is partially ascribed to inadequate initial bone ingrowth, which may

be improved by enhanced bone healing. Stimulation of local bone healing and bone formation could improve clinical results in the above mentioned situations. At present, all of the mentioned clinical problems with impaired bone healing are primarily solved by extensive use of bone graft material. Free vascular bone grafts are used in situations where mechanical stability is essential. However, such procedures require highly specialized surgical capacity and prolong operation times. Autologous bone graft from *crista iliaca* is the main source of trabecular bone grafting material and has a well documented osteoinductive capacity. The bone harvest procedure, however, prolong surgery and may subject the patient to postoperative discomfort and morbidity.[4] Allogenic bank bone is also extensively used, but the bone graft often disintegrates over time and provides only osteoconductive properties. However, in revision endoprosthetic surgery, new methods of impacted milled allograft have been used with success.[5]

A number of newly developed artificial bone materials are presently being evaluated as graft material over the last decade. These include bioactive glass ceramics, porous synthetic and coral hydroxyapatite ceramics, biodegradable calcium carbonate and combinations of collagen and ceramics. These products may provide osteoconductive properties and at least glass ceramics have some favorable biomechanical properties.[6] However, the final role of these new products in clinical use have not been reached.

The principle of *distraction osteogenesis*, as seen in the *Illizarov technique*, is an independent method for stimulation of bone formation with an enormous bone forming capacity.[7] This method has revolutionized the treatment for diaphyseal bone lengthening and the possibility of *bone transport* to fill large defects. Biochemical stimulation of bone healing is a new approach to the above mentioned clinical problems and this concept has become increasingly relevant with the discovery, more than a decade ago, of peptide regulator molecules called *growth factors*. Growth factors have been found in all tissues and are today known to regulate local cell-to-cell metabolism and to mediate cellular effects of various hormones. Bone matrix is now known to be a large reservoir for numerous growth factors that have been suggested as regulators of bone remodeling and initiators of the bone healing process.[8] In vitro studies have documented that bone growth factors exert numerous regulating effects on bone cells,[9] while in vivo studies have shown that a small number of growth factors can stimulate the bone healing processes in animals.[10-12] These data are promising for future possibilities for growth factor stimulated bone formation and bone healing in orthopedic surgery.

Aim of present studies

Basic mechanisms of osteoblast reactions to growth factors were investigated in *in vitro* studies.

The experiments performed aimed to investigate:

1) in vitro chemotactic effects of bone growth factors on human bone cells (II, III),
2) in vitro effects of growth factor combinations on human osteoblast meta bolism and chemotaxis (IV).

In vivo, our objective was to investigate the ability of transforming growth factor beta (TGF-ß) to stimulate bone healing in clinically related animal models:

1) in vivo effects of TGF-ß in an experimental tibial osteotomy in rabbits with continuous application of the growth factor (I),
2) to establish a new biological implant fixation principle with TGF-ß incorporated onto the ceramic coating of implants (V),
3) to characterize stimulative effects of the new implant fixation principle in canine models with different ceramic coatings and implant loading conditions (VI, VII).

Growth factors and bone physiology

Bone biochemistry

Bone consists of cells and extracellular matrix; the latter is comprised of 35% organic and 65% inorganic components.[13] The inorganic components are mainly calcium and phosphate as hydroxyapatite. The organic components of bone matrix are traditionally divided into collagen and non-collagenous proteins. Type I collagen constitutes more than 90% of the organic material in bone matrix and is the major structural protein of bone. The remaining 10%, the non-collagenous proteins, have different regulatory functions for mineralization, mediation of cell-to-matrix binding, and various interactions with structural proteins, such as collagen. (Table 2). Osteocalcin (or bone gla-protein, *BGP*) and osteonectin, the two most abundant non-collagenous proteins, have specific calcium binding properties; they have therefore been proposed as initiators of hydroxyapatite crystal growth during mineralization.[14,15] Osteopontin and bone sialoprotein contain special amino-acid sequences that connect to adhesion-receptors on bone cells.[16] Osteopontin may anchor osteoclast during bone resorption via vitronectin receptors.[17] Trombospondin also bind calcium and osteonectin.[18] Bone growth factors consist of less than 1% of the non-collagenous proteins.

Bone growth factors

Besides from structural proteins, bone matrix also contains small amounts of very potent regulators of bone cell metabolism. These proteins, called *bone derived growth factors*, are produced by osteoblasts and incorporated into the extracellular matrix during bone formation, but small amounts can also be trapped systemically from serum and incorporated into the matrix. The growth factors are located within the matrix until remodeling or trauma causes solubilization and release of the proteins.[19,20] After release, the growth factors are able to regulate osteoblast- and osteoclast metabolism during bone remodeling and may initiate and control a healing response after bone trauma. Thus, they are recognized to be the main regulators of bone-cell metabolism.

The bone growth factors exhibit their effects only in the local cellular environment, thereby stimulating neighboring bone cells to proliferate and increase matrix protein synthesis (*paracrine effects*). Likewise, the osteoblasts, which produce the growth factors, can stimulate themselves to additional metabolic activity (*autocrine effect*). The total number of growth factors which are able to affect proliferation, differentiation and secretive functions of bone-related cells is unknown, but the number is continually expanding as a result of the novel advanced research techniques in protein biochemistry and molecular biology.

Table 2. Organic components in bone tissue

The proteins in bone	Size (kD)	Fraction (%)	Function
Type I Collagen	320	90	Major structural protein, gives tensile strength
Type V, VIII, XII		<1	Associate with type I fibrils to control collagen fibril structure
Osteocalcin (Bone gla-protein)	6.5	1.5	Binds hydroxyapatite
Osteonectin	33	2.5	Binds calcium
Phosphoproteins			Bind calcium and cells
-Osteopontin	32	0.2	
-Bone sialoprotein	34	1.0	
Trombospondin	150	0.2	Binds calcium, osteonectin and cells
PS-S1, Biglycan	75	1.0	Modulates matrix organization
PS-S2, Decorin	120	0.2	Modulates fibril formation. Binds TGF-ß and modulates activity
Bone growth factors		<0.1	Regulate balance between bone formation and bone resorption. Initiate healing response after bone trauma

Modified after Sandberg[121]

Table 3. In vitro effects of growth factors on osteoblasts

	Proliferation	Alk. phos.	Coll. synth.	Non-coll. synth.	Chemotaxis
TGF-ß1	+++	—/+	++	+/–	+++
BMP-2, 3	0	++?	0	ND	+
BMP-7	+	+++	+	0	ND
PDGF-BB	++	0/–	0	0	+++
PDGF-AA	+	0	0	0	+
IGF-1	++	0	+	0	+
IGF-2	+	0	0	0	+
FGF-basic	+	0	0	0	+
FGF-acidic	0	0	0	0	0
EGF	+	0	0	0	0

Normal skeletal growth and bone remodeling results from a balance between the processes of bone-matrix formation and resorption. These activities are regulated both by systemic and local factors. Several systemic hormones are known to have important effects on bone metabolism and have been studied extensively.[21] The pathways triggered by these hormones in the target cells are however much less well-known. A number of polypeptide growth factors have significant effects on bone and cartilage metabolism; this suggests an important role for these growth factors in mediating hormonal responses locally. But they also suggest a local metabolic regulation of bone metabolism, without the influence of systemic hormones. Growth factors augment cell replication and contribute to stimulation of differentiation and metabolic functions of bone cells (Table 3). They exhibit their effects through binding to membrane-bound receptors. This leads to a cascade of intracellular events which affect the expression of genes that encode for such metabolic functions as cell division and protein synthesis.

The following growth factors are the most important in bone tissue: *Transforming growth factor- beta (TGF-ß)* exist in 5 different subtypes. Bone and platelets contain high amounts of this growth factor. TGF-ß is probably the most potent multifunctional regulator of bone cell metabolism. *Bone morphogenetic proteins (BMP's)* exist in 12 different subtypes and are the only growth factor known to stimulate the mesenchymal stem-cells to differentiate into osteoblastic and chondroblastic lineage. *Platelet-derived growth factors (PDGF)* exist in three isotypes and are potent stimulators of both proliferation and matrix protein synthesis. The *insulin-like growth factors (IGF I and II)* are produced by osteoblasts. IGF-II is the growth factor found at highest concentration in bone matrix. The synthesis of IGF-I is mediated through growth hormone. IGF's primarily stimulate the proliferation of undifferentiated osteoblasts. *Fibroblast growth factor (FGF's)* are present in bone matrix and are secreted by isolated osteoblasts. FGF-basic is found in bone matrix at a tenfold higher concentration than FGF-acid. FGF's are primarily a mitogen in normal bone cells.

Growth factors

Transforming growth factor-beta (TGF-ß)

TGF-ß's are multifunctional cytokines with a broad range of biological activities. These include regulation of growth and differentiation of many cell types, and in general, TGF-ß has stimulative effects on cells of mesenchymal origin and inhibitory effects of cells of ectodermal origin. TGF-ß belongs to a family of related proteins, which demonstrate various degrees of amino acid sequence homology; this protein family is called the *TGF-ß superfamily*.[22] Other members of this family include the bone morphogenetic proteins (BMP's) and the embryonical growth factors inhibin, activin and Müllerian substance.[8] The secreted precursor protein is biologically inactive and is called latent TGF-ß.[23] Activation and cleavage to the mature peptide occur both in acidic environment and in vivo also through an unknown enzymatical reaction. A total of 5 subtypes of TGF-ß have been found until now, and are named TGF-ß 1 to 5.[24-26] Since almost all cell types have TGF-ß receptors, the modulation of TGF-ß effects is probably regulated through activation of latent TGF-ß.

Bone and platelets contain almost 100 times more TGF-ß than any other tissue, and osteoblasts bear the highest amount of TGF-ß receptors.[27,28] These findings suggest that TGF-ß is of major importance for bone metabolism. TGF-ß has profound in vitro effects on osteoblasts (Table 3). In mouse calvarial osteoblasts and murine osteoblastic cell-lines, TGF-ß

inhibits proliferation and alkaline phosphatase activity.[29,30] In rat calvarial and human osteoblasts and corresponding cell-lines, TGF-ß increases cell proliferation.[31,32] The effects of TGF-ß on bone-cell differentiation is controversial. Both collagen production and collagen gene expression can be stimulated by TGF-ß.[33,34] But alkaline phosphatase activity and expression is generally decreased by TGF-ß stimulation,[30,34] and in vitro mineralization is also inhibited by TGF-ß.[35]

The first in vivo studies demonstrating stimulatory effects of TGF-ß on bone formation used injections of TGF-ß into rat and mice calvaria and revealed a marked increase in bone formation.[10,36] In a study using a calvarial defect model in rabbits, TGF-ß in a methyl cellulose gel carrier was able to stimulate bone-healing of otherwise non-healed defects.[37] However, a recent study in baboons found only minor bone stimulatory effect of TGF-ß in calvarial defects.[38] Systemic administration in rats and rabbits causes endosteal bone formation and generalized osteoblast hypertrophy with high matrix protein synthesis activity.[39] In monkeys, TGF-ß caused no effects on bone ingrowth into a titanium bone ingrowth chamber, but the newly formed bone demonstrated a markedly increased osteoblastic activity.[40] TGF-ß applied continuously to a healing osteotomy in rabbits stimulated increased callus formation and increased maximal bending strength of the osteotomy (I). Our own studies have demonstrated stimulatory effects of rhTGF-ß1 on bone healing to both unloaded and weight loaded TCP and HA ceramic coated implants in dogs (V–VII). The in vivo data on TGF-ß's ability to stimulate bone formation are very promising and TGF-ß, along with the bone morphogenetic proteins, are probably the most realistic candidates for growth factors to be used as stimulators of bone healing and bone induction in clinically related situations within orthopedic surgery.

