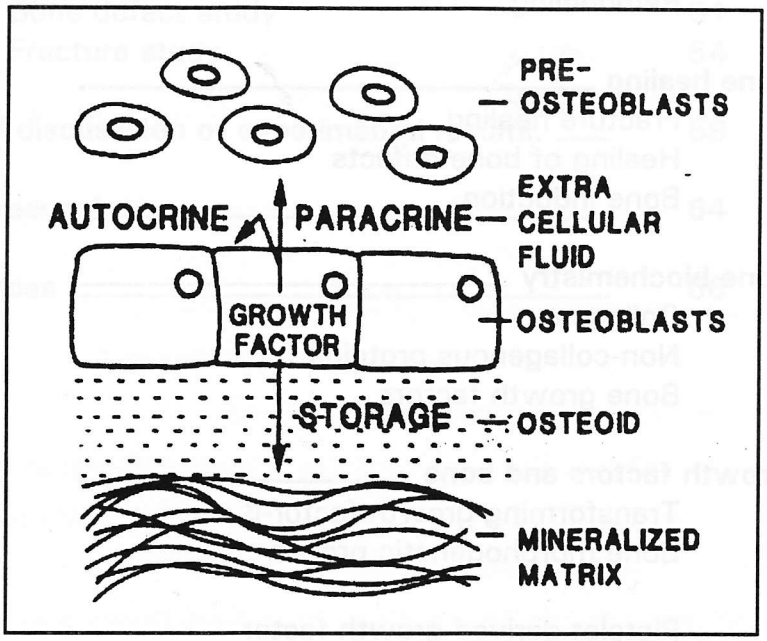
In vivo and in vitro stimulation of bone formation with local growth factors

Ph.D. Thesis



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PREFACE

This thesis is based upon the investigations performed during my research scholarships and fellowships at the Orthopedic hospital and the Institute of Experimental Clinical Research, University of Aarhus over the period from 1992 to 1994. I would like to express my gratitude my two supervisors Professor *Cody Bünger* M.D. D.M.Sc and Orthopedic Surgeon *Kjeld Søballe* M.D. D.M.Sc, for their introduction into the field of orthopedic research and for their belief and enthusiasm for my ideas during periods of success and disappointment. They are also thanked for their ever friendly atmosphere during work and at travels round the would.
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Definitions and abbreviations

BMP	Bone Morphogenetic Protein
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 for the immunological system.
Growth factor	Peptide regulator molecule that regulate cell to cell metabolism and with generally stimulative effects. Some growth factors acts as cytokines and some cytokines act as growth factors.
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked ImmunoSorbent Assay
IGF	Insulin Like Growth Factor
Northern Blot	Visualization of specific mRNA's by electroforetical separation of mRNA, blotting to nylon membranes and detection by specific radioactive labeled probes.
Osteoblast	The metabolic active bone forming cell. Derived from the mesenchymal cell lineage.
Osteoclast	The cell capable of bone resorption. Derived from the hematopoietic monocytical cell lineage.
PDGF	Platelet Derived Growth Factor
RIA	Radio Immuno Assay
TGF-ß	Transforming Growth Factor Beta

CHAPTER 1

GENERAL INTRODUCTION AND AIM OF STUDY

Inadequate bone healing is a problem in several clinical situations in orthopedic surgery. Complicated fractures often heal insufficiently with formation of pseudarthroses that are difficult to treat surgically and therefore causes great distress for the patients. Surgical treatment of bone tumors often leave large defects that heals inadequately. Total joint replacements have been a major success in orthopedic surgery, but late loosening of the prosthetic components is a problem for active young patients who need longtime survival of their prosthesis and for patients with poor bone quality such as patients with osteoporosis and rheumatoid arthritis. Stimulation of local bone healing and formation could improve the clinical results in the above mentioned situations.

Biochemical stimulation of bone healing is one approach to the problems and this concept has become increasingly relevant with the discovery of peptide regulator molecules called growth factors more than a decade ago. Growth factors has been found in all tissues and are today known to regulate local cell to cell metabolism and to mediate cellular effects of different hormones.

Bone matrix has been found to be a large reservoir for numerous growth factors that has been suggested as regulators of bone remodeling and initiators of the bone healing process. In vitro studies has documented that the bone growth factors exert numerous regulating effects on bone cells and in vivo studies have shown that a few growth factors can stimulate bone healing in animals. These result are promising for the future use of growth factors as stimulators of bone formation and healing in clinical situations.

This thesis will review bone cell biology, bone biochemistry, and especially the effects of growth factors on bone cells and bone tissue. The experiments performed during the Ph.D. study aimed to investigate in vitro effects of bone growth factors and to investigate the ability of transforming growth factor beta (TGF-ß) to stimulate bone healing in clinically relevant experimental animal models.

CHAPTER 2

BONE HEALING AND BONE INDUCTION: A SHORT HISTORICAL REVIEW.

Since the days of *Hippocrates* 500 B.C. in ancient Greece, it has been recognized that bone has considerable potential for regeneration and repair. Hippocrates is also credited with the concept that naturally occurring endogenous substances are superior therapeutic agents for clinical use [196].

Galen (130 -201 A.D.) gained experience of bone healing as a physian for the gladiator school in Pergamon. He postulated the existence of a "Succus ossificus" leaking from fractured bone ends and stiffening into the stabilizing fracture callus. Although Galen thought that the fracture callus was a lifeless substance, the knowledge of today supports the theory of "succus ossificus" where we now know that growth factors and other bioactive substances released from the fracture hematoma and fractured bone ends are of major importance for initiation and maintenance of the bone repair process.

More than a century ago, Senn described the use of decalcified bone implants as stimulators of bone defect healing in osteomyelitis and bone deformities [220].

In 1965 *Urist* discovered that when he implanted demineralized bone matrix subcutaneously new bone formation was induced [238]. This finding gave rise to the theory, that bone matrix contained a substance with bone inducing capability. This substance was named bone morphogenic protein. Later Urist was able to extract and partly purify a protein with the ability to form organized bone at ectopic locations [240]. And in 1988 *Wozney* was able to identify 7 different bone morphogenic proteins by the use of novel molecular biology techniques [251].

During the last two decades numerous proteins with stimulative effects on different tissues and cell types have been found. These proteins are called growth factors. Several of these growth factors are able to stimulate bone cells growth and metabolism in vitro. The growth factor Transforming Growth Factor- beta was in 1989 found to be able to induce significant increase of bone formation in rat calvaria [172]. Several other growth factors have been shown to exhibit stimulative effects on bone cells and bone tissue and will be reviewed in chapter 6 later in this thesis.

CHAPTER 3

BONE CELLS AND BONE REMODELING

OSTEOBLASTS

Osteoblasts are the bone forming cells; they are cuboidal plump cells that are organized in layers and act in a coordinated fashion to generate the bone matrix, which subsequently proceeds to mineralize extracellularly. Osteoblasts work together to produce the characteristic lamellar structure of bone.

Osteoblasts originate from mesenchymal stem cells, which can also give rise to cartilage, muscle and fat cells, tendon fibroblasts and possibly other types of fibroblasts [88]. The periosteum contains cells committed to the osteogenic and chondrogenic phenotype, since periosteal bone formation continues throughout life. Following fracture, the periosteum gives rise to cartilaginous callus, that develop into mature bone through endochondral ossification. Bone precursor cells are also found in the endosteum and the bone marrow. It is shown that stromal cells from the marrow produce bone when explanted in diffusion chambers in animals [179].

Differentiation is a stepvise process, during which the cells start producing characteristic products of the osteoblasts. These products include all the constituents of bone matrix, such as type 1 collagen, which is the major matrix constituent, and noncollagenous proteins, such as the bone specific osteocalcin, osteopontin, osteonectin, bone sialoprotein, biglycan, decorin, matrix gla-protein and bone acidic glycoprotein [215]. The cell membrane of the osteoblasts is very rich in alkaline phosphatase, which participate in the mineralization process. All these proteins are produced by the differentiated osteoblast during bone formation, and some of them spill over into the blood and can be used as markers of bone formation. Serum levels of osteocalcin and alkaline phosphatase are examples of such markers used both in research and in clinical situations.

Osteoblasts deposit 0.5 μm matrix pr day and their formative period lasts about 100 days [71]. Some of the osteoblast are buried within the matrix and are then called osteocytes. Others become flattened cells on the surface of bone and are called lining cells. The lining cells are osteoblasts in a state of activity quiescence. Osteocytes communicate with each other through projections, which join in gab junctions thus creating network of cellular communication across bone. Osteoblasts take part in both bone metabolism and bone remodeling. The precise mechanisms, which control these events are still not understood in detail, but osteoblasts are known to respond to systemic hormones and locally produced growth factors.

Osteoblasts respond to bone resorptive stimuli including parathyroid hormone, 1,25-vitamin D_3 and prostagladins [192]. The bone resorptive cytokines interleukin-1 and 6, tumor necrosis factor and lipopolysaccharides also acts on osteoblasts to inhibit proliferation and protein synthesis. But their main effect on bone tissue is stimulation of osteoclastic activity. Osteoblasts respond to a significant number of growth factors and also produce some of them, such as TGF-ß, IGF-I and IGF-II. This is probably an important aspect in autocrine/paracrine regulation of bone formation during bone remodeling, that could explain the coordinated activity of groups of osteoblasts during bone formation.

OSTEOCLASTS.

The cells which carry out bone resorption are the osteoclasts. Osteoclast originate from the pluripotent stemcells of the bone marrow, which generates all the blood cells. They are probably related to the macrophage/monocyte lineage [75]. When bone marrow cells are cultured in vitro, cells occur with osteoclastic characteristics. These cells are multinucleated, contain tartrate resistant alkaline phosphatase, have calcitonin receptors and resorb bone when seeded on bone slices in culture [94,221,233]. Microscopic examination of osteoclasts shows that resorption is carried out by a special surface of the osteoclast. Electron micrographs shows that this section of the membrane is in close contact to the mineralized surface of the bone. This membrane is highly convoluted, and forms a so called ruffled border. At the edge of the membrane, there is a ring a of membrane known as the sealing zone, which adheres tightly to the bone surface and seals off the resorption space. This results in a closed space inside which the bone resorption takes place. The osteoclasts create a special chemical environment that facilitates bone resorption. The pH is acid around 4 which dissolves the mineral [16]. Lysosomal enzymes that work at low pH degrade the matrix. The membrane-seal is accomplished by special membrane receptors called integrins that bind to extracellular matrix proteins of the bone surface [216]. Enzymes are required for maintaining the acid-base balance and the proton requirements during bone resorption. Carbonic anhydrase that produce protons from bicarbonate, is also very abundant in osteoclasts. [92]. In addition osteoclasts also have abundant sodium potassium ATPase [17].