Bone morphogenetic proteins (BMP's)

In 1965, Marshall Urist made the discovery that demineralized bone matrix (DBM) could induce bone formation when placed ectopically in subcutaneous tissue.[41] He observed that DBM caused formation of a complete ossicle with mineralized woven bone and bone marrow. The ability of demineralized bone matrix to induce bone formation was ascribed to a protein which Urist named "Bone Morphogenetic Protein (BMP)".[42,43] In 1988–1989 Elizabeth Wang and John Wosney from the Genetics Institute purified three different BMP's and characterized their amino acid sequences.[44,45] Later, nine additional BMP genes were identified by analogous techniques, so that today BMP's 1 to 12 have been identified.[46-48]

BMP's exert their effects through recently identified receptors. These receptors are heteromeric complexes of type I and II serine/threonine kinase receptors. BMP 2, 4 and 7 seem to use the same receptor complex, whereas the other BMP,s use different, as yet uncharacterized, receptor complexes.[49-51]

BMP's are the only growth factors with a known ability to stimulate differentiation of the mesenchymal stem-cell in a chondro- and osteoblastic direction.[42,52,53] The proteins should therefore be involved in the maintenance of a differentiated bone-cell population. Also, during a healing response, release of BMP's from traumatized bone tissue would stimulate a differentiation response of mesenchymal stem-cells, which would correspondingly participate in the healing process. Recent studies have demonstrated, that BMP's are expressed during the early phases of fracture healing.[54,55] The novel recombinant BMP's have intact bone-inducing capacity but need special carriers in order to exert their activity at low doses.[56-59] Functional carriers for BMP are collagen matrix, demineralized bone matrix and various synthetic polysaccharide matrices.[60] The function of the carrier matrix is to immobilize the bone-inducing protein at a particular site for a sufficient amount of time in order to allow bone induction to occur. In vivo studies have primarily focused on the usage of BMP's for stimulating healing of bone defects. In long bone defect models in rats, rabbits, sheep, and monkeys, BMP-2, 3 and 7 have proven to be powerful stimulators of bony healing in otherwise nonunion healing.[12,61-64] In simian muscle diffusion chambers, BMP-2 stimulates bone formation,[65,66] while BMP-3 was found to stimulate bony healing of large skull defects in monkeys.[59] A novel clinical approach has been performed by Cook, who used BMP-7 and collagen as a substitute for autologous bone in spine fusions in dogs.[67] Although the cellular mechanisms for BMP- stimulated bone induction are vaguely understood, the in vivo bone induction activity of this group of growth factors is unique and the bone morphogenetic proteins are very promising for clinical use in any situation where bone defects require stimulation for proper healing.

Platelet derived growth factors (PDGF's)

Platelet-derived growth factor (PDGF) was originally discovered in serum as the major mitogenic activity responsible for the growth of cultured mesenchymal cells.[68] The main effect of PDGF on bone cells is mitogenic. This effect has been found in both human and rat osteoblasts and various osteoblastic cell-lines.[69-71] The most potent isoform is PDGF-BB which can cause a sixfold increase in thymidine incorporation at

50 ng/mL.[72] PDGF is also a powerful chemotactic factor for mesenchymal cells and this effect is valid with respect to osteoblasts from both rat and human tissue[73,74] (II). PDGF has only vague effects on metabolic functions of bone cells. In vivo studies have shown varying potencies of PDGF for the stimulation of bone formation. Demineralized bone matrix treated with PDGF and implanted in muscle in rats, showed increased calcium content and alkaline phosphatase activity. In rat calvarial defects, PDGF inhibited BMP-3 stimulated bony healing by increased soft tissue formation.[75] One study has used a combination of PDGF and IGF in a gel formulation to stimulate bony ingrowth into dental titanium implants. Here, an increased bony ingrowth and new bone formation was found in the growth factor treated implants.[76]

Insulin growth factors (IGF's)

Two IGF's have been characterized: IGF-I and IGF-II. Their original designations were somatomedin-C and skeletal growth factor.[8] These peptides are synthesized by multiple tissues, including bone.[77,78] IGF-II is the growth factor found in highest concentration in bone matrix, whereas IGF-I is found in 10–20 times lower concentration.[79] IGF I and II have similar biological properties, but IGF-I is 4–7 times more potent than IGF-II. Bone cells also secrete IGF-binding proteins (IGF-BP's) which bind IGF and modulate biological activities. The precise role of the IGF-BP's is not fully understood; they may prolong the half-life of IGF, neutralize or enhance its biological activities, or be involved in the transportation of IGF to its target cells.[80-83]

IGF production in bone tissue is known to be stimulated by parathyroid hormone and growth hormone (GH).[84-86] The major effect of IGF in bone tissue is probably its potent effects on cartilage in the growth plate. Here it is assumed that GH controls longitudinal growth via local stimulation of chondroblastic IGF production and IGF subsequently regulates chondroblastic growth and metabolism.[87-89] Both IGF-I and II stimulate osteoblastic cell replication, which increases the number of cells capable of synthesizing bone matrix.[90,91] But their mitogenic effect is less pronounced than other growth factors such as TGF-ß or PDGF-BB.[92] IGF's also have independent effects on the differentiated functions of the osteoblast, increasing bone collagen production and inhibiting collagen degradation.[93,94] As a result of these effects, IGF's increase bone mass.

Several studies have investigated the use of IGF's for stimulation of in vivo bone-healing and systemic bone formation. However, IGF has had limited success as a local stimulator of bone-healing. One study has used a bone ingrowth chamber model with IGF-1-loaded bone matrix, but found no increased bone formation.[95] Another study used continuous local application of IGF-1 to a healing osteotomy, but no stimulatory effects were found [96]. Two studies performed on rats have investigated effects on bone formation after systemic administration of IGF-1. They found increased bone-formation activity after 9 and 14 days of stimulation as indicated by increased bone weight and increased osteoblastic activity.[97,98]

Fibroblast growth factors (FGF's)

Fibroblast growth factors are polypeptide growth factors that exhibit potent mitogenic activities for cells of mesodermal and neuroectodermal origin.[68] The FGF family currently consists of seven members FGF-1 through FGF-7. FGF-1 and FGF-2 are also designated acidic and basic FGF (aFGF and bFGF) respectively. FGF's mainly have a proliferative effect on osteoblasts and less effect on protein synthesis. Consequently they probably enhance bone formation by increasing the number of cells capable of synthesizing bone collagen.[99,100] The bFGF is generally more potent than aFGF.[101] TGF-ß synthesis by osteoblasts can also be stimulated by bFGF, and FGF may therefore exert some stimulatory effects through other growth factors.[102] FGF's are angiogenic factors which are important for neovascularization during a healing response and in conjunction with their effects on bone cell replication, FGF's may be important factors in the bone healing process.

Several studies have used bFGF for in vivo stimulation of bone formation and bone-healing. Basic-FGF has been incorporated into demineralized bone matrix and implanted intramuscularly in rats. In this study, FGF-loaded bone matrix exhibited increased new bone formation.[11] Bone graft incorporation can also be enhanced by bFGF in bone chambers both by treatment of the graft with bFGF and by continuous application by a miniosmotic pump.[103,104] Fracture healing in rats has been stimulated by 50 mg bolus doses of bFGF and caused increased callus formation and bone mineral content.[105] Additionally acidic FGF has been shown to stimulate callus formation during fracture healing in rats.[106] Systemic administration of bFGF to rats caused increased osteoblast proliferation and endosteal bone formation.[107] Basic-FGF has been used to stimulate angiogenesis in molded bone graft. In this study, bFGF was found to have less effect on osteogenesis than TGF-ß.[108] FGF's, along with BMP's and TGF-ß's, are interesting for possible future clinical use especially since FGF's have both osteogenic and angiogenic properties.

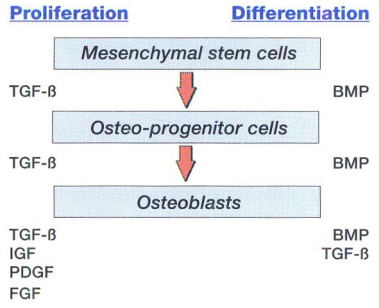


Figure 1. Growth factors that stimulate the different phases of osteoblast differentiation and proliferation.

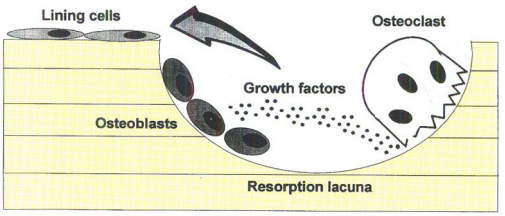


Figure 2. Growth factors released during osteoclastic bone resorption can stimulate osteoblasts and osteoprogenitor cells to migrate to the resorption pit and here form new bone matrix. This could be the so-called coupling effects between bone resorption and bone formation during bone remodeling.

Epidermal growth factors (EGF's)

The epidermal growth factor (EGF) was originally discovered in crude preparations of nerve-growth factor prepared from mouse submaxillary glands. EGF is a small polypeptide growth factor of 6 kD related to TGF-α and these two growth factors share the same 170 kD receptor. In vitro EGF is a mitogen for fibroblasts and endothelial cells and in vivo EGF induces epithelial development and promotes angiogenesis. EGF's have very modest effects on osteoblast in culture. Systemic administration of EGF to mice 200 mg/kg/day resulted in increased periosteal and endosteal bone formation and osteoblastic activity.[109]

Other cytokines and factors

Hematological cells secrete cytokines which mainly are regulators of immunological responses, but which also may also function as systemic regulators of bone-cell function. Because of the close proximity of the marrow cells to bone tissue, some cytokines could act as paracrine regulators of bone cell metabolism. The cytokines which have effects on bone cells can be divided into the interleukins (IL-1, IL-3, IL-6), the colony stimulating factors (M-CSF, GM-CSF) and the tumor necrosis factor (TNF-α). These factors can be produced by osteoblasts[101] and probably exert their main actions in osteoblast-osteoclast interactions, where colony-stimulating factors stimulate the monocyte/osteoclast lineage of cells, while the interleukins and tumor necrosis factors inhibit osteoblastic activity and stimulate osteoclastic activity. Their general effect on bone tissue is therefore stimulation of bone resorption, although some studies have indicated stimulative effects in low doses of IL-1 on isolated osteoblasts.[8,110] A non-peptide group of regulator molecules, the prostaglandins and especially PGE-2, have been demonstrated to be stimulate both bone formation and bone resorption.[111,112] Local application of PGE-2 in vivo leads to massive formation of immature bone and increased levels of IGF-1 in surrounding tissues.[113] However, in areas of bone resorption high levels of PGE-2 exist. Today, the precise mechanisms and influence of PGE-2 on bone physiological events are still unclear.

Actions of growth factors during bone-healing and bone-remodeling

Bone healing and bone remodeling are the two major bone physiological phenomena. Research over the last decade has revealed that growth factors regulate and control many of the complex cellular events during these processes. Besides having individual effects, the growth factors also mediate the effects of hormones.[19]

The two major events of bone remodeling, bone resorption and bone formation, are closely regulated and growth factors play a critical role in their control. The initiation of osteoclastic bone resorption is mainly under hormonal control of PTH.[114] During osteoclastic bone resorption, increasing amounts of growth factors are released from the resorbed bone matrix.[114] TGF-ß probably participates in the inhibition of continued osteoclastic activity.[115] In concert with other released growth factors, BMP's initiate the differentiation,[116,117] whereas other factors like TGF-ß and IGF's stimulate proliferation of osteoprogenitor cells on adjacent periosteal surfaces (Figure 1).[8] Then PDGF and TGF-ß stimulate chemotactic migration of osteoblasts to the resorption pit. The differentiated osteoblasts in the resorption pit maintain bone matrix synthesis through auto and paracrine stimulation of secretion of regulating growth factors (Figure 2).[8,118] Because of the stimulative effects of growth factors during the formative phase of bone remodeling, there is ongoing intensive research directed towards using systemic administration of growth factors to reverse the negative bone formation balance in patients with osteoporosis.

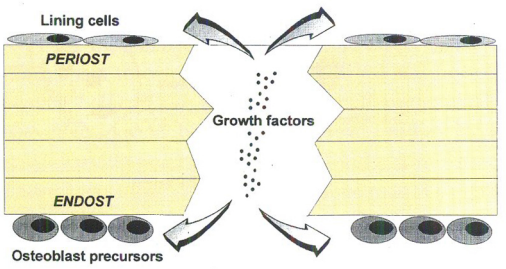


Figure 3. During bone-healing, growth factors released from both the blood clot and the traumatized bone ends can stimulate preosteoblasts from periosteal and endosteal surfaces to differentiate and proliferate in order to form a healing response.

During the bone-healing process, growth factors have important functions both for the initiation and maintenance of differentiation and proliferation of the osteoprogenitor cells and osteoblasts which contribute to the formation of new bone. In the early phases of healing, TGF-ß and PDGF released from platelets from the blood clot, initiate differentiation of osteoprogenitor cells towards an osteoblastic lineage.[119] Growth factors released from the traumatized bone ends can contribute to continued stimulation of osteoblastic activity as well (Figure 3). The initiation of healing by growth factor released from platelets and bone tissue stimulates specific expression and synthesis of growth factors by the osteoblasts participating in the healing process.[120] Very early, after 2 days, BMP is expressed by periosteal osteoblasts during fracture healing.[54] This synthesis could contribute to the continuous osteoblastic differentiation of mesenchymal stem cells. Later (after 7–12 days), other growth factors like TGF-ß, FGF and PDGF are synthesized by osteoblasts in order to maintain a high proliferative and metabolic level of the osteoblasts that are involved in the healing process.[20,121] FGF's are probably important for the angiogenesis in the newly-formed bone.[122]

In vivo effects of growth factors in clinically related studies

Due to the profound importance of growth factors for bone-healing, extensive efforts have been made to establish methods by which growth factors can be used to stimulate local bone-healing and bone formation in various clinical situations of relevance for orthopedic and dental surgery. The growth factors from the TGF-ß superfamily (TGF-ß's and BMP's) are virtually the only growth factors which have been demonstrated to possess significant in vivo bone stimulatory capacity (Table 4). Stimulation of bone formation in bone defects at various anatomical sites has been investigated for both BMP's and TGF-ß. Beck et al. demonstrated that a single dose of TGF-ß in methyl cellulose gel could stimulate bony healing of large calvarial defects in rabbits.[37] However, in rabbit femoral condylar defects TGF-ß had no effects on bone healing using the delivery system.[123] Ripomonti et al. demonstrated similar effects of BMP-3 in large calvarial defects in monkeys while TGF-ß exhibited modest effects.[38,59] Defects in long bones is another type of model, where especially different BMP's have demonstrated potent capacity for inducing bony healing in otherwise non-healing defects. BMP-2 has been shown to stimulate 100% bony healing and restore mechanical strength when applied in a collagenous matrix carrier to femoral defects in rats and sheep.[12,64] BMP-7 has exhibited similar potent stimulatory effects in ulna defects in rabbits and monkeys.[61,63] A recent study by Beck et al. has shown that a tibial defect fixated by an intramedullary nail demonstrates enhanced bone-healing when the defect is stimulated with TGF-ß adsorbed to a tricalcium-phosphate (TCP) ceramic.[124] In a spine fusion model in dogs, Cook et al. have shown that BMP-7 together with a collagenous matrix carrier can induce bone formation to an extent comparable to the bone formation seen after usage of autologous bone grafting.[67]

Biochemically enhanced fracture healing was an early expectation when the potency of growth factors first emerged, but only few experimental studies have successfully demonstrated enhanced fracture healing. In a plated rabbit tibiae osteotomy model stimulated by a continuous administration of TGF-ß, the growth factor stimulation leaded to increased mechanical strength and callus formation of the osteotomies after six weeks (I). Another fracture study used TGF-ß injections to rat tibial fractures and also demonstrated increased mechanical strength and callus formation as a result of the local growth factor application.[125]

Growth factor enhanced healing of implants is another interesting area for orthopedic research. An early study from 1991 by Lynch et al. demonstrated increased bone ingrowth and bone formation in dental implants stimulated by a combination of PDGF and IGF-1 in a canine model, where the implants were implanted in press fit in root canals.[76] The positive results from this study have neither been followed up nor reproduced. Recent studies have demonstrated that rhTGF-ß1 adsorbed to the ceramic coating of unloaded implants inserted into trabecular bone of mature dogs can stimulate mechanical anchorage, bone ingrowth and gap healing of the implants (V).[126] Also in dog models, similar stimulative effects of rhTGF-ß1 to unloaded implants coated with hydroxyapatite ceramic and to weight loaded tricalcium phosphate

Table 4. Important in vivo studies using growth factors in clinically related models

Author	Year	Animal	Model	Dose	Effects
Beck [37]	1991	Rabbits	Calvaria defects	3 µg, one dose 4 weeks	Induction of bony healing
Lynch [76]	1991	Dog	Dental implants	PDGF & IGF in gel	Improved bone ingrowth
Ripomonti [59]	1992	Monkey	Calvaria defect	100 µg BMP-3	Healing compared to non-union in controls
Yasko [12]	1992	Rat	Femoral defects	15 µg BMP-2	100% healing and restoration of strength
Cook [63]	1992	Rabbit	Ulna defect	3–400 µg BMP-7	Healing compared to non-union in controls
Lind (I)	1993	Rabbits	Tibial osteotomy	1 and 10 µg TGF-ß/d 6 weeks	Increased callusformation. Higher bending strength
Cook [61]	1993	Monkey	Ulna defect	1–5 mg BMP-7	Healing compared to non-union in controls
Nielsen [125]	1994	Rat	Tibial fracture	4 and 40 ng TGF-ß/d 40 days	Increased callus formation. Higher bending strength
Kirker-head [64]	1994	Sheep	Femur defect	1–5 mg BMP-2	Healing compared to non-union in controls
Cook [67]	1994	Dog	Spine fusion	1 mg BMP-7	Healing comparable to autologous bone
Beck [124]	1995	Rabbit	Tibial defects Intramedullary nail	TGF-ß in TCP ceramic	Enhanced healing
Lind (V)	1996	Dog	TCP coated unloaded implants	0.3 and 3 µg TGF-ß/d 6 weeks	Increased bone ongrowth, gap healing, and mechanical fixation
Lind (VII)	1996	Dog	HA coated unloaded implants	0.3 and 3 µg TGF-ß 6 weeks	Increased bone ongrowth
Lind (VI)	1996	Dog	TCP coated implants weight loaded	0.3 µg TGF-ß 6 weeks	Increased bone ongrowth
Ripomonti [38]	1996	Baboons	Calvaria defects 25 mm	5, 30, 100 µg TGF-ß 30 days	Sparse bone formation

coated implants, although to a lesser extent (VI,VII). Generally studies have used an observation period of less than two month, thereby investigating the effects of the applied growth factors in the early healing phase. In this critical phase rather high stimulative effects have been found, with up to 200% increases in bone formation or mechanical parameters. Whether these stimulative effects is maintained over a longer time period is still undetermined. Also the stimulative effects are found in very controlled animal studies with varying resemblance to clinically related situations, where patient related factors may dominate a healing response.

In conclusion, research during the last 5 years has shown increasing evidence that growth factors can be used as in vivo stimulators of bone-healing and bone formation and therefore has the potential to become a new therapeutic possibility in a variety of clinical situations in orthopedic surgery.

Methodological considerations

Cell culture

Primary osteoblastic cultures can be obtained by tissue explant method or sequential bone tissue digestion. With the *tissue explant method*, cells migrate from bone chips onto plastic or glass surface of a culture dish.[127] Most commonly, bone chips are first treated with collagenase to remove connective-tissue cells and bone marrow cells that adhere to the bone surface. This culture method gives an osteoblast culture with a high purity, but a varying degree of differentiation.[127] The explant method is suitable in situations where the available bone tissue for culture is limited or cell growth is slow, for example culture from adult bone tissue. Problems with this method is the growth of cells with a different proliferative capacity and different degree of maturation. The other method for primary culture uses *sequential digestion* of bone with collagenase (20–30 min steps). This causes the release of different bone cell populations.[128] The initially released cells are periosteal fibroblasts and immature osteoblast and osteoclasts, whereas the subsequently released cells are more mature osteoblasts. The phenotype of cells released by this method is closer to the osteoblasts seen in bone tissue. But there are a number of disadvantages with the technique; cell isolation is complicated and time-consuming. The number of cells isolated by this method is low and considering the slow growth of cells in vitro, it takes a long time (weeks to months) to obtain enough cells for experiments. Also, the advantage of stable cell phenotype is quickly lost if the cells are passaged more than a few times.

Osteoblasts can be released from bone from several animal species. The choice of species must depend on purpose of the study and which types of bone tissue are available for culture. Most of the in vitro studies that investigate effects of hormones and growth factors have used rodent primary cultures or cell lines.[129] Mainly because of the easy access to rats and mice in most biological laboratories and the fact that cells released from rodent bone tissue grow fast and are easy to maintain. If the objective is to relate in vitro data to human bone physiology, then is it essential to use human bone cells despite problems with limited availability and slow growth. We used human primary osteoblast cultures that were released by the tissue explant method (II–IV), and gained thereby the advantage of being as close to human bone physiology as possible. The explant method enabled us to produce cultures from limited bone resources obtained from hip and spine surgery and we were able to produce a homogenous culture with phenotype characteristics of osteoblasts e.g. osteocalcin producing and vitamin D_3 inducible alkaline phosphatase activity. We controlled the osteoblastic phenotype of random selected cultures to ensure a stable phenotype in our osteoblast cultures. Cells were characterized for osteoblastic phenotype by type I collagen and osteocalcin immunostaining for which cells were more than 95% positive. Vitamin D_3 stimulation caused increase in alkaline phosphatase activity and osteocalcin production. Parathyroid hormone stimulation caused an increase in intracellular cAMP levels.

Due to the known osteogenic potential of bone marrow, marrow stromal osteoblast-like cells were cultured and used in study (III). These cells are isolated as cells capable of plastic adherence in the mononuclear fraction of bone marrow cells. Although the cells generally show a large phenotypic variability, they express the characteristics of the osteoblastic phenotype. Marrow stromal osteoblasts represent undifferentiated osteoblasts permitting studies of the osteoblast differentiation process.[130]

In vivo model considerations

Experimental animals

Many animals species, from mice to monkeys, have been used for investigation of the in vivo effects of growth and healing promoting agents in bone. Many aspects must be considered before choosing an experimental animal. The phylogenetically lower animals like mice and rats have been used extensively for studies of basic bone physiology. Such animals are advantageous because large amounts of data and observations from previous related studies are available. Also, homogenous populations are readily available at low cost, and the experimental procedures can be relatively simple. For clinically related studies, mice and rats are, however, disadvantageous because their bone physiology with regard to bone remodeling and bone healing is different from that of human bone, and the amount of bone available for simulating various operative procedures is very small. Rabbits, with a larger bone mass that allows clinically related oper-

ative procedures while still having advantages such as low cost and simple handling at experimental procedures, are an alternative to rats and mice. For these reasons rabbits were used for the osteotomy study (I). Larger animals such as dogs, pigs, sheep and monkeys are all well suited for advanced experimental surgical procedures, and the bone physiology and structure of these animals is close to that of humans. For these reasons, dogs were used for our implants experiments (V–VII). However, these animals are expensive and their handling for experiments is generally technically demanding.

Design of in vivo studies: All in vivo studies were designed to have optimally controlled conditions for investigations of growth factor effects. In all dogs studies (V–VII), intra individual control was available due to the possibility of having several implants in each animal. In the osteotomy study in rabbits (I), however, intra individual control was not possible due to animal ethical considerations, where bilateral osteotomies would have caused unacceptable stress on the animals. In the canine studies, random alternating location of implants was performed in order to avoid the possible bias that the right/left and medial/lateral location could introduce due to regional differences in bone density and bone turnover.

In all in vivo studies the observation period was 6 weeks. This observation time was chosen on the basis of the wish to observe early effects of growth factor stimulation on the different healing situations. The 6 weeks represent in both rabbits and dogs a time period where new bone formation occurs and the healing process is still in an active phase. Also, bone remodeling is still only occurring to a limited degree. Such an observation period will theoretically be optimal for investigating enhancement of the osteogenetic processes, which exogenously applied growth factors effectuate.

The number of animals used in each study (n) was selected to be 10; this number enabled detection of significant effects of growth factor stimulation if 50% change in the investigated parameters was found (d). These calculations were based on a standard deviation (SD) of 50% of the mean values. This value was known from previous studies in the same models where SD's ranged from 0.2–0.3 of mean values for histological parameters and up to 0.5 of mean values for mechanical parameters.[131] (Z) level of significance = 1.96 SD.[132]

$$n > 2\ (\frac{(Z)(SD)}{d})^2 \Leftrightarrow n > 2\ (\frac{1.96 \times 0.5}{0.5})^2 \quad n > 7.7$$

With 10 animals we were able to demonstrate significant difference even if one or two animals were lost during the study.

The risk for an error of the first degree (α) was for two-tailed significance analysis = 1.96 SD: corresponding to the chosen level of significance = 0.05.

The risk for an error of the second degree (ß) with above mentioned assumptions was:

($ß_{tb}$) SD value of (ß) in a normal distribution.[132]

$$(ß_{tb}) = \frac{(\sqrt{n/2}) \times d}{SD} - Z$$

$$(ß_{tb}) = \frac{(\sqrt{10/2}) \times 0.5}{0.5} - 1.96 = 0.276 \text{ SD}$$

in a normal distribution table.

$$ß = 0.394$$

This is unfortunately a relatively high risk for false negative result, but with the given variation in the data and the resources for including animals in the study, we could not achieve any better risk for an error of the second degree.

Growth factor for in vivo studies

The BMP's have recently become commercially available but are patent protected. They are produced by only two companies, both of which have very strict policies for engagements in preclinical research projects. Our initial contact to both companies inviting to collaboration was unsuccessful. For this reason, we used natural TGF-ß purified from human platelets (I), and recombinant human TGF-ß1 for our implant studies provided by Genentech Inc. San Francisco, USA for our in vivo studies (V–VII).

The natural TGF-ß used in study I was produced by our group according to the methods described by Assoian.[133] The technique was based on protein extraction from extensive amounts of human platelets and purification by sequential gelfiltration columns. Purity and identification of TGF-ß was based on silver stained SDS-page electrophoresis and reverse-phase HPLC electrophoresis. With this method we were able to obtain a biological active extract which was tested for biological activity in a fibroblast collagen production assay.[134] Since the TGF-ß in study I was released from pumps over a period of 3 weeks, we tested the bioactivity after 3 weeks at 37 °C, and found intact biological activity using the fibroblast collagen assay. The recombinant TGF-ß was produced by Genentech Inc. San Francisco, USA. Recombinant human TGF-ß type 1 was produced by a Chinese hamster ovary cell expression system and processed to 98% purity.