The resorption process produces several products of degradation of the bone matrix. These degradation products can be used as biochemical markers of bone resorption. Hydroxyproline excretion in the urine is proportional to collagen breakdown and, since most of body collagen is situated in bone, this measure provides an estimate of bone resorption. An even better estimate is provided by hydroxy- or deoxy-

pyridinoline, an aminoacid crosslink of collagen that can be determined in urine by reverse phase HPLC [31].

BONE REMODELING

Regulation of bone volume is maintained through a continuous balance between its formation and destruction. Bone remodeling occurs in anatomically distinct foci that remain active for 4-8 month. Two sets of mechanisms control bone remodeling : systemic regulation by calcium and phosphate-regulating hormones and local regulation. The systemic hormones primarily affect osteoclastic activity, whereas osteoblastic activity is regulated mainly by local factors. The local regulation involves the actions of growth factors that act as autocrine and paracrine effectors of bone formation by stimulating osteoblast proliferation and matrix protein synthesis. Also cytokines stimulate osteoclastic differentiation and activity. The remodeling sequence is effectuated by a set of osteoblasts and osteoclasts and their precursors that form a bone physiologic entity called "Basic Multicellular Unit (BMU)"[79]. These different bone cells work as a team to accomplish the main bone physiological events which is primarily bone remodeling but also bone healing and bone modeling. In remodeling the BMU undergoes a set of phases: Activation, Resorption and Formation, the A-R-F sequence.

A: Activation is the preparation of a certain small area of bone surface for osteoclastic resorption. It includes migration of osteoclasts to the bone area and activation of resorption activity.

R: When osteoclasts have been attracted to the bone area they adhere tightly to the exposed bone surface through specific cell-membrane proteins called integrins. They thereby seal off a closed space enabling formation of a local environment with low pH and high concentration of lysozymes. The osteoclast erode a cavity with a depth of 70 μm called Howships lacunae in trabecular bone and the Cutting cone in cortical bone [71,180].

F: Bone formation is initiated by migration of osteoblast and osteoblast precursors to the resorption cavity. The osteoblasts are activated by local factors that ensure proper mitogenic activity, correct cell arrangement and stimulate matrix protein synthesis.

Quiescence phase: 80 % of trabecular bone surface and 95 % of inner cortical bone surface are inactive with no remodeling taking place. During this quiescence phase the bone surface is covered by a thin layer of inactive osteoblasts called lining cells.

CHAPTER 4.

BONE HEALING

BONE REPAIR (FRACTURE HEALING)

Under optimal conditions, bone tissue has the capability of repairing an injury without scar tissue formation. Fracture healing is a typical example this process and a brief summary of bone repair during fracture healing will here be given.

The process of bone repair can be divided into three sequential phases.

Inflammatory phase. Following accumulation and coagulation of hematoma within the fracture space an acute inflammatory response occurs with vasodilatation and exudation of plasma and leukocytes will migrate to the area followed by macrophages.

Reparative phase. In this phase the fracture hematoma is invaded by fibrovascular tissue (revascularization) which replaces the hematoma. Devitalized necrotic bone is removed by recruited osteoclasts. Mesenchymal cells probably already determined for osteoblastic and chondroblastic differentiation are located at the periosteum and endosteum. These cells further differentiate and proliferate stimulated by local factors released during the initial healing process. This creates a fibrous and cartilaginous callus that envelops the bone ends and increases the mechanical stability of the fracture fragments. The callus is subsequently replaced by woven bone formed by intramembranous or endochondral ossification [217]. *Remodeling phase.* Following woven bone formation, an internal organization (remodeling) starts where new lamellar bone is formed with a functional organization that eventually brings the mechanical strength and geometry of the fractured bone back to that of uninjured bone.

If a fracture is stable and undislocated and with bone fragments at very close approximation *primary bone healing* take place. This situation rarely occurs in nature but is the goal of different types of surgical osteosynthesis techniques. Bone is here formed within weeks from a collagenous matrix laid down by osteoblasts and fibroblasts with a minimum of external callus. If fracture ends are subjected to compression with a gab less than 10 μm union can occur by direct Haversian remodeling [4].

The inflammatory phase take place during the first few days of an injury or fracture. The reparative phase with formation of woven bone occurs after two weeks and the remodeling phase begins after 4 weeks [80,232]. A complete repair with structural

normalization occurs as soon as 6 to 8 months [80].

HEALING OF BONE DEFECTS (GAP HEALING).

Healing of cortical bone defects is very different from fracture healing [155]. Small holes may be integrated in micro repair phenomenon which may be a part of normal bone remodeling. Healing of larger bone defects occurs via endochondral ossification with a cartilaginous phase which is later replaced by bone [155]. In a study by Johner [111], healing of cortical bore holes were investigated in rabbits. Holes of 200 μm were filled exclusively by lamellar bone. Larger holes of 400 μm was initially filled with woven bone [111]. Healing of holes greater than 1 mm in diameter is considerably delayed, because woven bone cannot bridge the gap but has to be formed by endochondral ossification with an initial cartilaginous phase before bone is formed [217].

BONE INDUCTION

Already a century ago Senn [220] described the use of decalcified bone implants in the healing of bone defects. In 1965 Urist made the discovery that demineralized lyophilized bone matrix was able to induce new bone formation when placed ectopically, indicating the osteoinductive potential of bone matrix components [238]. These components first to be identified in the eighties were called Bone Morphogenetic Proteins [239] and they are described in detail later in the chapter on bone growth factors.

Bone induction is a multi step cascade involving migration, mitosis and differentiation of cells that participate in the bone formation process [195]. The cellular and biochemical changes during bone induction have been well investigated by implantation of demineralized bone matrix [194]. (Table 4.1 describes this process with regard to time, cellular events, molecular processes and growth factors involved.)

Table 4.1.

MULTI STEP PROCESS OF BONE INDUCTION

TIME AFTER IMPLANTATION	CELLULAR EVENTS	MOLECULAR EVENTS	GROWTH FACTORS
1 min	Blood clot formation Platelet release	Fibrin network formation Binding of fibronectin to bone matrix.	Release of PDGF
1 hour	Arrival of polymorphonuclear leukocytes (PMN) by chemotaxis.	Release of proteolytic enzymes collagenase and elastase. Release of collagenous peptides	IL-1, IL-6
3-18 hours	Accumulation of PMN Adhesion of cells	Proteolysis and release of chemotactic factor for Fibroblasts.	PDGF and TGF-ß
Day 1	Chemotaxis of fibroblasts Cell attachment to implanted bone matrix.	Release of fibronectin Increased cell motility	PDGF and TGF-ß
Day 2	Continued fibroblast chemotaxis. Signal transduction from matrix to cell surface.	Initiation of protein and DNA synthesis Release of growth factor	TGF-ß, BMP, IGF-II.
Day 3	Cell proliferation	DNA synthesis Collagen type III synthesis	TGF-ß, BMP, IGF-II.
Day 5	Differentiation of chondroblasts	Proteoglycan synthesis	BMP and TGF-ß.
Day 7	Synthesis and secretion of matrix by chondrocytes	Collagen Type II synthesis Cartilage specific proteoglycan synthesis.	BMP and TGF-ß
Day 9	Hypertrophy of chondrocytes.	Calcium accumulation Alkaline phosphatase activity.	TGF-ß
	Vascular invasion	Type IV collagen synthesis laminin synthesis.	unknown
Day 10-12	Osteoblasts: bone formation and mineralization.	Type I collagen synthesis Bone proteoglycan synthesis Peak Ca incorporation	BMP and TGF-ß
day 12-18	Osteoclast: bone remodeling Dissolving of matrix	Increase in lysomal enzymes Accumulation of osteocalcin	IL-1, IL-6 and TNF. TGF-ß
Day 21-	Bone marrow differentiation	Increase in Fe in heme. Type III collagen synthesis	Colony stimulating factors

Modified from Reddi [196].

CELLULAR EVENTS

Chemotaxis in the early phases of bone induction may be defined as the directed migration of cells in response to a chemical gradient. Implantation of demineralized bone matrix promotes chemotaxis of cells to the matrix. Plasma fibronectin binds extensively to the implanted bone matrix [245]. Fibronectin has a high affinity for collagen, fibrin and heparin all of which are major components released in case of any skeletal trauma. Fibronectin is also chemotactic and perhaps mitogenic and probably attract bone precursor cells. The next major phase of bone induction is mitosis. Proliferation of newly attached mesenchymal cells indicates that the bone matrix is a local mitogen [193]. The mitogenic phase of bone induction is followed by the differentiation of cartilage, vascular invasion and bone differentiation. By day 3, most leukocytes have disappeared, and numerous elongated fibroblast-like mesenchymal cells appear in close proximity to the implanted matrix and proliferate. Matrix to cell interaction results in the transformation of mesenchymal cells into chondroblasts on day 5, and numerous chondrocytes are present on days 7 to 8. After capillary invasion on day 9, chondrocytes hypertrophy and the first signs of mineral formation are seen in the matrix of the hypertrophied chondrocytes. By day 10 to 11 numerous multinucleated chondroclasts appear close to the regions of chondrolysis. Concurrently, osteoblast appear close to the vascular endothelium, and new bone is formed by appositional growth on the surface of the calcified matrix and the implanted non-living matrix. Multinucleated osteoclast with the characteristic ruffled border remodel the newly formed bone by day 12 to 18, resulting in the selective dissolution of implanted matrix and the formation of an ossicle consisting essentially of the newly formed bone. Between day 16 to 21, there is further remodeling of bone and the roughly ovoid ossicle is filled almost entirely with bone marrow elements. [196]

CHAPTER 5

BONE BIOCHEMISTRY

Bone consist of cells and extracellular matrix, the latter comprises of 35 % organic and 65 % inorganic components [144]. The inorganic components are mainly calcium and phosphate as hydroxyapatite crystallites [$Ca_{10}(PO_4)_6(OH)_2$] and other calcium/phosphate compounds. The bone calcium comprises more than 99 % of total body calcium. Also Sodium and Magnesium are stored in small amounts in bone. The organic components of bone matrix are traditionally divided into collagen and non-collagenous proteins. (Table 5.1)

COLLAGEN

Type I collagen constitutes more than 90 % of the organic material in bone matrix. Also human osteoblasts produces virtually only type I collagen [206]. Collagen molecules are formed by the assembly of three polypeptide chains, the α1 and α2 chains. These chains are folded into rod-like triple helical molecules 300 nm long. In bone matrix the collagen molecules are packed end to end with small gaps between them. These gabs probably provides sites for hydroxyapatite deposition during mineralization. Small amounts of other collagen types are also present in bone matrix. Collagen type V, VII and XII are associated with type I fibrils, where they control fibril linkage and spatial dimensions [25,86]. Type VI collagen mediates attachment of cells to type I collagen [58].