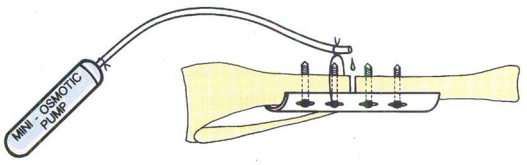


Figure 4. The experimental osteotomy model consisted of a plated unilateral mid-tibial osteotomy. The continuous application of growth factor was accomplished by a mini-osmotic pump placed subcutaneously on the thigh.

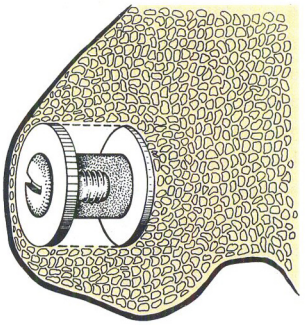


Figure 5. The unloaded implant is centralized in the drilled canal in the femoral condyle surrounded by a 2 mm gap. The deep part of the implant is fixed by the footplate in press-fit. A titanium washer centralizes the implant superficially. From Søballe et al., Clin Orthop 1992; 274: 282–93.

Growth factor doses

In the osteotomy study, a dose of 1 and 10 µg TGF-ß daily was used. These doses were chosen on the background of studies in rats which had found stimulative effects on bone formation of 0.3 to 1.0 µg TGF-ß daily.[10,36] With the large bone size in rabbits and the continuous application method of TGF-ß to the osteotomy line, the above mentioned doses were chosen.

In the canine studies, single doses of 0.3 and 3.0 µg rhTGF-ß1 were adsorbed to the ceramic coating of the implants. These doses were chosen on the background of pilot studies in sheep performed by DePuy Inc. Warsaw, Indiana, USA, which had demonstrated that higher doses on ceramic coatings lead to fibrous tissue overgrowth. These doses of study V–VII are relatively low compared to previous in vivo studies and study I. But the ceramic coatings were expected to act as a favorable carrier of the growth factor, thereby increasing the bioavailability and bioactivity. This phenomenon has been described experimentally when rhTGF-ß1 where adsorbed to hydroxyapatite crystals. In this system nanogram doses stimulated bone formation.[135]

Osteotomy model

Osteotomy models are interesting because of their close resemblance to the clinical fracture healing situation. They can be performed in both small and larger animals.[125,136,137] Mid-diaphyseal fractures or osteotomies are typically used. Fracture fixation by means of osteosynthesis or intramedullary nailing is necessary and a standardized fixation procedure is essential for reproducible results. Studies that investigate stimulation of fracture healing encounter problems with the application of stimulating agents. Systemic administration is possible for some agents (e.g. growth hormone) while other agents (e.g. growth factors) require local application by pumps or injections.[125,138,139]

We chose a plated tibial mid-diaphysial osteotomy model in rabbits as a model for testing the ability of TGF-ß to stimulate bone formation in a clinically related model (I). This model had previously been used in our laboratory for studies of exogenous application of bone healing promotive agents and we therefore used the experience of those previous studies regarding surgical procedures and data analyses.[113,140] The model employed miniosmotic pumps to deliver the growth factor continuously to the osteotomy site in a buffer solution (Figure 4). We considered this application method advantageous because it enabled precise application to the area where stimulation of bone healing was desired. Daily injections have also been used as growth factor administration method to bone surfaces,[10,36] but the method creates a minor trauma with each injection which may disturb the healing process; such minor trauma is avoided with pump application.

Implant models

Unloaded model (V, VII). In the unloaded model the hole in the trabecular bone was 4 mm larger than the implant diameter so permitting a 2 mm gap around the implant (Figures 5 and 6). The position in the drilled hole was ensured by a 10 mm footplate and top washer. The implants were inserted extraarticulary perpendicular to the long axis of the femur and humerus, which eliminated any change of loading during animal motion. The model was developed by Søballe et al. and has been described in detail previously.[141] The advantage of this model is the possibility to investigate bone formation in the gap and bone ingrowth to the implant under very controlled circumstances. The bone healing is not affected by weight loading and takes place in a purely osseous environment. A disadvantage is the lack of similarity to the clinical situation where such controlled situations are not found.

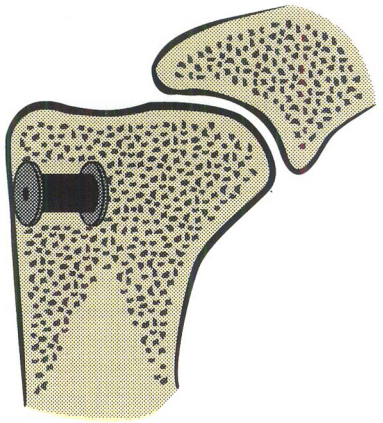


Figure 6. The unloaded implant is centralized in the drilled canal in the proximal humerus surrounded by a 2 mm gap. The deep part of the implant is fixed by the footplate in press-fit. A titanium washer centralizes the implant superficially.

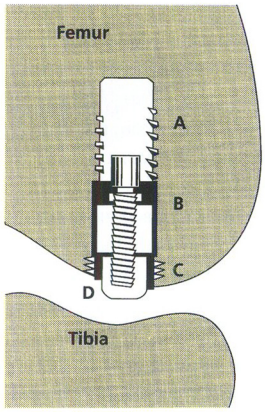


Figure 7. The implant device and test implant is positioned in the weight-bearing part of the femoral condyles. (A) The deep part of the implant device is fixed by the threaded titanium cylinder. (B) The test implant is mounted on the threaded piston of the implant device surrounded by a 0.75 mm gap. (C) A titanium ring inserted in the subchondral part of the condyle serves as a support and centralizer for the polyethylene plug. Note protrusion of the polyethylene plug (D), which transmits the load from the tibial plateau of the knee-joint to the implant. From Søballe et al., J Orthop Res 1992; 10: 285–99.

Weight-loaded model (VI). In the loaded model weight-loading was achieved by intraarticular placement of the implant. Furthermore the implant was mounted on a special implant-device, which ensured deep stable fixation in condylar bone with a subchondral position of the implant which was surrounded by a 0.75-mm gap. Loading from the tibial plateau was transferred to the implant-device and the implant by a polyethylene plug at distal end of the implant device (Figure 7). This model was developed by Søballe et al. and has been described in detail previously.[142] This model is very clinical relevant since there is both a gait controlled weight-loading and access joint fluid from joint cavity to the gap around the implant. The disadvantages of the model is that numerous factors apart from the stimulative agent and endogenous bone healing capacity affect the bone formation. Weight loading might vary between animals due to different locomotive activity during the observation period. Also joint fluid contains numerous bioactive substances that potentially can modulate the activity of growth factor stimulation.

Placement. Implants can be placed in either cortical or trabecular bone. We used implants placed in trabecular bone, as it mimics the placement of most endoprosthetic components. Also, the high metabolic activity of trabecular bone makes this tissue more susceptible to growth factor stimulation. The unloaded implants were placed either, in the medial and lateral side of the femoral condyles or in the proximal humerus (Figures 5 and 6). These placements were optimal due to the easy surgical access and minimal trauma to the research animal. Previous studies have further shown good bone structure in these regions and possibility for an enhanced healing response with stimulation by ceramic coatings.[131]

Gap. In all the studies we had an initial gap around the implants; 2 mm around unloaded implants and 0.75 mm around weight loaded implant (Figure 7). The gap model was chosen because of its clinical relevance, where gaps around endoprosthetic components lead to impaired bone-healing or even fibrous tissue healing, that probably contributes to the later failure of the prosthetic components. In a gap model, extensive new bone formation occurs. Bone formation and bone anchorage of the implants will therefore be by newly formed bone. Any stimulation of the osteogenesis by growth factor application will be more accessible for discovery.

Implant texture. In all the studies, a relatively smooth implant surface was chosen. The implants were all grit-blasted, which provided roughness only for improved application of the ceramic coatings. We did not use a porous coating since this texture has independent osteoconductive properties and causes a mechanically strong bone/implant interface.[143] These properties could make it difficult to demonstrate effects of growth factors application.

Ceramic coating. For the first implant study (V) and the weight loaded model, tricalcium phosphate was chosen as the ceramic coating. The ceramic coating functioned as a carrier for the growth factor which

is essential for the principle of local growth factor application for these studies. TCP is resorbed readily in a physiological environment but still has initial osteoconductive and osteogenetic properties.[144,145] Resorption leads to a mechanically weaker bone/implant interface, in comparison with more stable ceramics like hydroxyapatite and fluorapatite.[146] However, this weaker interface is favorable for demonstrating effects of growth factor stimulation on implant anchorage. After we had demonstrated stimulative effects of local rhTGF-ß1 application to TCP coated implants (V–VI), we proceeded on to experiments on stable hydroxyapatite coatings (VII). This coating is the most relevant for clinical use and by itself form a very strong bone/ceramic implant interphase which apriori might not be susceptible for further stimulation.

Growth factor adsorption. The loading of RhTGF-ß onto the implants was done by direct adsorption. To adsorb different doses of rhTGF-ß1 to the ceramic surface, the implants were incubated for two hours with a 2.0 mL solution containing 1.6 mg/mL and 12 mg/mL rhTGF-ß1 in 20 mM sodium acetate buffer. The dose adsorbed was ensured by measuring rhTGF-ß1 content of the buffer before and after incubation. Release of rhTGF-ß1 from ceramic coated implants into serum and saline was tested in vitro by performing the adsorption procedure using ^{125}iodine labeled rhTGF-ß1. In this assay 90% of the rhTGF-ß1 was released to serum within 4 hours and virtually no release was seen to physiological sodium chloride solution. The incubation and release analysis was performed by Genentech Inc. South San Francisco, USA. The fast release of rhTGF-ß1 from the ceramic surface could be potentially problematic due to biodegradation before any biological effect were achieved. However, in calvarial defects in rabbits, rhTGF-ß1 has been shown to be present in surrounding tissue for up to a week after delivery from gel carrier.[147] During such a time period there is sufficient time for initiation of the different biological actions of the growth factor. The growth factor prepared implants were transported from USA to Denmark for two days and typically a week passed before operative insertion. During this period the implants were stored between 2 and 5 °C at which the growth factor is known to maintain it biological activity for months.

EVALUATION

In vitro assays

Cell proliferation (IV)

Cell division is an important parameter when investigating osteoblast metabolic activity. Several methods exist for quantitating cell-division activity. The most simple method is to count cells in the culture wells under a microscope. Another more convenient method is staining a cell layer with methylene blue.[148] This basic dye binds predominantly to the phosphate groups of nucleic acids and absorbance measured on an ELISA plate reader at 650 nm is therefore a good measure for total nucleic acid in the cells. This method is very reliable and easy to perform and is highly correlated with cell number. Two methods exist which determine number and activity of cells which synthesize DNA (S-phase cells). The bromodeoxyuridine assay utilizes the thymidine analog bromodeoxyuridine and a recognizing antibody for labeling and detecting S-phase cells. Labeled cells are visualized with a secondary antibody and peroxidase and cells are counted.[149] This method is very time consuming due to the counting technique and we therefore used an alternative method that uses labeling of cells with ^{3}H- thymidine (IV). This assay is easy to perform and has the advantage of being a more sensitive measure of cell proliferation than cell count measurements and methylene blue measures, since it quantitates DNA synthesis rather than number of cells. A disadvantage of the technique is that fluctuations in the intracellular thymidine concentration often cause considerable intra individual variations in radioactive counts.[150] This problem can be overcome by adding a sufficient concentration of radioactive thymidine and by having triplet or quadruplet determinations of each test unit.

Enzyme activity (III,IV)

Alkaline phosphatase (AP) is a membrane bound enzyme which is present in many cell types but is most abundant in osteoblasts.[151] It is a marker of the osteoblastic phenotype and thought to be of importance in the mineralization process by increasing the concentration of inorganic phosphate leading to precipitation of calcium-phosphate crystals in the mineralization front.[152] AP is a marker of the highly differentiated osteoblast. AP can be visualized in a cell-layer by histochemical staining and enzyme activity can also be quantified by incubating a cell-layer with p-nitrophenyl-phosphate for a standardized time-period and then measuring absorbance in a spectrophotometer at 405 nm. This method is easy and reliable when performed in triplicates and quadruplicates. AP activity is increased in differentiated cells stimulated with $1,25(OH)_2D_3$ and responsiveness to other stimulating agents is also increased, therefore analysis of AP activity should be performed with and without $1,25(OH)_2D_3$ stimulation. We therefore tested AP activity in the cell-layer with p-nitrophenyl-phosphate

with and without $1,25(OH)_2D_3$ stimulation.