Markers of collagen metabolism are used as determinants for bone formation both in vivo and in vitro. Collagen type I is synthesized from a precursor molecule called pro-collagen type I, where the C-terminal and N-terminal propeptides are split off before the collagen molecule are incorporated into the extracellular matrix. The procollagen C-terminal propeptide (PICP) are secreted into supernatant medium in vitro and into serum in vivo and can be detected by radioimmunoassay [156]. In vivo serum levels of PICP correlates with bone formation as measured by histomorphometry [70].

NON-COLLAGENOUS PROTEINS

The remaining 10 % of organic components in bone matrix consist of proteins called *non-collagenous proteins.* (TABLE 5.1)

Osteocalcin also called BGP (bone gla-protein) is found in serum as well as bone. And the concentration in plasma correlates with formation of new bone [189]. Plasma

osteocalcin levels can therefore be used as indicators of osteoblastic activity [52].Osteocalcin contains three modified amino acid residues of γ-carboxyglutamic acid (Gla), which provides the protein with calcium binding properties. Thus it has been suggested that osteocalcin participate in the regulation of hydroxyapatite crystal growth [187]. Osteocalcin expression is stimulated by $1,25(OH)_2D_3$ one of the few well established cellular effects of hormones on bone cells [23]. Also osteocalcin is exclusively produced by osteoblast and odontoblasts and can therefore be used as specific marker for osteoblasts . Matrix gla-protein (MGP) is another gla-containing protein in bone matrix [97,188]. It has homologies to osteocalcin and is strongly associated with bone morphogenetic proteins and may serve as an in vivo biological carrier.

Osteonectin or SPARC (Secreted Protein, Acidic and Rich in Cysteine) is the most abundant non-collagenous protein in bone matrix. Osteonectin is highly concentrated in bone compared to other connective tissues, but probably have important actions in human fetal tissue, that also contains high concentrations of the protein Human osteoblasts produce osteonectin and the protein bind strongly to colla surfaces and interacts with calcium and phosphate. These properties has led to hypothesis that osteonectin are involved in the mineralization process by initia hydroxyapatite crystal nucleation and growth [27].

A group of calcium binding proteins are *phosphoproteins*, which are very a proteins that are phosphorylated; *osteopontin* and *bone sialoprotein* belong to group. Osteopontin and bone sialoprotein contain the amino-acid sequence R (Arg-Gly-Asp) along with *trombospondin* and fibronectin. This tripeptide seque is typically found in proteins with cell-binding properties. The cell-binding proc involves matrix proteins with the RGD sequence that connects to specialized receptors called integrins [105]. Osteopontin may anchor osteoclast during bo resorption via vitronectin receptors [197]. Tromspondin also bind calcium a osteonectin [207].

BONE GROWTH FACTORS

Apart form structural proteins, bone matrix also contains small amounts of v potent regulators of bone cell metabolism. These proteins called *bone deri growth factors* are produced by osteoblasts to the extracellular matrix during bo formation, but small amounts can also be trapped systemically from serum and incorporated into the matrix. The growth factors are located within the matrix un remodeling or trauma cause a solubilization and release of the proteins [38,112]. After release, the growth factors are able to regulate osteoblast and osteoclast

homeostasis during bone remodeling and may initiate and control a healing response after bone trauma . The bone growth factors exhibit their effects only to the local cellular environment, thereby stimulating neighboring osteoblasts to proliferate and increase matrix proteins synthesis (*paracrine effects*). Likewise the growth factor producing osteoblasts stimulate themselves and cause 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 are unknown, but the number is still expanding as a result of the novel advanced techniques in protein biochemistry and molecular biology.

Table 5.2 shows known bone related growth factors, their size, amount in bone and source. TGF-ß exist in 5 different subtypes and bone and platelets contains high amounts of this growth factor. TGF-ß is probably the most potent regulator of bone cell metabolism. BMP exist in 9 different subtypes and is the only growth factor known to stimulate the mesenchymal stem-cells to differentiate into osteoblastic and chondroblastic lineage. PDGF exist in three isotypes and is a potent stimulator of both proliferation and matrix proteins synthesis. The IGF I and II are produced by osteoblasts, and 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 proliferation of undifferentiated osteoblasts. FGF's are present in bone matrix and secreted by isolated osteoblasts. FGF-basic is found in bone matrix at a tenfold higher concentration than FGF-acid. FGF are primarily a mitogen on normal bone cells. A detailed review of the various growth factor effects on bone tissue and bone cells are given in chapter 6.

TABLE 5.1 ORGANIC COMPONENTS IN BONE

The proteins in bone	Size	Fraction	Function (Ref.)
Type I Collagen	320 kD	90 %	Major structural protein, gives tensile strength. [82]
Type V, VIII, XII - Collagen		<1%	Associates with type I fibrils to control fibril structure. [25,86]
Type VI Collagen -		<1%	Mediates cell attachment. [58]
Osteocalcin (Bone gla-protein)	6.5 kD	1,5 %	Binds hydroxyapatite [78,98,187]
Matrix gla-protein	10 kD	0.5 %	Binds Bone morphogenic proteins [96].
Osteonectin	33 kD	2,5 %	Binds calcium [127,235]
Phosphoproteins			Binds calcium and cells. [78]
-Osteopontin	32 kD	0.2 %	
-Bonesialoprotein	34 kD	1.0 %	
Trombospondin	150 kD	0.2 %	Binds calcium, osteonectin and cells. [207]
PS-S1, Biglycan	75 kD	1.0 %	Modulate matrix organization. [77]
PS-S2, Decorin	120 kD	0.2 %	Modulate fibril formation Bind TGF-ß and modulate activity. [122]
Fibromodulin	-	0.3 %	Modulate fibril formation.
Bone Growth factors		<0.2%	Regulate balance between bone formation and bone resorption. Initiate healing response after bone trauma.
-TGF-ß	25 kD		
-BMP's	16-30 kD		
-PDGF	36 kD		
-IGF's	7 kD		

Modified after Sandberg,M. [215].

TABLE 5.2 GROWTH FACTORS IN BONE MATRIX.

The growth factor in bone	Size	Bone content	Source in bone (Ref.)
TGF-ß* 1-5	25 kD	200 µg/kg	Osteoblasts [48]
BMP's* 1-9	16-30 kD	2-5 µg/kg	Osteoblasts [166]
PDGF's AA.AB,BB	36 kD	50 µg/kg	Osteoblasts, Serum
IGF's I,II	7.6 kD	400 µg/kg (IGF-II)	Osteoblasts, Serum [152]
FGF's Acidic, basic 1-6	16-17 kD	20 µg/kg	Fibroblasts,

* Members of the TGF-ß super family.

ABBREVIATIONS:

TGF-ß:	Transforming growth factor- beta
BMP:	Bone morphogenetic protein
PDGF:	Platelet derived growth factor
IGF:	Insulin like growth factor
FGF:	Fibroblast growth factor

CHAPTER 6.

GROWTH FACTORS AND BONE

Normal skeletal growth and maintance result from a balance between the processes of 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 [191]. The pathways triggered by these hormones in the target cells are however much less well known. The significant effects of a number of polypeptide growth factors on bone and cartilage metabolism suggest an important role for these in mediating hormonal responses locally. But they also suggest a local metabolic regulation of bone metabolism without influence of systemic hormones. The large content of these growth factors in bone matrix also enables bone to initiate a local healing response upon any degree of trauma (Table 5.2). Growth factors augment cell replication and contribute to stimulation of differentiation and metabolic functions of bone cells. They act primarily as local regulators on adjacent cells (paracrine factors), or on the secreting cell itself (autocrine factors). They exhibit their effects through binding to membrane bound receptors. This leads to a cascade of intracellular events often leading to a rapid expression of certain regulating genes called proto-oncogenes (ex. c-fos and c-jun) [69,102], which in turn affect the expression of other genes that encode for metabolic functions, such as cell division and protein synthesis. This chapter will review the important growth factors that have been found in bone or have been shown to possess effects on bone forming cells and bone tissue. The effect of growth factors on bone resorption and osteoclasts is a large area with extreme interest for bone physiology and metabolic bone diseases, but this review focus on bone formation and growth factors.

TGF-ß (Transforming growth factor-beta)

Classification: The TGF's are polypeptides that were originally isolated from tumor extracts and identified by their ability to induce non-neoplastic cells (NKR-49 cells) to exhibit neoplastic behavior by forming colonies in anchorage independent, soft agar suspension culture [205,227]. This finding was thought to be a biochemical key to neoplastic transformation [164,203], but additional research have shown that TGF's are multi functional cytokines with a broad range of biological activities. These include regulation of growth and differentiation of many celltypes, and in general TGF-ß has

stimulative effects on cells of mesenchymal origin and inhibitory effects of cells of ectodermal origin. TGF's have been catagorize into TGF-α and TGF-ß, but TGF-α has not been isolated from bone tissue and is not considered a bone growth factor. TGF-α belongs to an other growth factor family along with EGF and uses different receptors for its actions than TGF-ß. TGF-ß belongs to a family of related proteins that show various degree amino acid sequence homology, this protein family is called the *TGF-ß superfamily* [33]. Other members of this family includes bone morphogenetic proteins (BMP's) and the embryonical growth factors inhibin, activin and müllerian substance [162].

Structure: TGF-ß was originately purified from human platelets [9], and identified as a homodimeric peptide with molecular weight of 25 kD. TGF-ß is first synthesized as 390 precursor protein and later cleaved to generate mature C-terminal protein of 112 amino-acids, which sequence is conserved in all known animal species. The secreted precursor protein is biologically inactive and is called latent TGF-ß [128]. The activation and cleavage to the mature 112 aminoacid peptide occurs in acid environment pH < 2 and in vivo also through an yet unknown enzymatical reaction. A total of 5 subtypes of TGF-ß have been found until now, and are named TGF-ß 1 to 5. TGF-ß1 is identical to the TGF-ß originately purified from human platelets and bone matrix. TGF-ß2 have been purified from bovine and porcine bone matrix [124]. TGF-ß3 was identified from cDNA libraries and is expressed by many mesenchymal cells [66]. TGF-ß4 was also originally identified from cDNA, but has now also been purified from chick embryo chondrocytes [109,128]. TGF-ß5 is found to be expressed by Xenopus (an amphibian species) [212].

Receptors: Almost all celltypes appear to have receptors for TGF-ß and three types of receptors have been described [53,147]. The type I receptor has a molecular weight of 65 kD, Type II receptor of 90 kD and Type III receptor of 250-400 kD. The lower weight receptors has the highest affinity for TGF-ß and probably exert the main biological effects of the growth factor. Since almost all celltypes have TGF-ß receptors, the modulation of TGF-ß effects is probably regulated through activation of latent TGF-ß.

In vitro Effects: Bone and platelets contain almost 100 x more TGF-ß than any other tissue, and osteoblasts bear the highest amount of TGF-ß receptors [208,227]. These findings suggest a major importance of TGF-ß for bone metabolism. TGF-ß produces a number of species specific actions on bone cells (Table 6.5). In mouse calvaria osteoblasts and murine osteoblastic cell-lines TGF-ß inhibit proliferation and alkaline phosphatese activity [173,182]. In rat calvarial and human osteoblasts and corresponding cell-lines TGF-ß increases cell proliferation [34,47,50,198], but generally the

effect is biphasic, with stimulation at low levels 0.01-1 ng/ml and no effects at higher levels. The effects of TGF-ß on bone cell differentiation is controversial. Both collagen production and collagen gene expression is stimulated by TGF-ß [46,252]. Production of non-collagenous proteins is variously stimulated by TGF-ß, osteocalcin production and gene expression is most often reported decreased by TGF-ß [171,246]. In contrast to this, osteonectin, fibronectin, and osteopontin production and their gene expression, is stimulated by the growth factor [252,260]. Alkaline phosphatase activity and expression is generally decreased by TGF-ß stimulation [173,213,246,252] but in low density cultures TGF-ß increased the activity and even act synergistically with vitamin-D_3 [246]. Apart from stimulating alkaline phosphatese activity in some situations in bone cells, TGF-ß also stimulate matrix vesicle enzyme activity and can thereby further enhance bone mineralization [28]. Distinct from the described numerous effects on bone cell metabolism, TGF-ß has also been found to be a potent chemotactic factor towards osteoblasts of both rat and human origin [104,133,185](Own data see experimental section part I). This effect is found at very low concentrations from 1-100 pg/ml. The chemotactic effect of TGF-ß make this factor important for recruitment of osteoblasts to resorption lacunae during bone remodeling and to areas of bone formation upon bone trauma. Although there exist some controversy of the specific activities of TGF-ß on bone cell metabolism, TGF-ß is generally a very potent stimulating factor compared with the other osteotropic growth factors.

In vivo effects: TGF-ß has been used in several in vivo models for stimulation of new bone formation and seem to have potent abilities for this purpose (TABLE 6.1). The first studies used injections into rat and mice calvaria and found a marked increase in bone thickness of up to six times control thickness [113,172]. Other studies in similar models suggested that this effect was mediated through Prostaglandin-E_2, but the results were controversial [137,140]. In a study, using a calvarial defect model in rabbits, TGF-ß in methyl cellulose gel carrier was able to stimulate bone healing of otherwise non-healed defects [19]. Systemic administration in rats and rabbits causes endosteal bone formation and generalized osteoblast hypertrophy with high matrix protein synthesis activity [159]. One study have used phylogenetically higher animals, monkeys, to study TGF-ß effects on bone ingrowth into a titanium bone ingrowth chamber. After 22 days of stimulation with 1 and 10 μg TGF-ß in methyl cellulose gel carrier, no stimulation of the amount of new bone was formed, but the newly formed bone showed a marked increased osteoblastic activity [11]. In our own in vivo studies presented in this thesis, we were unable to find any stimulatory effect in both fresh and inhibit rabbit femoral defects. In a second study TGF-ß was

applied continuously to a healing osteotomy There it stimulated increased callus formation and increased maximal bending strength of the osteotomy (See experimental section part II and III).

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 growths factors to be used as stimulators of bone healing and bone induction in clinically related situations in orthopedic surgery.

TABLE 6.1
EFFECTS OF TGF-ß BONE IN VIVO.

Author, Ref	Year	Animal	Model/Dose	Effects
IN VIVO				
Noda,M. [172]	1989	Rat	Calvaria injections 1μg/day for 2 weeks.	6 x thickness increase
Joyce,M.	1990	Rat	Femur injection 0,2 μg/day for 2 weeks.	Induction of bone and Cartilage.
Marcelli,C. [140]	1991	Mice	Calvaria injections 5 μg for 5 days.	5 x thickness increase Inhibition by Indometacin.
Mackie,EJ. [137]	1991	Mice	Calvaria injections 2 μg for 7 days.	2 x thickness increase No inhib. by Indometacin.
Beck,SL. [19]	1991	Rabbits	Skull defects 3 μg in one dose 4 weeks.	Induction of bony healing.
Aufdemorte,TB. [11]	1992	Monkeys	Bone ingrowth chamber 1 & 10 μg TGF-ß 2 weeks	Increased osteoblast activity.
Lind,M. (II)	1993	Rabbits	Femoral defects 5 μg in one dose. 4 weeks	No stimulation of bone formation.
Lind,M. (III)	1993	Rabbits	Tibia osteotomy 1 and 10 μg/day. 6 weeks	Increased callus formation Higher bending strength.
Terrel,TG. [236]	1993	Rat/Rabbits	Systemic adm. 10-1000 μg/kg/day, 2 weeks.	Endosteal bone formation Osteoblast hypertrophy.

BONE MORPHOGENETIC PROTEINS (BMP's)

In 1965, Marshall Urist made the discovery that demineralized bone matrix (DBM) could induce bone formation when placed ectopically [238]. He observed that DBM caused formation of a complete ossicle subcutaneously with mineralized woven bone and bone marrow.

Classification: The ability of demineralized bone matrix to induce bone formation was ascribed to a low molecular weight protein, which was named Bone Morphogenetic Protein (BMP) by Urist [239]. Urist later reported the purification of a BMP from bovine bone in 1984 [240]. In 1988 Elizabeth Wang purified 3 different BMP's [243] of 30, 18 and 16 kD. By the use of novel molecular biological techniques John Wosney [251] generated oligonucleotide probes derived from the aminoacid sequence of fragments from the three purified BMP,s. From these oligonucleotide probes, it was possible to identify 3 genes coding for three BMP's called BMP-1, 2 and 3. Later additional six BMP's genes were identified by same techniques, so that today BMP 1 to 9 have been identified [45,65,250]. The BMP's have been grouped according to their homology into four groups. The first group consist of BMP-1, which appears to be genetically unrelated to the other BMP,s. It is believed that BMP-1 may be involved in binding and activation of other BMP's. BMP-2 and 4 are closely related and form a second group. BMP-3 (Osteogenin) has only minor homology to other BMP's and form the third group. BMP-5, 6 and 7 is synthesized from same precursor molecule and show 60 - 70 % homology and are thereby the fourth group. BMP-7 was simultaneously cloned by others who named the protein Osteogenic protein-1 (OP-1)[214].

Receptors: BMP's exert their effects through newly discovered receptors. These receptors are heteromeric complexes of type in and II serine/threonine kinase receptors. BMP 2, 4 and 7 seem to use the receptor complex, whereas the other BMP,s uses different receptor complexes not yet characterized [73,138,234].

In vitro effects: The physiological effect of the BMP's is still largely unknown. But from the data of numerous in vitro experiments several theories have evolved. BMP's are the only growth factor with a known ability to stimulate differentiation of the mesenchymal stem-cell into a chondro- and osteoblastic direction [54,239,253]. The proteins should therefore be involved in the maintance of a differentiated bone cell population. Also during a healing response release of BMP's from traumatized bone would stimulate a differentiation response of mesenchymal stem-cells, that will participate in the healing process. Mature osteoblasts are able to produce BMP and store the protein in bone matrix, which enables a storage of osteoblastic differentiating ability [239]. BMP's have also been shown to have several effects on

mature bone cells (Table 6.5). BMP-7 stimulated alkaline phosphatase activity, cell proliferation and collagen and osteocalcin synthesis in rat calvaria osteoblasts in 10-100 ng/ml [214]. BMP-2 and 3 were very potent stimulators of alkaline phosphatase activity but not a proliferative stimulator in a murine osteoblastic cell-line, and no stimulation of collagen gene expression and production was seen in bovine periosteal cell [15,101].

In vivo effects: Most strikingly are the in vivo effects of the BMP's . As mentioned earlier BMP activity was identified and is still assayed by its ability to form bone ossicles in vivo [238]. The novel recombinant BMP's have intact bone inducing capacity but need special carriers to exert their activity at low doses [64,135,199,244]. Actually BMP-2 at very high concentration can by itself induce bone formation [244]. Carriers for BMP are demineralized bone matrix and various synthetic polysaccharide matrices [255]. The function of the carrier matrix is to immobilize the bone inducing protein at a particular site for a sufficient amount of time to allow bone induction to occur. In addition the biocompability of the matrix allow cellular ingrowth and provide an environment conductive for cellular attachment and proliferation. Also matrix geometry is important since subcellular matrix particles inhibit BMP activity [214].

In vivo studies have primarily focused on the usage of BMP's in stimulation of defect healing (TABLE 6.2). In long bone defect-models in rats, rabbits, sheep, and monkeys, BMP-2, 3 and 7 have proven to be power-full stimulators of bony healing in otherwise non-union healing [60,61,124,229,255]. In muscle diffusion chambers BMP-2 stimulates bone formation in monkeys [7,158]. And also in monkeys BMP-3 were found to stimulate bony healing of large skull defects [199]. 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 [62,63]. Although the cellular mechanisms for BMP stimulated bone induction are vaguely understood, the in vivo bone induction activity of this group of growth factors are unique and the bone morphogenetic proteins are very promising for clinical use in any situation where bone defects need stimulation for proper healing.

TABLE 6.2
EFFECTS OF BMP's ON BONE HEALING IN VIVO

Author, Ref	Year	Animal	Model/Dose	Effects
IN VIVO				
Urist,M. [238]	1965	Rat	Muscular pouches with DBM.	Ectopic bone formation.
Yasko,A. [254]	1992	Rat	Femoral defects 15 μg BMP-2	100 % healing and restoration of strength.
Cook,SD. [60]	1992	Rabbit	Ulna defect 3-400 μg BMP-7	Healing compared to non-union in controls.
Cook,SD [61]	1993	Monkey	Ulna defect 1-5 mg BMP-7	Healing compared to non-union in controls.
Kirker-head,H. [124]	1994	Sheep	Femur defect 1-5 mg BMP-2	Healing compared to non-union in controls.
Cook,SD [62,63]	1994	Dog	Spine fusion 1 mg BMP-7	Healing comparable to autologous bone.
Ripomonti ,U. [199]	1992	Monkey	Calvaria defect 100 μg BMP-3	Healing compared to non-union in controls.
Aspenberg,P. [7]	1993	Monkey	Muscle Pouches 5 and 50 μg BMP-2	Ossicle formation.
Miyamoto,S. [158]	1993	Monkey	Diffusion chamber in muscle. crude BMP.	New bone formation

PLATELET DERIVED GROWTH FACTOR'S (PDGF's)

Platelet-derived growth factor (PDGF) was originately discovered in serum as the major mitogenic activity responsible for growth of cultured mesenchymal cells [226]. Subsequently its was found that the growth factor was derived from platelets, where it is stored in the α-granules and released upon activation along with other factors. PDGF are generally a power full mitogen for most mesenchymal cells, but also a chemotactic factor for fibroblasts, smooth muscle cells and osteoblasts.