Matrix protein synthesis (IV)

A key aspect in osteoblastic formation of new bone is synthesis of matrix proteins. In bone, collagen type I is the major structural protein, and measurement of this protein is therefore crucial for evaluation of in vitro bone formation. Today the most frequently employed method is determination of pro-collagen 1 C-terminal propeptide (PICP) in supernatant medium which was used in study IV. This peptide is split off from the mature collagen molecule and can be recognized by an antibody and quantitated by RIA or ELISA technique.[153,154] Osteocalcin is one of the most abundant non-collagenous proteins in bone matrix and is produced exclusively by bone cells. Osteocalcin is probably of importance for cell attachment and bone mineralization.[121] Osteoblast expression of osteocalcin in culture can be visualized by an immunohistochemical technique. We used this technique for characterization of osteoblastic phenotype. Osteocalcin is produced to the supernatant medium and can be quantitated by RIA or ELISA technique.[155]

Numerous other proteins exist in bone matrix (see Table 5) but no available assays for determination of protein production exists today. An indirect approach exists using measurement of mRNA synthesis for a specific protein using molecular biology techniques.

Chemotaxis (II,III,IV)

Biochemically controlled cell migration is an important aspect in osteoblast biology both during bone remodeling and bone healing. Two methods exist for investigating cell chemotaxis: The first method used was the *Boyden chamber technique* that used cell migration into a thick gel membrane in which a concentration gradient of the test agent was created. Cell migration was quantified by counting cells that had reached a standardized level in the membrane.[156,157] The Boyden chamber technique used single well chambers which caused extensive screening of several concentrations and test agents to be very time consuming. This problem has recently been overcome by the *multiwell chamber technique*, which consists of up to 48 wells in each chamber (Figure 8).[158] This chamber consists of a two level system separated by a thin membrane with pores allowing cell migration. Test agent is placed in the bottom level and cell-suspension in the top level and a concentration gradient is created over the membrane. Cell migration is quantified by counting cells which have migrated to the bottom side of the membrane. Correct incubation time and cell-concentration for the cell type tested are essential for reproducible results. Typically non-

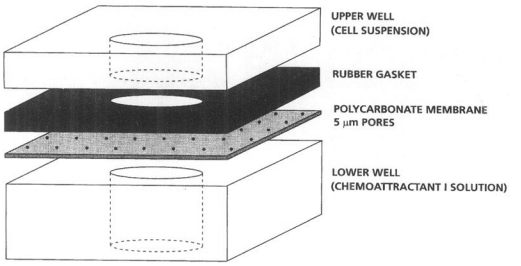


Figure 8. The chemotaxis model consisted of a micro chemotaxis chamber with 48 wells. Each well had an upper section for the cell suspension and a lower section for the growth factor solution. These were separated by polycarbonate membrane with 5 μm pores. If the growth factor in the lower well has chemotactic properties, increased number of cells will migrate through the pores of the membrane and attach to the collagen coating on the lower surface of the membrane.

transformed cells, like primary human osteoblasts, show large inter-individual differences in absolute number of migrated cells. Data must therefore be normalized as cell-counts in stimulated wells divided by cell-counts in control wells, a measure designated Chemotactic Index (CI).

In order to verify that a stimulation of cell migration is truly chemotactic (concentration directed) and not chemokinetic (random migration in all directions), a control experimental must be performed. In this experiment, a concentration found to stimulate migration is eliminated by adding the test agent together with the cell-suspension.[159] For true chemotaxis this will reduce migration to control levels. We chose to use the multiwell chemotaxis chambers in our osteoblast migration studies due to the ability of this techniques to perform several test units under identical conditions and because data sampling could be performed fairly simply under a microscope.[158]

Primary human osteoblast culture are known to express heterogenic phenotypes; therefore, fibroblastic contamination could be a problem in the used chemotactic assay where only a minor part of the seeded cells migrated through the membrane. We found, however, that a majority of the migrated cells stained positive for osteocalcin which is one of the most specific osteoblast markers (Figure 9).

Mechanical testing (in vivo studies)

Osteotomy model (I)

Biomechanical testing of fractured bone presents several problems: Standardized specimens cannot be prepared because of the irregular geometry of fracture callus and inter-individual differences of bone struc-

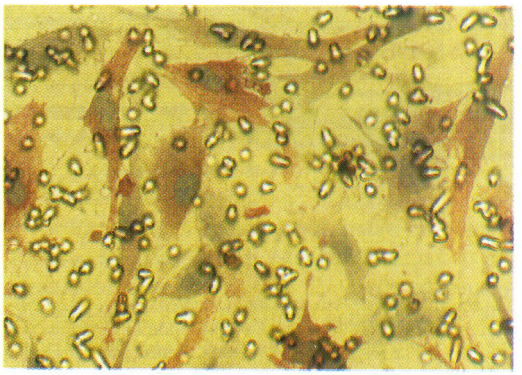


Figure 9. Microphotograph of a polycarbonate membrane after osteoblast migration through the 5 µm pores. The cells have been immunostained for osteocalcin (red staining). Note that the majority of the cells stain positive for osteocalcin, indicating the osteoblastic phenotype of the migrated cells, original magnification 40x.

ture. The mechanical testing procedure is another concern. Tensional and torsional tests have the advantage, at least in theory, of creating an even distribution of stress around the fracture callus. Also, such tests will determine the weakest point of the entire bone.[160] But considerable practical problems with fixation and alignment in the testing apparatus causes the test type to be rarely used for fracture healing studies, despite the superiority of test. Bending tests, (three or four point), are also suitable for mechanical testing of fracture strength. Four-point bending test involves, to a degree, adjacent cortical bone. This is a disadvantage when, in the later stages of fracture repair, the strength of the callus equals or exceeds the strength of the cortical bone. Another disadvantage is that a small degree of rotation of the test specimen is difficult to avoid and standardization of the test can therefore be impaired. We used a three-point bending test in our mechanical analysis; since this test type enables a precisely located load placement, thereby testing the mechanical properties at the fracture line.[139]

Implant models (V–VII)

The aim of the mechanical push-out test for the implant experiment was to evaluate the bony fixation of the implant in order to test how adjuvant therapy (growth factor application) can improve this fixation and hopefully, in the clinical situation lead to improved implant survival. In the clinical situation, however, a prosthetic component is subjected to both bending, shearing and compressive forces and no experimental mechanical test exist that can mimic this situation. We chose a push-out test because such a test measures the strength between the implant and the surrounding tissue in the load direction that an implant is mostly subjected to in the patient. Also, this test had in numerous previous studies demonstrated its suitability for testing bone-implant specimens from animal studies and furthermore a good correlation exist between data from push out test and histomorphometrical bone ingrowth analysis.[131] An alternative to the push out test would be a torsional test, but such a test would measure pure shear at the bone-implant interface.

In order to minimize variation in test conditions, all specimens from each study were tested on the same day and by the same investigators. All specimens were frozen before mechanical testing, a procedure which might have influence on the mechanical properties. However, prolonged freezing up to 100 days and repeated thawing and freezing, have been shown not to influence the mechanical properties of trabecular bone.[161]

The thickness of the specimens for push-out test varied from unloaded implant studies (V,VII) to the weight loaded study (VI) due to different size of the test implant. In the unloaded studies the thickness of the specimen for mechanical testing was 4 mm, whereas it was 3 mm for the weight loaded study. However, all reported data are normalized for surface area.

Two different coatings were used in the studies; tricalcium phosphate and hydroxyapatite. These two coatings have very different resorption characteristics in vivo; where TCP is readily resorbed and HA is very stable and resorbs slowly. Moreover, HA forms very strong bonds to the surrounding bone. These proper ties produce different interface conditions during mechanical testing. For TCP, which is nearly resorbed at 6 weeks, the mechanical properties of the bone-implant interface is tested, whereas for HA coated implants, the implant-ceramic and ceramic-bone interfaces are so strong that the push-out test to some extent will measure the strength of woven bone trabeculae in the gap. This is problematic, since the result of test to some extent do not represent the strength of the bone/implant interphase, but instead the strength of the woven bone trabeculae.

Finite element analysis of the push-out test have established that difference between implant diameter and the hole supporting setup is important, with a reduction in stress concentration if this difference becomes to large.[162] A study suggests an optimal difference of 0.7 mm, which was close to the 0.5 mm difference used in the present studies.[163]

Histology, bone histomorphometry (in vivo studies)

Osteotomy model

Quantitative histomorphometry was an important technique for evaluation of new bone formation in the osteotomy study (I). In this study, we used 200 µm thick sections cut 5 mm distal to the osteotomy line. These sections were cut unembedded and undecalcified on a precision diamond saw. On these sections, area of fracture callus, thickness of cortical bone and cortical bone porosity were calculated. The callus area represented the new bone formation during fracture healing, and cortical bone thickness and porosity were used as parameters for remodeling activity in preexisting diaphyseal bone. Callus area and cortical thickness were measured using a computer assisted tracing devise. This provided very reproducible results with low coefficient of variations. Cortical thickness and haversian canal diameter were determined by a digitizer based measuring system. Twenty measurements per section were performed.

Reproducibility. To eliminate inter-individual observer variation, the same investigator performed all histomorphometric analyses. To eliminate biased data sampling all analyses were performed blindly. The intra individual variation was estimated by calculation of coefficient of variation from 10 double measurements. These were 0.03, 0.02 and 0.06 for callus area, cortical thickness and haversian canal diameter respectively.

Implant models

All histomorphometric analyses were performed on sections embedded in methyl methacrylate and cut to 200 µm on a precision diamond saw and then ground to 50 µm thickness. This thickness allowed for discrimination of different tissue types and materials; bone, fibrous tissue, bone marrow, titanium implant and ceramic coating. Bone tissue was visualized by light green staining,[164] whose properties as a surface staining enhanced the sharpness of the bone interfaces against other tissues and materials. A drawback of this preparation method is that analyses at cellular levels are impossible due to the large thickness of the sections. For cellular analyses a thickness less than 10 µm is required. At the time of the studies, no available technique for preparation of so thin sections existed for bone tissue containing metal implants. Subsequently a more technically advanced method has been developed in Holland, the Leiden microtome saw, which can produce sections down to 10 µm.[165]

Only one transverse section from each implant was used for histomorphometrical analysis. This represents only a small fraction of the entire bone/implant complex which could be a problem for data sampling. However, a previous study has shown that bone density only changes 12% (intersection variance) between serial sections in trabecular bone.[166] If similar variation is present for the bone parameters in woven bone in the present studies, the use of only a single section should not affect the precision of the data significantly.

Vertical sections based on the stereology principle would be the optimal sampling technique and is required for completely unbiased sampling and measurements. This is relevant for bone tissue, since trabecular bone is not of isotropic nature due to weight loading that orientates the trabeculae according to Wolff Law.[167] We had a relatively small sample size since a large sample was used for mechanical testing. Due to this situation, we did not use vertical sectioning which would have been the optimal principle for data sampling. Our histomorphometric data are therefore partly biased due to the anisotropic structure of trabecular bone. Studies performed after the studies included in the present thesis have used vertical sections on the same size sections. Data from these studies were found be consistent and reproducible.[168]

Reproducibility. To eliminate inter-individual observer variation, the same investigator performed all histomorphometric analyses. To eliminate biased data sampling, all analyses were performed blindly. The intra individual variation was estimated by calculation of coefficient of variation from 10 double measurements. These were 0.032 for bone ongrowth and 0.051 for bone density in the gap.

STATISTICS

Mean and standard deviation or standard error of mean were calculated from all parameters. All data sets were tested for normal distribution by Kolmolgorov analysis and probit plots. Comparison between two data sets was done by means of a Students T-test for parametric data sets and Wilcoxon tests for nonparametric data sets. Comparisons between several data sets were done by one-way ANOVA and Fishers LSD tests for comparisons between control and stimulation data sets. Differences were considered significant when the p-value was less than 0.05. For definition of synergistic effects in study (IV), an arithmetic principle for synergistic effect was defined as a combined effect that was greater than the arithmetical sum of the individual growth factor plus the mean value of the standard error of mean (SEM).[169]

Results of own studies

Chemotactic effects of growth factors (II,III)

Previous studies have focused on a single growth factor such as TGF-ß or PDGF when bone cells have been investigated for chemotactic responsiveness in rodent osteoblast culture.[74,170] The present studies indicate that numerous growth factors are able to stimulate osteoblast migration. In vivo, bone cell migration could therefore be regulated by combinations of growth factors rather than a single growth factor. TGF-ß and PDGF-BB, both of which are present in bone matrix, induced a significant chemotactic response in human osteoblasts (Figure 10). Both showed a fourfold stimulation of migrated cells and these growth factors probably serve as key regulators of osteoblast migration in bone tissue. Several other growth factors, PDGF-AA, IGF-1, IGF-2, and BMP-2, have less but nevertheless significant chemotactic effects on osteoblasts. Additionally BMP-2, has a strong chemotactic effect on osteoprogenitor cells, as demonstrated on the bone marrow stroma osteoblasts investigated in study (III).

Thus, the findings in our studies support that different growth factors could play a role in the regulation of osteoblast recruitment during both bone healing and bone remodeling. This is of major importance for bone physiology since migration of osteoblasts is a crucial event in both situations.