Structure: PDGF is a disulfide-linked dimer with a molecular weight of approximately 30 kD. The subunits of the dimer are two related polypeptides designated the A and B chains. These chains are products of distinct genes and PDGF can be either homodimeric (PDGF-AA, PDGF-BB) or heterodimeric (PDGF-AB). Human platelets consists of 70 % PDGF-AB and 30 % PDGF-BB, whereas the PDGF-AA isoform primarily is secreted by malignant cells.

Receptors: The PDGF's use two different receptors, the α-receptor that have equal affinity for all three isoforms and the ß-receptor that have a tenfold higher affinity for the PDGF-BB isoform.

In vitro effects: The main effect of PDGF on bone cells is mitogenic (Table 6.5). This effect has been found in both human and rat osteoblasts and various osteoblastic cell-lines [1,39,43,51,87,186,257]. The most potent isoform is PDGF-BB which can cause a sixfold increase in thymidine incorporation at 50 ng/ml [257]. PDGF is also a power full chemotactic factor for mesenchymal cells and this effect is valid towards osteoblasts from both rat and human tissue [104,237](Own data; experimental section part in). PDGF have few other effects on the differentiated osteoblast. Several studies have found no effect on collagen and osteocalcin synthesis [1,39,51], but one study indicate a negative effect on alkaline phosphatase activity [51].

In vivo effects: In vivo studies have shown various potency of PDGF for stimulation of bone formation (TABLE 6.3). Demineralized bone matrix treated with PDGF and implanted in muscle in rats, showed increased calcium content and alkaline phosphatase activity. Increased ash-weight of heterotropic bone in rat was seen after continuous stimulation with PDGF-BB [201]. In rat calvarial defects PDGF inhibited BMP-3 stimulated bony healing by increased soft tissue formation [141]. One study have 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 [136].

TABLE 6.3
EFFECTS OF PDGF's ON BONE HEALING AND BONE FORMATION IN VIVO

Author, Ref	Year	Animal	Model/Dose	Effects
IN VIVO				
Howes,R.[103]	1988	Rat	DBM + PDGF in muscle	Increased calcium content and AP-activity.
Piche,JE.[123]	1989	Rat	Heterotropic bone formation Continuous administration.	Increased ash weight.
Lynch,SE.[136]	1991	Dog	Dental implants PDGF+IGF in gel. 5 μg each. 1 & 3 weeks.	Increased bony ingrowth and new bone formation.
Marden,LJ.[141]	1993	Rat	Calvarial defect, PDGF+BMP-3	Increased soft tissue formation decreased bony healing.

INSULIN GROWTH FACTOR'S (IGF's)

Structure and classification: Insulin-like growth factors are growth hormone-dependent polypeptides with a molecular weight of 7.6 kD. Two IGF's have been characterized; IGF-in and IGF-II and their original designations were somatomedin-C and skeletal growth factor [162]. These peptides are synthesized by multiple tissues, including bone [151,154]. IGF-II is the growth factor found in highest concentration in bone matrix, whereas IGF-in is found in 10-20 x less concentration [76]. IGF in and II have similar biological properties, but IGF-in is 4-7 times more potent than IGF-II.

Receptors: There are two receptors that mediate IGF's effects, type in of 450 kD and type II of 250 kD. The type II receptor has a high affinity for IGF-1 and the predominance of this receptor on osteoblasts cause IGF-1 to have the major metabolic effects on osteoblasts [162].

Binding Proteins: Bone cells also secrete IGF binding proteins (IGFBP's) that bind and modulate IGF activities. So far, six different IGFBP's have been identified and termed IGFBP 1 to 6, and osteoblasts synthesize all of them except IGFBP-1 [36,37,93]. The precise role of the IGFBP's is not fully understood. They may prolong the half-life of IGF, neutralize or enhance its biological activities, or be involved in transport of IGF to its target cells [160]. IGFBP-1 and 2 mainly have transport functions, whereas IGFBP-3 increases IGF half-life considerably [72,163]. IGFBP-4 is mainly an

inhibitor of IGF bioactivity [161] and IGFBP-5 potentiates the proliferative effects of IGF's mainly in bone cell systems [36,59].

Hormones: IGF production in bone tissue is known to be stimulated by PTH and Growth Hormone (GH) [35,57,225]. PTH may therefore mediate its anabolic effect on bone via IGF, that in an auto and paracrine fashion stimulates osteoblasts. Other hormones regulate the synthesis of IGFBP's e.g. estradiol and growth hormone are stimulators and cortisol is an inhibitor [153,163]. The major effect in bone tissue of IGF is probably its potent effects on the cartilage in the growth plate. Here it is assumed that GH control longitudinal growth via local stimulation of chondroblastic IGF production and IGF thereafter regulate chondroblastic growth and metabolism [106-108,170,218].

In vitro effects: With respect to bone cells (Table 6.5) both IGF-in and II stimulate preosteoblastic cell replication, which increases the number of cells capable of synthesizing bone matrix [219,241]. But their mitogenic effect is less pronounced than those of other growth factors such as TGF-ß or PDGF-BB [184]. IGF's also have independent effects on the differentiated functions of the osteoblast, increasing bone collagen production and inhibiting collagen degradation [149,230]. As a result of these effects IGF's increase bone mass.

In vivo effects: Several studies have investigated the use of IGF's for stimulation of in vivo bone healing and systemic bone formation (FIG 6.4). 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 [5]. Another study used continuous local application of IGF-1 to a healing osteotomy, but no stimulatory effects were found [123]. 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, this was indicated by increased bone weight and increased osteoblastic activity [222,224].

TABLE 6.4
EFFECTS OF IGF's ON BONE HEALING AND BONE FORMATION IN VIVO

Author, Ref	Year	Animal	Model	Effects
IN VIVO				
Aspenberg,P.[5]	1989	Rat	Bone chamber	No effects.
Kirkeby,OJ.[123]	1992	Rabbit	Osteotomy	No effects.
Skottner,A.[222]	1990	Rat	Systemic adm.	Increased bone weight.
Spencer,EM.[224]	1991	Rat	Systemic adm.	Increased osteoblastic activity.

FIBROBLAST GROWTH FACTOR'S (FGF's)

Classification: The fibroblast growth factors are polypeptide growth factors that show potent mitogenic activities for cells of mesodermal and neuroectodermal origin [226]. The FGF family currently consist of seven members FGF-1 through FGF-7. FGF-1 and FGF-2 are also designated acidic and basic FGF (aFGF and bFGF) respectively. These two growth factors were the first FGF's to be characterized and they were named for their different isoelectric points and they are by far the most extensively studied. FGF-3 to FGF-6 have been identified as oncogenes, which are intracellular regulators of gene expression, and FGF-7 is also called keratinocyte growth factor. Both acidic and basic FGF have a molecular weight of 18 kD and exhibit 55 % aminoacid homology. FGF's are synthesized by cultured osteoblast and the secreted growth factor is stored in bone matrix [84]. The secreted FGF in bone matrix consist of 90 % basic FGF and 10 % is acidic FGF.

Receptors: FGF's bind to heparin sulfate proteoglycans before they are capable of binding to one of the three high-affinity FGF-receptors identified today.

In vitro effects: FGF's have mainly a proliferative effect on osteoblasts and less effect on protein synthesis (Table 6.5), consequently they probably enhance bone formation by increasing the number of cells capable of synthesizing bone collagen [150,211]. The bFGF is generally more potent than aFGF[40] . TGF-ß synthesis by osteoblasts can also be stimulated by bFGF and FGF may therefore exert some stimulatory effects through other growth factors [176]. FGF's are angiogenic factors

that are important for neovascularization during a healing response and in association with its effects on bone cell replication FGF's may be important factors in the bone healing process.

In vivo effects: A few 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 matrix stimulated more new bone formation after three weeks than controls [6]. Fracture healing in rats have been stimulated by 50 μg bolus doses of bFGF and caused increased callus formation and bone mineral content [117]. Systemic stimulation of rats with bFGF caused increased osteoblasts proliferation and endosteal bone formation [148].

EPIDERMAL GROWTH FACTOR'S (EGF's)

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 have very modest effects on osteoblast in culture (Table 6.5), but two studies suggest a proliferative effect on immature bone cells [3,241]. A potentiating effects of other growth factors on bone cell proliferation have been reported [121]. Systemic administration of EGF to mice 200 μg/kg/day resulted in increased periosteal and endosteal bone formation and osteoblastic activity [142].

TABLE 6.5
IN VITRO EFFECTS OF GROWTH FACTORS ON OSTEOBLASTS

Growth factor	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	0
FGF-acidic	0	0	0	0	0
EGF	+	0	0	0	0

OTHER CYTOKINES AND FACTORS

Hematological cells secrete cytokines, that mainly are regulators of immunological responses but also may function as systemic regulators of bone cell function. Because of the close proximity of the marrow cells and bone, some cytokines could act as paracrine regulators of bone cell metabolism. The cytokines that 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 [40] and probably have their main actions in osteoblast-osteoclast interactions, where the colony stimulating factors stimulate the monocyte/osteoclast lineage of cells and the interleukins and tumor necrosis factors inhibit osteoblastic activity and stimulate osteoclastic activity. Their general effect on bone tissue is therefore stimulations of bone resorption, although single studies have indicated stimulative effects in low doses of IL-1 on isolated osteoblasts [85,162]

GROWTH FACTOR INTERACTIONS

Since multiple growth factors are contained in bone and produced by bone cells, it is obvious that more than one growth factor may be present at any one time in the bone-cell micro environment. A limited number of studies have shown that growth factors have interactive relations. In bone cells systems growth factor combinations

often potentiate proliferative or differentiated effects. And some growth factors are able to stimulate synthesis and regulate activity of other growth factors. For example TGF-ß, PDGF and bFGF decrease IGF gene expression in rat calvarial cells [41]. Whereas in an osteosarcoma cell line bFGF increased TGF-ß gene expression [176]. Studies of growth factor combinations have shown that IGF-II act synergistically with bFGF and TGF-ß on proliferation of rat calvaria osteoblasts [115]. Additive effects on proliferation of osteoblastic cell lines has been demonstrated for TGF-ß and bFGF [83]. And on human osteoblasts synergistic effects on proliferation were found for combinations of TGF-ß, PDGF-BB and EGF [121]. TGF-ß have also been found to inhibit osteoblast production of IL-1 and TNF-α, thereby inhibiting their stimulative effect on osteoclasts. The exact physiological significance of growth factor interactions in the modulation of osteoblast metabolism at the local cell environment in vivo remains to be determined.

GROWTH FACTORS AND OSTEOTROPIC HORMONES

The effects of osteotropic hormones on bone formation are complex in the respect that no single hormone stimulates bone formation. Regulation of bone formation is therefore a complex set of interactions between hormones and locally produced factors.

The bone content of TGF-ß can be modulated by several systemic hormones, suggesting a possibility for systemic regulation of this very important growth factor for local bone metabolism [48]. The secretion of TGF-ß from rat calvarial bone in culture is increased by PTH, 1,25-dihydroxyvitamin-D_3, Estradiol and Calcitonin [49,178,183]. The two first hormones are well established stimulators of osteoclastic bone resorption, but the effect on osteoblastic TGF-ß secretion may represent a hormonal axis for simultaneous stimulation of bone formation, in this way coupling bone formation and bone resorption. IGF production in bone tissue is known to be stimulated by PTH and Growth Hormone (GH) [35,57,72,225] and IGF's are one of few local factors were a hormonal regulation axis have been identified. PTH may therefore mediate its anabolic effect on bone via IGF, that in an auto and paracrine fashion stimulates osteoblasts. Other hormones regulate the synthesis of IGFBP's e.g. estradiol and growth hormone are stimulators and cortisol is an inhibitor [153,163]. Regulation of IGF activity can by this mechanism be regulated indirectly by other systemic hormones. The above mentioned hormone to growth factor relations only represent a few recent discoveries and the exact mechanisms and relationships between osteotropic hormones and growth factor regulated metabolism in bone still needs to be established.

CHAPTER 7

METHODOLOGICAL CONSIDERATIONS

MODELS FOR EVALUATION OF GROWTH FACTOR EFFECTS ON BONE TISSUE.

In Vitro Models

Cell cultures

Several cell culture systems exist for the study of osteoblasts (Table 7.1)

TABLE 7.1

IN VITRO MODELS FOR THE STUDY OF OSTEOBLASTS :

Species	Culture Type, Reference
1.	**Primary Culture**
Human trabecular bone	21,143,206
Rat Calvaria	20,55,181
Mouse Calvaria	248,249
Chick Calvaria	81
Bovine trabecular bone	247
2.	**Osteosarcoma derived cell lines**
Human	
Saos-2	169
U-2 os	100
Rat	
UMR-106	145
ROS 17/2	210
3.	**Non-transformed immortalized cell lines**
MC3T3-E1	231
4.	**Marrow stromal osteoblasts**
Human	99
Rat	139

Modified from Kassem,M. Ph.D. Thesis. [116]

1. Primary osteoblastic cultures can be obtained by two methods: 1) With the *tissue explant method*, cells migrate from bone chips onto culture-dish plastic or glass surface [206]. 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 and degree of differentiation [206]. The explant method is suitable in situations where the bone available is limited or cell growth is slow, such as adult bone. Problems with the method is that it probably selects cells with a high proliferative capacity and degree of maturation. 2) The other method for primary culture uses *sequential digestion* of bone with collagenase (20-30 min steps). This causes release of different bone cell populations [248]. The early released cells are periosteal fibroblasts and immature osteoblast and osteoclasts, whereas the later released cells are more mature osteoblast. The phenotype of cells released by this method are 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 long time (weeks - months) to obtain enough cells for experiments. Also the advantage of stabile 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 what 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 [42]. This is probably done because of the easy access to rats and mice in most biological laboratories and the fact that cells released from rodent bone grow fast and are easy to maintain. If it is wanted to relate in vitro data to human bone physiology, then it is important to use human bone cells despite problems with limited availability and slow growth.

In the in vitro studies described in experimental section part in, we used human primary osteoblast cultures that were released by the tissue explant method. We thereby gained 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.

2 Osteosarcoma tissue have been used to create cell-lines with various characteristics of the differentiated osteoblast while retaining a high proliferative activity. This has made it possible to establish cell lines at different stages in the differentiation process. Osteosarcoma derived cells has the advantage that large numbers of homogenous cells can easily be obtained. The disadvantages are the different cell biology and responses to stimuli when comparing to normal untransformed cells.

3 Non malignant immortalized cell-lines have been made by a virus transfection of a well characterized osteoblast clone. Non transformed celllines do not have the unknown genomic lesions causing malignant transformation of osteosarcoma cell lines, while still possessing the advantages of minimal phenotype variation and large available cell numbers. However phenotypical drift and subclonal heterogeneity has been found.

4 Marrow stromal osteoblast-like cells were cultured due to the known osteogenic potential of bone marrow. These cells are isolated as cells capable of plastic adherence in the mononuclear fraction of bone marrow cells. They express the characteristics of the osteoblastic phenotype although the cells generally show a large phenotypic variability. Stromal osteoblasts represent undifferentiated osteoblasts permitting studies of the osteoblast differentiation process.

Organ cultures

Bone organ cultures has been described for many animal and bone types [67,202,228]. Most commonly used are calvaria and intact femora from newborn mice and rats. Since bone is a heterogenous tissue, organ cultures have the advantage of having intact celltype to celltype and cell to matrix interactions, while still enabling precise control of external stimuli and culture conditions. The disadvantages of organ cultures is that numerous animals needs to be sacrificed if more extensive studies with several stimulating agents and concentrations are to be completed. Furthermore is it difficult to maintain the cultures for longer observation periods.

In vitro assays for bone formation

Cell proliferation:

Cell division is an important parameter when investigating osteoblast metabolic

activity. Several methods exist to quantitate cell division activity. The most simple method is to simply count cells in the culture wells under microscope. Another more convenient method is staining a cell layer with methylene blue [177]. This basic dye binds predominantly to the phosphate groups of nucleic acids and absorbance measured on a 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 that determine number and activity of cells that synthesize DNA (S-phase cells). The Bromodeoxyuridine assay utilizes the thymidine analog bromodeoxyuridine and a recognizing antibody to label and detect S-phase cells. Labeled cells are visualized with a secondary antibody and peroxidase and cells are counted [134]. This method is very time consuming due to the counting technique and therefore is an alternative method that used labeling of cells with ^{3}H-thymidine the most commonly used technique. This assay is easy to perform and have 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 that number of cells. A disadvantage of the technique is that fluctuation in the intracellular thymidine concentration often cause considerable intra individual variations in radioactive counts [190]. This problem can be overcome by adding a sufficient concentration of radioactive thymidine and by having triplet or quadruplet determinations of each testunit.

Enzyme Activity

Alkaline phosphatase (AP) is a membrane bound enzyme that is present in many cell types but is most abundant in osteoblasts [210]. 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 [29]. AP can be visualized in a cell-layer by histochemical staining and enzyme activity can also be quantitated by incubating a celllayer with p-nitrophenyl phosphosfate 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 $1,25(OH)_2D_3$ co-stimulation.

Matrix protein synthesis

A key aspect in osteoblastic formation of new bone is synthesis of matrix proteins.

In bone, collagen type in is the major structural protein, and measurement of this protein is therefore crucial for evaluation of in vitro bone formation. Today the most used method is determination of pro-collagen 1 C-terminal propeptide (PICP) in supernatant medium. This peptide is split off the mature collagen molecule and can be recognized by an antibody and quantitated by RIA or ELISA technique [156,200]. 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 [215]. Osteoblast expression of osteocalcin in culture can be visualized by immunohistochemical technique. Osteocalcin is produced to the supernatant medium and can be quantitated by RIA or ELISA technique [68].

Numerous other protein exist in bone matrix (see Table 5.1) but no available assays for determination of protein production exist today. An indirect approach exist by measuring mRNA synthesis for a specific protein using molecular biology techniques. Northern blot analysis is used to detect a specific mRNA and this technique can be made semi-quantitative by measuring gel staining intensity and compare it to staining intensity of mRNA's of proteins that are produced at constant levels (housekeeping enzymes) [175,176,209]. If only small amounts of total mRNA can be obtained the polymerase chain reaction (PCR) technique can be used to amplify very small amount of mRNA to detectable levels [24]. The described molecular biological techniques are generally very labor intensive and technically demanding for the investigator, also methodological problems and problems for quantitative analysis are considerable.

Chemotaxis

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 quantitated by counting cells that had reached a standardized level in the membrane [89,223]. 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 new *multi well chamber technique* that consist of up to 48 wells in each chamber [74]. This chamber consist of a two level system that is 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 quantitated by counting cells that has migrated to the bottom side of the membrane. Correct incubation time and cell-concentration for the cell type tested is essential for reproducible results. Typically non-transformed cells show large interindividual differences in absolute number of migrated cells. Data therefore need to be normalized as cellcounts in stimulated wells divided by cellcounts 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 need to be performed. In this experiment a concentration found to stimulate migration is eliminated by adding test agent together with the cell-suspension [261]. For true chemotaxis this will reduce migration to control levels. We chose to use the multi well chemotaxis chambers in our osteoblast migration studies due this techniques ability to perform several test units under identical conditions and because data sampling could be performed fairly simple under microscope [74].

IN VIVO MODELS

Experimental animals

Many types of animals from mice to monkeys have been used for investigation of the in vivo effects of growth and healing promoting agents on bone. Many aspects must be considered before choosing type of 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 also the amount of bone available for simulating various operative procedures is very small. Rabbits are an alternative to rats and mice with a much larger bone mass that allow clinically related operative procedures, while still having advantages such as low cost and simple handling at experimental procedures. For these reasons rabbits where used for the two studies described in the experimental section [129,130]. Larger animals such as dogs, pigs, sheep and monkeys are all well suited for advanced experimental surgical procedures, and the bone physiology of these animals is close

to that of humans. Unfortunately are these animals expensive and their handling for experiments generally technically demanding and labor consuming.

In vivo experimental models.

Several models have been used to study the effects of growth factors on bone. Three main model systems can be described.

A. Injections into bone.

B: Bone defect models.

C: Fracture models.