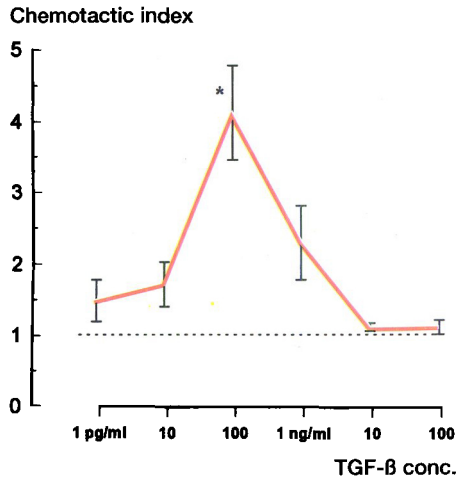


*Figure 10. Dose response curve of osteoblast chemotaxis to rhTGF-ß1. The chemotactic index (mean ± SEM) of six independent experiments is shown. *P = 0.01 for stimulated groups compared to control group.*

The chemotactic properties of TGF-ß, BMP-2 and PDGF-BB also make these growth factors promising candidates for use in growth factor induced osteogenesis due to the stimulation of recruitment of bone forming cells. TGF-ß has already been proven to possess bone-forming abilities in other in vivo studies.[37,171] The chemotactic abilities of TGF-ß combined with the antiinflammatory properties of TGF-ß [172] make this growth factor a possible inhibitor of inflammatory bone resorption as seen in diseases such as rheumatoid arthritis.

Effects of growth factor combinations on osteoblast metabolism (IV)

In study IV, the effects of growth factors both as single stimulation and in different combinations were investigated for effects on human osteoblast metabolism and chemotaxis. Metabolic effects were evaluated by determination of proliferation measured as DNA synthesis. State of differentiation was evaluated by alkaline phosphatase activity and collagen synthesis. Responsiveness to chemotactic stimuli was measured by cell migration in microwell chemotaxis chambers as in study II and III.

Proliferation is a metabolic activity, considered to be characteristic for the less differentiated osteoblasts, in order to obtain sufficient amounts of cells for the production of bone matrix components. This occurs in the early phases of healing response. We found, for the 10 ng/mL dose tested, that TGF-ß was most stimulative in single stimulation. For double combinations TGF-ß and PDGF-BB was the most stimulative combination with a 250% increase in DNA synthesis. Adding EGF to TGF-ß and PDGF-BB resulted to a further increase in DNA synthesis (Figure 11). The highly stimulative effect on proliferation by TGF-ß, PDGF-BB and EGF is physiologically interesting since platelets contain these three growth factors. In any traumatic bone lesion, a blood clot is formed and the disintegration of platelets in this blood clot will release these growth factors to the local environment, this leads to a high level of proliferative activity among osteoblasts close to the bone lesion. For stimulation of chemotaxis a similar pattern was found. However, for single growth factor stimulation, PDGF-BB was the most active. This was due

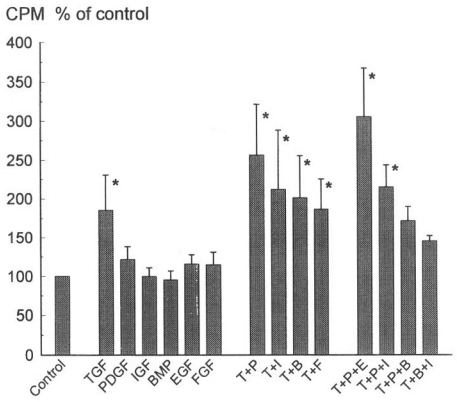


*Figure 11. Osteoblast cell proliferation assessed by [^{3}H]-thymidine incorporation into DNA. Results of the different growth factors and combinations are presented as percentage of control values (mean ± SEM) of six independent experiments. *P < 0.05 (Fisher test); ANOVA = 0.007. Abbreviations are T: TGF-ß, P: PDGF-BB, I: IGF-1, F: FGF-b, B: BMP-2, E: EGF*

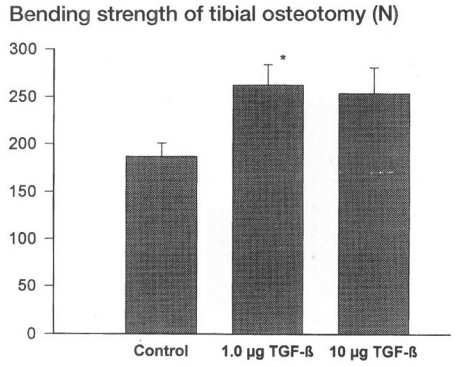


Figure 12. Effect of TGF-ß on maximal strength in 3 point bending test after 6 weeks of stimula tion. The group receiving only solvent (0 µg TGF-ß/day) serves as the control. The intact tibia group illustrates the strength of the uninjured tibia. Values are expressed as mean (SEM).

to non-optimal 10 ng/mL dose for TGF-ß which has its optimal chemotactic effect at 100 pg/mL (II). For the growth factor combinations, TGF-ß and PDGF-BB double and triple combinations, were the most stimulative, with osteoblast migration stimulated from 250–300% compared to control stimulation without growth factor. The finding that the growth factors contained in platelets also have powerful chemotactic effects is advantageous in the early phases of the bone healing process together with the similar proliferative effect.

The human osteoblast required vitamin D in order to be responsive to growth factor stimulation of alkaline phosphatase activity. Vitamin D serves as a differentiative factor that makes the heterogenic culture of human osteoblasts more homogenic with respect to a more highly differentiated state where enzyme activity is expressed. The exact function of alkaline phosphatase during bone healing is not fully established, but it is speculated that the enzyme catalyzes the chemical processes during initiation and maintenance of the mineralization process which occurs in the later phases of bone healing. Again, single stimulation and double combinations with TGF-ß and PDGF were the most stimulative, but interestingly, triple combinations containing TGF-ß and BMP exhibited the highest stimulative effects on alkaline phosphatase activity. BMP is found in bone matrix and may be released to the local environment after the early fracture healing phase, where the traumatized bone ends are remodeled and growth factors in the resorbed matrix will gain access to the local surroundings. Thus, the increase in BMP levels could trigger osteoblastic differentiation facilitating the initiation of mineralization.

We could not demonstrate any effects on collagen synthesis as a result of the growth factor stimulation neither as single stimulation nor in combination, despite, the fact that the osteoblasts were matured with vitamin D during the experiments. This finding in human osteoblast deviates from studies in rat osteoblasts where both TGF-ß, PDGF, IGF-1 and the combination of these growth factors were able to stimulate collagen synthesis.[92] However, the rat osteoblasts were derived from fetal tissues and, in comparison to the human osteoblasts, which were derived from adult donors, fetal cells are known to be far more responsive to growth factors than cells from adults. This difference in donor type could explain why bone matrix synthesis exhibits lack of responsiveness to growth factor stimulation.

Stimulation of fracture healing with TGF-ß (I)

A sixfold increase in calvarial bone thickness has been demonstrated in rats that were given local daily calvarial injections of 1 µg TGF-ß,[36] and a considerable stimulation of new bone and cartilage formation was found when 0.2 µg TGF-ß was injected subperiosteal in femurs of newborn rats.[10] We were able to demonstrate that TGF-ß could enhance the mechanical strength and stiffness of a tibial osteotomy after six weeks of local continuous application of 1 and 10 µg TGF-ß daily (Figure 12). This general stimulatory effect of TGF-ß on fracture healing might be ascribed

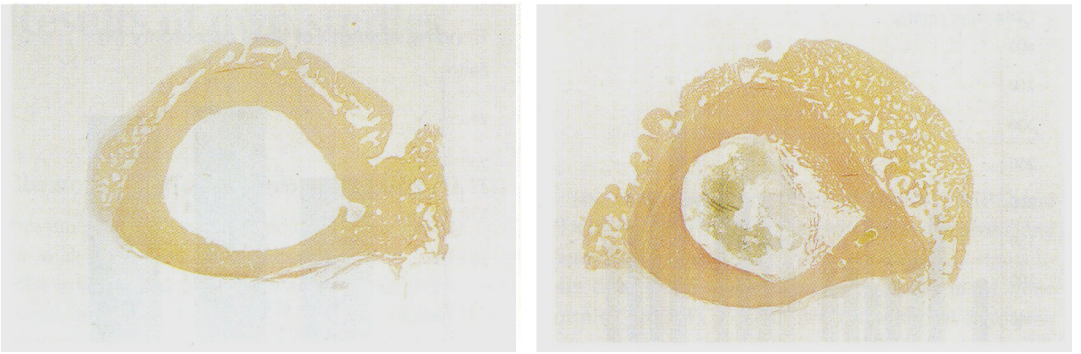


Figure 13. Decalcified transverse section 5 mm from the osteomy-line showing diaphyseal cortical bone and callus formation in a specimen from the control group (A) and 10 µg/day group (B). A moderate callus formation is seen in the control and massive callus formation in the stimulated specimen. The callus free area represents the AO-plate position (HE, x5)

to the stimulation of a larger callus formation around the osteotomy. The increased callus envelope around the diaphyseal bone-ends increases the mechanical strength of the osteotomy (Mark Bolander, Mayo Clinic, personal communication). We found an increased callus formation with increased doses of exogenous TGF-ß (Figure 13). Although callus formation was further enhanced by 10 µg TGF-ß/day compared to 1 µg TGF-ß/day, a greater mechanical strength was not demonstrated for increasing doses of TGF-ß. One explanation might be that the extensive callus formed at the highest TGF-ß dose was too immature to enhance the mechanical strength of the osteotomy, a phenomenon seen in other experiments (Steven Beck, Genentech, personal communication). The stimulation of callus formation found in the present study is in agreement with previous in vitro studies on callus tissue, which have shown stimulatory effect of several growth factors.[125,171,173] Our finding of increased strength and callus formation of the osteotomy supports the theory that TGF-ß stimulates both proliferation and differentiation of the cell types involved in the healing process of bone.[171,174]

We investigated remodeling in the cortical bone using the parameters cortical thickness and haversian canal diameter. No changes in these parameters were shown in the groups stimulated with TGF-ß. Thus, exogenous application to the osteotomy site did not demonstrate any effect on existing cortical bone remodeling. The lack of effect on the cortical bone remodeling could be due to the local application of the growth factor. The growth factor concentration in the cortical bone might therefore not be altered enough to change bone remodeling. Another explanation could be that cortical bone turnover generally is very slow compared to trabecular bone and healing woven bone or that the growth factor simply did not gain entrance to cortical bone tissue. Six weeks of growth factor application might therefore not be long enough to observe changes in cortical bone remodeling. Bone mineral content in callus tissue and cortical bone around the osteotomy was not altered in the groups stimulated with TGF-ß. This could be explained by the fact that the cortical bone contained the majority of mineral, and since cortical bone remodeling was not influenced by TGF-ß stimulation, a significant increase in bone mineral content could not be expected.

Effects of rhTGF-ß1 on bone healing to implants in dogs (V,VI,VII)

TGF-ß1 stimulation to unloaded TCP coated implants

In study V, the effect of 0.3 µg and 3.0 µg rhTGF-ß1 was investigated when adsorbed to TCP coated implants inserted unloaded into trabecular bone with a 2 mm gap. The observation period was 6 weeks and the material was skeletally mature dogs.