A: Injections into normal or healing bone in newborn mice and rats have been used as a first line model to test possible bone stimulative effects of a newly discovered growth factor [113,172]. Newborn animals are generally very responsive to growth factors and the results obtained in these models are often difficult to reproduce in adult and phylogenetically higher animals. Injections of growth factors into both calvaria and femur have been successful for demonstration of new bone formation [113,137,140,172].

B Bone defect models are very attractive for investigation of bone stimulating agents since it is possible to create models where control defects have no or slow spontaneous bone healing [18]. The most used locations for defects are in calvaria or distal extremity bone [18,61,254]. Such defects can be created without any need for internal or external fixation, a major advantage for surgical procedures and animal handling.

We choose a defect model in the femoral condyles of rabbits since a similar model in rabbit calvaria had previously been used with success for studies of growth factor effects on bone healing [18,129]. We placed the defect in trabecular bone of the femoral condyles because this location was more clinically relevant for growth factor stimulation of bone healing into cementless endoprosthetic components. A calvarial defect investigates membranous bone healing and we were interested in healing of trabecular bone that typically surrounds cementless endoprosthesis components. The femoral condyle defect model provided very easy access to standardization of defect placement and to access of the bone surface when the animals were reoperated

C Fracture models are interesting because of their close resemblance to the clinical fracture situation. They can be performed in both small and larger animals [4,12,118].

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 application of stimulating agents. Systemic administration is possible for some agents (e.g. growth hormone) while other agents (e.g. growth factors) need local application by pumps or injections [13,14].

We choose a plated tibial mid-diaphysial osteotomy model in rabbits as a model to test the ability of TGF-ß to stimulate bone formation in a clinically related model [130]. This model was previously used in our laboratory for studies of exogenous application of bone healing promoting agents and we therefore used the experience of those previous studies regarding surgical procedures and data analyses [119,120]. The model used miniosmotic pumps to deliver the growth factor continuously to the osteotomy site in a physiological buffer solution. We considered this application method advantageous because it enabled precise application to the area where stimulation of bone healing was desired. Daily injections creates a minor trauma for each injection that may disturb the healing process and such minor trauma is avoided with pump application [113,172].

Evaluation of in vivo models.

Mechanical testing:

Several problems exist for biomechanical testing of fractured bones. Standardized specimens cannot be prepared because of the irregular geometry of fracture callus and interindividual differences of bone structure [12]. The mechanical testing procedure is another matter of concern. Tensional and torsional tests have the advantage of at least in theory to create an even distribution of stress around the fracture callus. Also such tests will determine the weakest point of the entire bone [32]. But the practical problems with fixation and alignment in the testing apparatus are generally so big that despite the superiority of test the test types are not widely used for fracture healing studies. Bending tests, three or four point, are also suitable for mechanical test 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 small degrees of rotation of the test specimen is difficult to avoid and standardization of the test can therefore become impaired. We

used three-point bending test in our mechanical tests since this test type enables a precisely located load placement thereby testing the mechanical properties at the fracture line [13].

Histology, bone histomorphometry

Quantitative histomorphometry was an important technique for evaluation of new bone formation in both the fracture and bone defect study [129,130].

In the fracture study we used sections 5 mm distal to the osteotomy line. On these sections area of fracture callus, thickness of cortical bone and cortical bone porosity were evaluated. 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 existing bone. Callus area and cortical thickness were measured using a computer assisted tracing devise. This provided very reproducible results with low coefficient of variations (table 7.2). Cortical thickness and Haversian canal diameter was determined by a digitizer based measuring system. Twenty measurements pr section was performed.

In the bone defect study we determined new bone formation in the created defect. This was done by measuring the area of woven bone in the defects using point counting technique. We also measured bone forming activity at cellular level by quantitating the osteoblast, osteoid and osteoclast density on the woven bone surface. This was done by using the line-intercept histomorphometric technique. The histomorphometry was performed on one 8 μm section from the central part of the defect. If serial sections had been used a more precise determination of the various parameters could have been achieved. But since to both preparation of section and data retrieval was very resource consuming we choose 6 random specimens on which we evaluated the variation of bone formation through the defects.

Bone histomorphometry is highly valuable for evaluating biological responses in bone both at tissue and cellular level. Data sampling needs to be performed according to a set of rules that ensure sufficient numbers of sampled data and standardized histological sections. These rules need to be followed for precise and reproducible results [90,91]. For all studies we estimated the precision of the histomorphometric analysis by series of double measurements and calculation of coefficient of variation from following formula: SD(Δ)/Mean (table 7.2).

TABLE 7.2

Coefficient of variations for histomorphometric analyses.

Tibial Osteotomy study in rabbits.

Callus area	Cortical thickness	Haversian canal diam.
0.031	0.026	0.061

Bone defect healing in rabbit femoral condyles.

Woven bone area	Osteoblast fract.	Osteoid fract.	Osteoclast Fract.
0.035	0.061	0.073	0.12

Chemotactic effects of bone growth factors

Chemotactic Index
0.15

Bone mineral content Scanning (BMC Dual Photon Scanning)

In the fracture study we tried to measure the degree of mineralization of the healed fracture. For this purpose we used dual photon scanning, a well established technique with a high reproducibility (coefficient of variance 0.03). We scanned 16 mm of the fracture site but the results showed no difference between groups although histology had demonstrated increased callus in the TGF-ß stimulated groups. This can probably be explained by the fact that diaphyseal cortical bone contains the majority of the mineral in the scanned area compared to the callus tissue. A better method for evaluation of mineralization would be determination of ash-weight of free-dissected callus. By this method the results would be based solely on the callus mineral.

CHAPTER 8
EXPERIMENTAL WORK

INTRODUCTION TO EXPERIMENTAL MODELS

Studies on stimulation of bone healing can be approached by various experimental models. In in vitro investigations a specific celltype can be studied under very controlled situations. It is thereby possible to investigate numerous stimulating agents for their effects on several bone cell metabolic functions. A disadvantage of cell culture systems is celltype to celltype (e.g. Osteoblast-Chondroblast and Osteoblast-Fibroblast), and cell to matrix interactions are lost. These interactions are of great importance for the cellular events of a healing response such as bone healing. In in vivo models is it very difficult to control the experimental situation at the cellular level and therefore also difficult to interpret cellular parameters. The most valuable parameters are therefore best determined at the macro tissue level, such as mechanical properties of a whole bone or new bone formation in a defect. For the present studies both in vitro and in vivo models were used to study the effect of growth factors on bone healing. The in vitro study investigated the ability of a panel of bone growth factors to stimulate chemotactic migration of human osteoblasts (in). This recruitment of osteoblast is a very important event in the bone healing process and osteoblast chemotaxis has only been sparely investigated. The in vivo studies were performed using different bone healing models and growth factor application methods. One study tested TGF-ß in gel preparation as stimulators of bone healing in rabbit femoral defects (II). The second in vivo study used continuous application of TGF-ß to a healing osteotomy in rabbit tibiae (III).

In vitro study: Chemotactic effects of growth factors and cytokines have been studied extensively in neutrophils, mononuclear cells and fibroblasts [2,89,126,242]. Transforming growth factor-ß (TGF-ß) and Platelet derived growth factor (PDGF) and the matrix proteins collagen, fibronectin and elastin have all been shown to be chemotactic for fibroblasts [95]. A number of studies have described the chemotactic migration of osteogenic cells both in vitro and in vivo. As early as 1965 Urist observed the migration of mesenchymal cells into ectopically placed demineralized bone matrix to form bone and cartilage [167]. Rat osteoblast-like cells and rat osteosarcoma cells have been investigated for chemotactic reaction to TGF-ß, PDGF, IL-1ß, PTH and bone gla protein (BGP) [104,132,185,237]. In these studies TGF-ß, PDGF and BGP were shown to be chemotactic bone cells. However, it is not known if these findings are valid for human osteoblasts and a detailed investigation of larger number of osteotropic growth factors and their effects on human osteoblasts

have not been performed in same setup. Neither has any quantitative comparison between the chemotactic abilities of these growth factors been performed. More detailed knowledge about the biochemical regulation of osteoblast migration is needed in order to understand the recruitment of osteoblasts in coupling between bone resorption and bone formation during bone remodeling. Understanding of the recruitment of bone forming cells is also of great importance in an attempt to use growth factors as stimulators and inductors of bone repair in clinical situations [19,130]. The aim of this in vitro study was to quantitate and compare the chemotactic effects of the osteotropic growth factors: TGF-ß, PDGF-AA, PDGF-BB, FGF-acidic, FGF-basic, IGF-1 and IGF-2 on normal human osteoblasts.

Bone defect model: A major perspective for growth factors in relation to bone healing is the possible clinical use as promoters of noncemented endoprosthetic fixation, healing of complicated fractures and pseudarthroses and stimulation of bone healing in large bone defects. TGF-ß has been found to be a potent stimulator of bone formation in numerous in vivo studies in rat and rabbits [113,130,137,140,172]. These studies have used injection or pump application as delivery system. A more clinically attractive method for application of TGF-ß is to use methyl cellulose gel. One study have used single dose of up to 2 μg TGF-ß in a methyl cellulose gel to stimulate bony healing of large calvarial defects in rabbits [19]. The purpose of the present study was to investigate the effect of single dose application of 5 μg natural TGF-ß in a 3 % methyl cellulose gel and its ability to enhance the bony healing in condylar defects. One problem when studying bone healing in rabbits is the high endogenous healing capacity. We therefore created a model with inhibited bone defects in order to study the isolated stimulative effects of the applied growth factor.

Osteotomy model: Fracture healing is a complex process that involves numerous cellular events such as chemotaxis, proliferation, differentiation and extracellular matrix protein synthesis. These cellular events are primarily regulated and stimulated by growth factors [26,110,112,114]. The role of growth factors as stimulators of cell metabolism and healing process maintenance at fracture healing. This have lead to the hypothesis, that exogenous application of growth factors could be used as possible stimulators of fracture healing. Previously the growth factors TGF-ß and PDGF-BB have demonstrated potent stimulative effect on cell proliferation in fracture callus organ cultures (238,241). The next step for us was to study the effect of growth factors on fracture healing in vivo. This study was therefore performed to investigate if continuous local dosage of exogenous TGF-ß could promote healing of rabbit tibia osteotomies.

(I) CHEMOTAXIS OF HUMAN OSTEOBLASTS IN RESPONSE TO OSTEOTROPIC GROWTH FACTORS

MATERIAL AND METHODS

Cell culture

Osteoblasts were isolated from human trabecular bone derived from orthopedic surgical procedures such as hip arthroplasty or spine surgery. Trabecular bone chips were plated after collagenase digestion for 120 min as described previously [10,22,206]. Cells were grown in Gibco's Minimal Essential Medium (MEM) supplemented with 10 % heat inactivated fetal calf serum (FCS), 50 μg/ml streptomycin and 100 μg/ml penicillin G. The medium was changed weekly and the cells were grown to confluence in 3-4 weeks. Cell were released by 120 sec. trypsinisation (0.01 % trypsin in saline citrate) and shaking.