Mechanical fixation of the implants was enhanced in both rhTGF-ß1 stimulated groups. The 0.3 µg dose was the most efficient, improving shear strength almost threefold, whereas the 3.0 µg dose caused a two fold increase in mechanical fixation (Figure 14). Similar trends were found for stiffness and energy absorption. The greatest amount of bone ingrowth was also found in the 0.3 µg rhTGF-ß1 group, where bone ingrowth was increased from 25% in the control group to 41% in 0.3 µg rhTGF-ß1 group. (Figure 15). Interestingly total bone area in the gap was maximally stimulated in the 3.0 µg rhTGF-ß1 group where bone content in the gap was increased from 9% in the control group to 18% in 3.0 µg rhTGF-ß1 group (Figure 16). A group with titanium implants without TCP coating and rhTGF-ß1 adsorption was also included and these implants were virtually without any bone

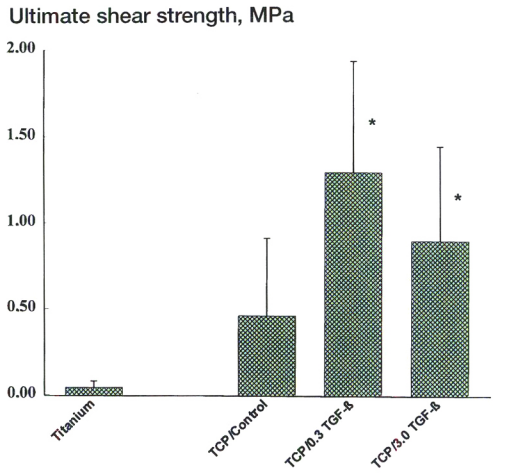


*Figure 14. Ultimate shear strength of the implant bone interface of unloaded TCP coated implants with and without rhTGF-ß1 stimulation as measured by push-out test six weeks postoperatively. *P < 0.05. The error bars indicate standard error of mean.*

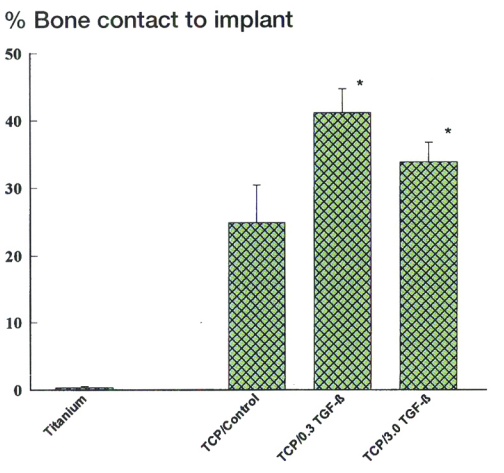
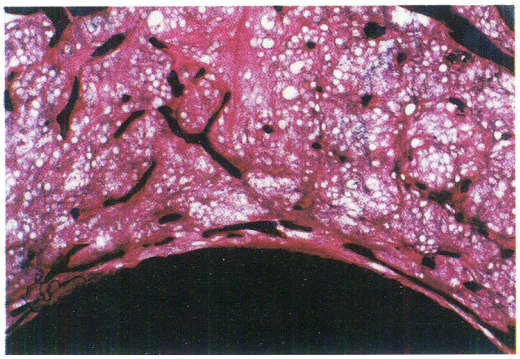


*Figure 15. Bone ongrowth to the implant surface of unloaded TCP coated implants with and without rhTGF-ß1 stimulation, as measured in percentage by histomorphometry 6 weeks postoperatively. *P < 0.05. The error bars indicate standard error of mean.*

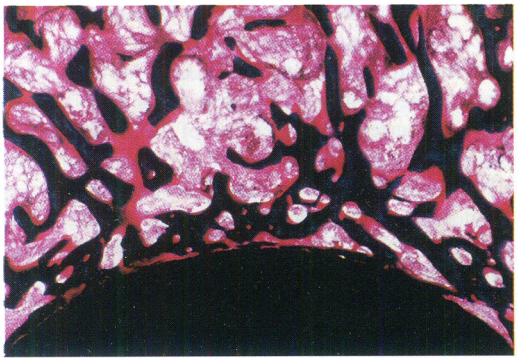
ingrowth and were thus very poorly fixated mechanically.

It is interesting to note that the lower dose of 0.3 µg rhTGF-ß1 proved to be the most efficient stimulator of bone healing. Previous studies have demonstrated stimulative effects of local TGF-ß application when a daily dose of 1–10 µg was administered (I).[36] An explanation for the high potency of rhTGF-ß in the presents studies could be that the ceramic coatings, TCP and HA, acts as a favorable carriers for the growth factor and thereby potentiates the biological activity. This phenomenon has been described previously in bone tissue after implantation of HA particles that were pre-incubated with TGF-ß.[135] A biological explanation could be that the growth factor is accessible to bone cells when adsorbed to the ceramic surface and might be protected from biodegradation as well. In a study by Sumner et al, which employed a design very similar to the present study, a 10-fold higher dose of rhTGF-ß was found to be most efficient for enhancement of bone ingrowth to ceramic coated implants with a porous surface.[126] However, the gap volume in this model was approximately 10 times greater than in our model, which could explain the difference in optimal rhTGF-ß1 dose. This could implicate problems for clinical application since the optimal dose for bone formation is dependent on the gap size.

Figure 16. TCP-coated implants showing bone formation in the gap (basic fuchsin and light green; original magnification x10).



A control implant without rhTGF-ß1 stimulation. Note the sparse new bone formation consisting of thin lamellae of woven bone.



An implant loaded with 3.0 µg rhTGF-ß1. Note the higher new bone density with thicker bone lamellae.

Bone ongrowth and mechanical fixation were optimally stimulated by the 0.3 µg rhTGF-ß1 dose, whereas bone formation in the gap was maximally stimulated by the 3.0 µg rhTGF-ß1 dose. It is interesting, why the increase in bone formation in the gap did not lead to an improved mechanical fixation. Previous in vitro studies have demonstrated that high doses of TGF-ß can inhibit mineralization.[175] It could be speculated, that the increased amount of woven bone in the gap in the 3.0 µg rhTGF-ß1 group is structurally more immature, so that mechanical properties of the bone deteriorate.

TGF-ß1 stimulation of weight loaded TCP coated implants

In study VI, the effect of 0.3 µg rhTGF-ß1 was investigated when adsorbed to TCP coated implants inserted weight loaded and intraarticularly surrounded by a 0.75 mm gap in skeletally mature dogs. The observation period was 6 weeks.

Bone ongrowth was increased 59% as a result of the rhTGF-ß1 stimulation, from 22% bone coverage of the implants in the control group to 36% in the rhTGF-ß1 group. Total bone volume in the gap was increased from 23% in the control group to 27% in the rhTGF-ß1 group, but this increase was not significantly different from the control. Also, mechanical fixation exhibited no difference between the control group and the rhTGF-ß1 group.

The stimulative effects of 0.3 µg rhTGF-ß1 was less pronounced in this weight loaded model than in the unloaded model (V). There can be different reasons for these findings. In the weight-loaded model, a smaller gap of 0.75 mm was used. In contrast to the 2 mm gap in unloaded model. This difference in the used experimental model causes less need for new bone formation, which probably is the major effect of rhTGF-ß1 and therefore bone ongrowth and mechanical fixation could be affected to a lesser extent by the rhTGF-ß1 stimulation. An other explanation could be that in the weight-loaded model, there is access of joint fluid to the implants interphase. This connection could cause a leakage of growth factor from the gap and a concurrent reduced effect of the growth factor. Also, a level of synovitis exists in the knee joint due to the surgical trauma and due to the protruding polyethylene plug, which contacts the tibial plateau during each gait cycle. The synovitis causes increased levels of inflammatory cytokines such as IL-1, IL-6 and TNF-α,[176] and these cytokines have been shown to be capable of abolishing the stimulatory effect of TGF-ß. Only bone ongrowth was significantly increased as a result of rhTGF-ß1 stimulation without any enhanced mechanical fixation of the implants. This could be due to the relative increase in bone ongrowth of 59%, which was considerably smaller than the 200% increase found in study V. The increase in bone ongrowth could be too small to result in any increase in mechanical fixation, since the mechanical test is a more crude analysis, in comparison to histomorphometry. Another explanation could be that the TCP ceramic was largely resorbed after the six week observation period, so that bone ongrowth to a porous remnant of TCP ceramic was frequently seen. The partly resorbed TCP ceramic probably has a poor contact to the implant surface and therefore it may be hypothesized that an increase in bone ongrowth to TCP ceramic does not necessarily lead to a corresponding increase in mechanical fixation.

TGF-ß1 stimulation to unloaded HA coated implants

In study VII, the effect of 0.3 µg and 3.0 µg rhTGF-ß1 when adsorbed to HA coated implants inserted unloaded with a 2 mm gap was investigated. The observation period was 6 weeks and the material was again skeletally mature dogs.

Bone ongrowth was increased 36% as a result of the 0.3 µg dose rhTGF-ß1 stimulation, but the 3.0 µg dose failed to enhance bone ongrowth. Total bone volume in the gap was increased 22% in the 3.0 µg rhTGF-ß1 group, but this increase was not significantly different from the control. Mechanical fixation exhibited minor increased values in rhTGF-ß1 groups, but none of these differences was found to be significantly different from the control groups.

The purpose of study VII was to elucidate whether or not rhTGF-ß1 could further enhance bone ongrowth and mechanical fixation in HA coated implants. This was of interest because HA ceramic coating on implants is known to stimulate bone ongrowth by bidirectional bone formation, where new bone is formed both from the surrounding trabecular bone and by new bone formation directly on the apatite layer. Also HA is known to improve mechanical fixation considerably due to a high chemical stability which leads to formation of strong HA-bone interfaces. We were able to demonstrate a significant increase of bone ongrowth amounting 36% relatively as a result of the 0.3 µg dose of rhTGF-ß1. This enhancement was smaller than when the similar dose was used on TCP ceramic; this finding is probably due to HA capability of forming bone by bidirectional formation. Higher levels of mechanical fixation were observed in the 0.3 µg rhTGF-ß1 group, but these increases did not reach significant levels despite the fact that they were of the same magnitude as the increase in bone ongrowth. This could be explained by the high abso-

lute levels for shear strength in the control group, which was 2.4 MPa, approximately 5 times higher than control levels for TCP coated implants. The strong fixation caused by HA coating makes it difficult for rhTGF-ß1 stimulation to result in a further enhanced mechanical fixation. We demonstrated a significant 36% increase in ongrowth but failed to demonstrate significance for a 41% increase in energy absorption after rhTGF-ß1 stimulation. This finding can probably be explained by the fact that the mechanical test is a more crude analysis than the histomorphometry.

General discussion

Basic cellular effects of growth factors

In the present in vitro studies, we investigated a large number of different growth factors with known metabolic effects on bone cells for their possible chemotactic effects on osteoblasts. We also investigated different growth factor combinations for effects on osteoblast metabolism.

In the chemotaxis studies, we found that several growth factors exhibited chemotactic effects on osteoblasts (II,III). Osteoblasts are likely to be affected by several growth factors locally in bone tissue, especially during bone healing and bone remodeling. Therefore, osteoblast migration and osteoblast recruitment to an area of bone healing might be regulated by several factors and probably by the synergistic chemotactic effects of some growth factors. TGF-ß and PDGF-BB were the most powerful chemoattracants in single stimulation but we also demonstrated a synergistic effect on osteoblast migration for the combination of these two growth factors (IV). This finding is consistent with studies performed in rodent osteoblasts and osteoblastic cell-lines with TGF-ß and PDGF-BB also have been demonstrated to stimulate chemotaxis.[73,74,170,177] For mature human osteoblasts the combination of TGF-ß and PDGF-BB could therefore be a very important regulator of osteoblast migration. Especially after a bone trauma, high levels of TGF-ß and PDGF-BB are present due to release from platelets and from the traumatized bone tissue. Thus, in the early phase of bone healing, these two growth factors could attract osteoblasts to the traumatized bone area.

In study III, we investigated the chemotactic effects of different bone morphogenetic proteins on different osteoblastic cell types. We found that less differentiated osteoblasts such as bone-marrow-derived osteoprogenitor cells were more responsive to BMP-2 than the mature human osteoblast. This could indicate that the state of osteoblast differentiation is important for chemotactic responsiveness of the osteoblasts. BMP-2 could therefore be an important regulator of migration for immature osteoblasts, whereas for mature osteoblasts BMP-2 has only moderate effects on migration at the same level as IGF's and PDGF-AA. Another indication that BMP-2 is important for immature osteoblasts is that these cells do not respond more to chemotactic stimuli from TGF-ß and PDGF-BB than mature osteoblasts (M. Lind, unpublished data). Previous studies have shown substantial effects of BMP's on osteoblastic stemcells cell-lines.[178,179]

In study IV, we investigated effects of growth factor combinations on human osteoblasts, not only on chemotaxis, but also on different parameters of metabolism, DNA synthesis, alkaline phosphatase activity, and collagen synthesis. Again, the platelet growth factors TGF-ß and PDGF-BB, both in single stimulation and in combination, had the most stimulative effects on DNA-synthesis and alkaline phosphatase activity. This further adds to the importance of growth factor release from blood platelets after bone trauma for the initiation and maintenance of the cellular processes in the healing response. Only a few studies have investigated growth factor combinations and their effects on osteoblast metabolism. Piche et al. demonstrated additive effects of PDGF-BB, TGF-ß and EGF on human osteoblast proliferation.[180] A study by Kasperk demonstrated that insulin growth factor II (IGF-II) acts synergistically with both basic fibroblast growth factor (bFGF) and TGF-ß on proliferation of rat calvaria osteoblasts.[169]

Interestingly, the addition of BMP-2 to other growth factor combinations further increased alkaline phosphatase activity. This effect of BMP-2 indicates an importance for differentiation of osteoblasts by BMP-2, especially in the presence of other growth factors. A problem for our growth factor combination study was that only one concentration of growth factor was tested and different effects might have been achieved if the growth factors were combined in different concentration. However, such studies would have been very extensive and that is why we chose one dose where the growth factors had a near maximal metabolic effect.

The application of in vitro data to in vivo physiological situations reveals several problems. In vitro studies typically employs outgrowth of a selected celltype. After considerable multiplication of cell number, the isolated cells are activated in a controlled manner and alterations in metabolic functions are measured. In vivo, a cell is positioned in functional matrix consisting of neighboring cells and intracellular matrix, which all can exhibit possible numerous effects on the celltype of interest. Therefore, can the found effects of the different growth factors on isolated osteoblasts not be expected to be found after exogenous application of the same growth factors to bone

tissue. In vitro investigations should, due to these limitations, only be used as screening technique, when growth factors for stimulation of bone healing are investigated.

In vivo stimulation of bone healing

The in vivo studies have demonstrated that TGF-ß, alone and in conjunction with ceramic coatings on implants, is able to enhance different aspects of bone healing in relevant situations for orthopedic surgery.

The continuous application of TGF-ß to a plated tibial osteotomy stimulated the callus bone formation to such a degree that the enlarged callus bridge caused increased mechanical strength of the osteotomy. The growth factor application in this situation primarily leads to increased osteogenesis, whereas the bone healing processes probably are not accelerated.

When rhTGF-ß1 was applied to the ceramic coatings of titanium implants we could demonstrate enhanced bone ongrowth to the implants in unloaded models with both TCP coatings and HA coatings and with weight-loaded implants coated with TCP. This again demonstrates the potent effects of rhTGF-ß1 on the osteogenetic processes, especially when it is considered that the ceramic coatings, and especially hydroxyapatite, have independent stimulatory effects on bone healing due to osteoconductive properties. This is consistent with the only other study which has used growth factor stimulation of bone healing to implants surrounded by a gap. Here increased bone formation and bone ingrowth were found.[126] Two other studies have used dental extraction cavities for press-fit insertion of implant stimulated either with a combination of IGF-1 and PDGF-bb or with BMP-7. In both studies increased bone ingrowth were found.[76,181]

Hydroxyapatite coatings and weight loading are known to stimulate bone healing to implants.[142,182] In models where these stimulative factors were included, we could only demonstrate moderate increased bone ongrowth to the implants as a result of the rhTGF-ß1 stimulation. The relative increase in bone ongrowth in these models was not of a sufficient magnitude to cause increased mechanical fixation of the implant. This might be explained by the fact that the mechanical test is a more crude method compared to histomorphometry due to relative higher standard deviations, and that the modest increase in bone ongrowth found in the present study might be to small to result in statistically significant enhanced mechanical fixation. Histomorphometry and mechanical tests were performed on two adjacent but separate sections, and it could be speculated that this also could explain the lack of correlation between the two tests. However, several previous studies using the same methods have shown correlation between histomorphometry and mechanical parameters.[182,183] For weight unloaded TCP coated implants, however, a 100% increase in bone ongrowth was observed and this lead to a significant increase in mechanical fixation of the implant.

Apart from the demonstrated stimulatory effects on osteogenesis in callus tissue and in woven bone in the gap around implants, we examined in study I effects of local TGF-ß application on the bone turnover in the adjacent preexisting bone tissue. In study I, we could not demonstrate any alterations in diaphyseal bone remodeling close to the osteotomy site. This result point towards a very localized effect on the bone healing processes during new bone formation and very little if any effect on preexisting bone tissue when local TGF-ß application is used. This finding indicates that local TGF-ß application is a safe method for enhancement of bone healing and formation since no side effects on preexisting bone were demonstrated.

Possible clinical applications of growth factors—gains and problems

The growth factors from the TGF-ß superfamily, BMP's and TGF-ß, have demonstrated potent stimulatory capability for bone-healing and bone formation in vivo. The major problem for clinical use of these growth factors is appropriate delivery systems to ensure sufficient biological activity for optimal effects on bone-healing and formation. BMP's have very little biological effect in solution and a carrier is needed for adequate in vivo activity. Demineralized bone matrix, collagen matrix, hydroxyapatite ceramic and various polysaccharide matrices are some of the known effective carriers for BMP's.[184] The autoinductive capacity of BMP's for bone formation makes this group of growth factors ideal for bone-filling of large bony defects. BMP's could therefore successfully be used to fill large bone cavities after tumor resections and to facilitate bony healing of pseudarthroses or complicated long bone fractures, where bone loss can compromise normal healing. For these purposes, BMP can be used alone in an appropriate carrier or as an adjuvant to a biological or non-biological graft material if initial mechanical stability is desired. Autologous and allogenic bone graft material is extensively used in orthopedic surgery, and especially for spinal surgery. The use of autologous bone has several disadvantages; these include additional postoperative pain, long-term discomfort from the donor site, limited resources and amounts and finally prolonged operative time. For allogenic bone grafts, risks of in-

fection exist. In the future, the use of bone grafting could probably be reduced considerably by the use of growth factors in collagenous or other matrices. Animal experiments have demonstrated that BMP's can form bone to the same extent as autologous bone for spine fusions in dogs.[67]

TGF-ß appears to have biological activity without the same carrier requirements as the BMP's. Additionally, TGF-ß has biological activity at much lower doses than BMP's. TGF-ß exerts its optimal effects on bone-healing at µg levels where as BMP's is required in mg levels. The low dosage requirements has made it possible to adsorb TGF-ß to ceramic coatings of implants and to ceramic beads that can stimulate bone-healing of defects.[124,126] This delivery method makes it possible to produce ceramic coated cementless prosthetic components, which have TGF-ß adsorbed to the ceramic surface for enhanced bone ingrowth. This new technique could become a breakthrough for cementless endoprostheses, which until now have failed to prove their superiority to cemented techniques. For revision arthroplasties, both TGF-ß and BMP's could be used to enhance bone formation in the compacted bone graft where the revision implant is placed, cemented or uncemented, in a sheath of packed bone powder. However concern exist, to what ceramic coating and implant surface texture that should be used with the growth factor. The smooth surfaced TCP coated implant certainly has so poor mechanical anchorage compared to HA coated implants that clinical usage should be avoided. We have used implants without porous coating for all implant studies. In the clinical situation, only porous coated implants are used for endoprosthetic components. A possible future clinical usage of growth factors for improved implant healing is therefore dependent on demonstration of enhanced bone ingrowth and mechanical fixation when growth factors are used with porous coated implants in preclinical animal studies.

The biological state of the bone tissue which is stimulated with growth might be important for any stimulatory effects. Ours and others studies have been performed in healthy bone with good vascularization. It should be speculated that impaired vascularization and poor bone quality both could reduce the stimulatory effects of growth factors. Additional studies are needed to answer these questions.

At present, only preclinical experimental data exist concerning in vivo growth factor effects in clinically related animal models. An increasing number of positive results from such studies exist which could indicate a possible future success for growth factors within clinical orthopedic practice (I,V).[12,67,126] However, it is not known whether or not the results from the animal studies can be transferred to humans with equal success. It is probable that a large number of studies have failed to demonstrate positive effects of growth factors on bone healing and many of these studies are not presented in the literature.[96,123] This is mainly due to the high sensitivity of growth factor stimulation to proper delivery systems and correct doses. A few studies has indicated possible side effects of short-term local growth factor stimulation. Healing of defects in dogs have demonstrated heterotopic bone formation and bone-cyst formation when high doses of rhBMP-2 were used.[185] In rabbits subjected to subperiosteal injections of rhTGF-ß2, bone stimulatory effects were present only in neonatal animals and oedema was evident in surrounding connective tissues.[186] Finally, growth factors could have long-term side effects that first will be revealed several decades into the future. BMP's are expressed by some tumor cell lines and this has caused concern that BMP treatment could be carcinogenic. No data that relating to this problem exist today.

Commercial interest for an orthopedic use of growth factors is currently focused on the BMP's. Two companies, Genetics Institute (who developed and now promote BMP-2) and Creative Biomolecules (who promote BMP-7, also designated Osteogenic protein-1) have initiated clinical trials for the use of BMP's in defect healing and for spine fusions. The first data from these clinical trials will probably be presented in the near future and BMP's will likely be commercially available within a few years. The use of growth factors will open a new set of treatment modalities which ideally will enable the surgeon to obtain improved bone formation and bone-healing in situations where the natural healing capacity of bone tissue is inadequate for proper clinical results. The coming decade will hopefully define indications for this new clinical tool in orthopedic surgery.

Conclusions

I) Continuous application of 1 and 10 µg natural TGF-ß to a plated tibial osteotomy in rabbits caused increased mechanical bending strength and callus formation after 6 weeks observation time. Diaphyseal cortical bone remodeling was not affected by the local growth factor application.

II) Several growth factors exhibited chemotactic effects towards human osteoblasts. TGF-ß1 and PDGF-BB had the strongest chemotactic effects with their ability to increase migration up to 4 times the control levels. Also PDGF-AA, IGF-1, and IGF-2 had chemotactic effects and stimulated migration up to 2

times the control levels. TGF-ß1 exhibited the highest chemotactic potency with maximal activity at 100 pg/mL, whereas the other growth factors had maximal effects at 10–100 ng/mL.

III) BMP-2 was found to have chemotactic effects toward human osteoblasts, human bone marrow osteoprogenitor cells, and U2-OS osteosarcoma cells. BMP-4 and BMP-6 were without any chemotactic effects towards these celltypes. Human bone marrow osteoprogenitor cells were the most responsive celltype to BMP-2 stimulation, with migration stimulated 3 times in comparison to control levels.

IV) Combinations of growth factors resulted in synergistic stimulative effects on different metabolic functions on human osteoblasts. Combinations with TGF-ß1 and PDGF-BB strongly stimulate proliferation and chemotaxis. Combinations with TGF-ß1, PDGF-BB and BMP-2 strongly stimulate a differentiation parameter (alkaline phosphatase activity). The different growth factor combinations had no effect on collagen synthesis in human osteoblasts.

V) 0.3 µg rhTGF-ß1, adsorbed to gritblasted tricalcium phosphate coated unloaded implants in skeletally mature dogs, was able to enhance mechanical fixation 3-fold and bone ingrowth and gap bone formation 2-fold. 3.0 µg rhTGF-ß1 had also stimulative effect, but was less effective than the 0.3 µg rhTGF-ß1 dose except for gap bone formation, which exhibited the highest stimulation for the high dose of rhTGF-ß1. The establishment of a biological implant fixation concept with growth factor absorbed to ceramic coatings of implants was successful.

VI) 0.3 µg rhTGF-ß1, adsorbed to weight loaded gritblasted tricalcium phosphate coated implants was able to enhance bone ingrowth, without enhancement of mechanical fixation.

VII) 0.3 µg rhTGF-ß1, adsorbed to unloaded gritblasted hydroxyapatite coated implants was able to enhance bone ingrowth, without enhancement of mechanical fixation. 3.0 µg rhTGF-ß1 had no stimulative effects.

Future research

Results of the studies described in this thesis and results from other studies suggest that growth factors can be used as in vitro and in vivo stimulators of new bone formation if correct dose and application method is used. Future in vivo research should focus on models with clinical relevance.

We have only used TGF-ß for our in vivo studies. A subfamily of growth factors in the TGF-ß superfamily, namely the BMP's, have demonstrated substantial capacity for stimulation of bone formation when applied locally in bone defects and as a substitute for bone graft for spine fusion. It would be very interesting to investigate BMP's in the implant models described in this thesis.

Combinations of growth factors is another approach which could be advantageous for enhanced ingrowth. By combining growth factors, different properties of the individual growth factor can be used for accelerated bone formation. As demonstrated in study IV, combinations of growth factor can elicit synergistic effects on different metabolic functions. For example, the mitogenic actions of TGF-ß and PDGF can be combined with osteoinductive properties of BMP's and the angiogenic properties of FGF's.

We have used implants without porous coating for all implant studies. In the clinical situation, only porous coated implants are used for endoprosthetic components. Preclinical animal studies with growth stimulation of porous coated implants is therefore needed.

An animal model with a full hip prosthesis would be the ultimate clinically relevant model to investigate the possible future use of growth factors for enhancement of bone ingrowth and fixation to prosthetic components. In such a model it would be possible to quantitate migration of the implant by roentgen stereophotogrammetric analysis to test long-term stability. Implant migration has been shown to be a predictor of long-term survival of a prothesis using this technique.

In the present studies we have focused on primary bone healing to implants. Clinically, bone healing during the prosthesis revision situation is more vulnerable due to the lack of bone stock and the inflammatory osteolytic environment caused by implant micromotion and particulate debris. Growth factor enhanced bone healing in this situation could clearly be advantageous and the recent development of implant revision models makes this an interesting objective for future studies.[187]

Stemcell mediated stimulation of bone ingrowth is another principle that could be utilized for enhanced bone ingrowth to implants. Bone marrow is a natural source of osteogenic cells and freshly aspirated bone marrow could be placed in a gap around implants where the excessive osteoprogenitor cells in the marrow could contribute to accelerated bone formation during the healing response. Another principle could be to culture the osteoprogenitor cells from the bone marrow in vitro, and then apply the multiplicated cells in the gap around the implants: these cells could possess a significant bone forming capacity. Finally, the principle of gene therapy could be used to transfer growth factor genes into osteoprogenitor cells, which then can over express growth factors and thereby functions as cellular growth factor factories.

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