Growth factors

Seven human recombinant growth factors were studied: Transforming Growth factor-ß1 (TGF-ß). platelet derived growth factor-AA (PDGF-AA), platelet derived growth factor-BB (PDGF-BB), fibroblast growth factor-acidic (FGF-a), fibroblast growth factor-basic (FGF-b) were all purchased from British Biotechnologies (Great Britain). Insulin like Growth factor-in (IGF-1) and insulin like Growth factor-II (IGF-2) were supplied by Novo Nordisk (Denmark). All growth factors were examined in ten fold dilutions with a concentration range 0.001 - 100 ng/ml.

Chemotaxis assay

Cultures from six different patients were used for the chemotactic experiments. All growth factors were tested in duplicate for each concentration. Chemotactic responses were measured by a modified Boyden chamber assay using a 48-well micro chemotaxis chamber (Neuroprobe) with polycarbonate filters with 5-μm pores (Millipore)[74]. Filters were coated with 30 μg/ml type in collagen (Sigma) for 45 min. Growth factor dilutions in Gibco RPMI medium containing 0.5 % bovine serum albumin (BSA)(Sigma) were added to the lower well in 27 μl aliquots. The filter was secured in place and 50 μl of osteoblast suspension in RPMI medium (2 x 10^5 cells/ml) were added to each of the upper wells of the chamber. After 17 hours incubation the chamber was disassembled and the filter was carefully removed. The cells on the upper aspect of the filter was removed by rinsing in cold PBS pH 7.0 and scraping over a rubber wiper. The remaining cells on the lower aspect of the filter were then fixed in 4 % formaldehyde for 4 min. and stained with Toluidine Blue. Control analysis was performed to distinguish between concentration dependent cell migration (chemotaxis) and random migration (chemokinesis). The analysis was performed by eliminating the concentration gradient by adding the chemoattractant to the upper chamber with the cells [261]. Bottom wells were examined for cells, which had migrated through the filter, but did not attach to the membrane.

Mitochondria dehydrogenase assay for cytotoxity

Growth factor concentration found to be chemotactic for osteoblast were tested for cytotoxity

by the colorimetric mitochondria dehydrogenase MTT (Dimethylthiazol-Tetrazolium Bromide) assay [165]. Ethanol was used as positive control.

Immunohistochemical osteocalcin staining of migrated cells.

Membrane bound osteoblasts were stained with an rabbit anti-osteocalcin antibody. Membranes with the adhesive cells were blocked in 2% BSA, and incubated overnight with the rabbit anti-osteocalcin IgG (1:100), washed in TBS and incubated with a biotinylated Fab_2 goat anti-rabbit Ig 1:100 (Dako A/S Denmark) for 30 min. at 37 °C. This was followed by washing in TBS and incubation in streptavidine-alkalinephosphatase 1:150 (Amersham, Denmark). The membranes were then incubated with fast red (Sigma) and levamisole (Sigma), and the reaction was stopped with distilled water. Counter staining was done in Mayers haematoxylin. Exclusion of the primary antibody served as a control of antibody specifity.

Collection and analysis of data

Four control wells with medium only in the bottom well were applied for each growth factor experiment. The number of migrated cells in control and stimulated wells was counted in 12 random fields in each well at 100x magnification. To allow comparison of data between different cell strains, the results were normalized by expressing them as a chemotactic index (CI).

$$CI = \frac{\text{Number of migrated cells in test well}}{\text{Number of migrated cells in control well}}$$

This index was determined as the average number of migrated cells in stimulated wells divided by average number of migrated cells in control wells. Coefficients of variation was determined by 16 double measurements .

Statistics

All data are presented as mean (SEM). One-way ANOVA followed by Fishers LSD tests were used to compare differences between control and stimulation groups. P values less than 0.05 were considered significant.

RESULTS

None of the growth factors were found to be cytotoxic at the concentrations used in the osteoblast chemotaxis assay. No cells had dissociated from the membrane into the bottom well. Coefficient of variation for determination of chemotactic indexes were 0.15.

TGF-ß, PDGF-AA, PDGF-BB, IGF-in and IGF-II all induced a chemotactic response in human osteoblasts although to a different degree. TGF-ß stimulated the migration in a bell-shaped manner (max CI = 4.2 ± 0.8) with migration reduced to background levels at concentration gradients exceeding 1 ng/ml (Figure 8.1 A). PDGF-BB

stimulated migration with a different profile in a dose-dependent manner with a maximum at 10 ng/ml CI = 3.7 ± 0.6 (Figure 8.1 B). PDGF-AA, IGF-in, IGF-II and FGF-b stimulated migration with the same chemotactic profiles and CI's of 1.8 - 2.5 at 100 ng/ml (Figure 8.1 C-E). FGF-a did not stimulate cell migration in the concentration range tested (Figure 8.1 F). Average cell counts in control wells were 55 ± 6.4 in the 12 fields counted. The 12 fields counted represented 2.5 mm^2 of the total well area of 8 mm^2. The average total number of migrating cells in control wells was therefore 176 ± 20.5 which represents approximately 2 % of the cells seeded.

Control analysis revealed that the migration induced by TGF-ß, PDGF-AA, PDGF-BB, IGF-in, and IGF-II was due to directed cell migration and not chemokinesis (TABLE 8.2). FGF-b stimulated migration was however due to chemokinesis.

Of the cell migrated in control wells 60 % stained positive for osteocalcin.

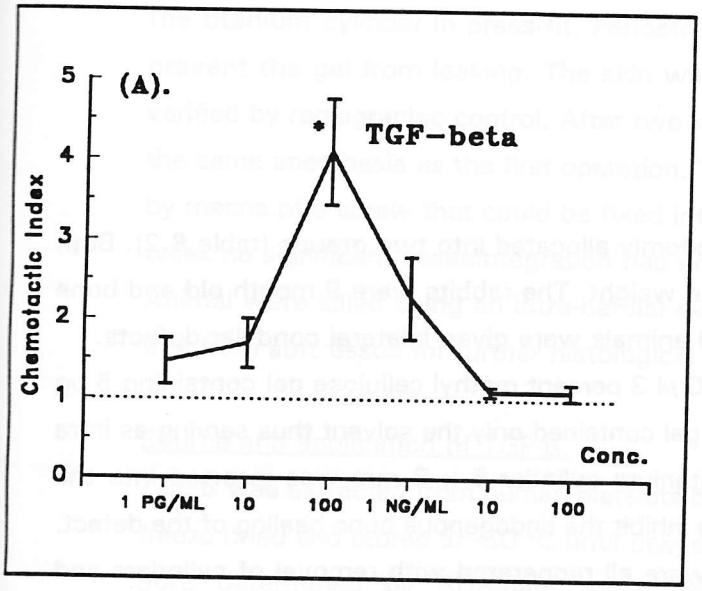
Table 8.2. Control Analysis.

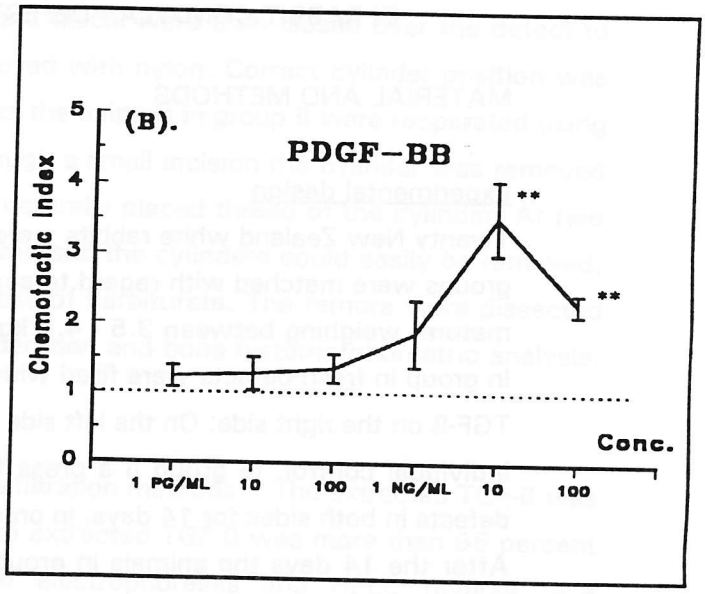
(ng/ml) Growth factor	Growth factor in upper well	Chemotactic index at conc. in lower well 1.0	0.1	0.01 (
TGF-ß	-	2.4	3.3	2.6
	+	1.4	1.2	1.3
		100	**10**	**1 (ng/ml)**
PDGF-AA	-	2.5	1.6	1.2
	+	1.1	1.1	1.1
PDGF-BB	-	2.3	2.0	1.8
	+	1.1	1.2	1.2
FGF-b	-	1.6	1.2	1.2
	+	1.8	1.3	1.3
IGF-I	-	2.1	2.0	1.4
	+	1.0	1.4	1.1
IGF-II	-	1.7	1.6	0.9
	+	0.9	0.8	0.9

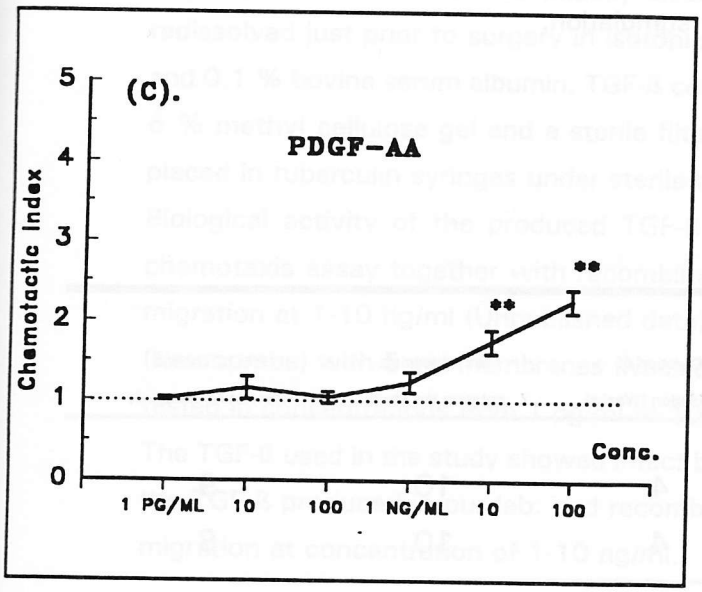
Figure 8.1:

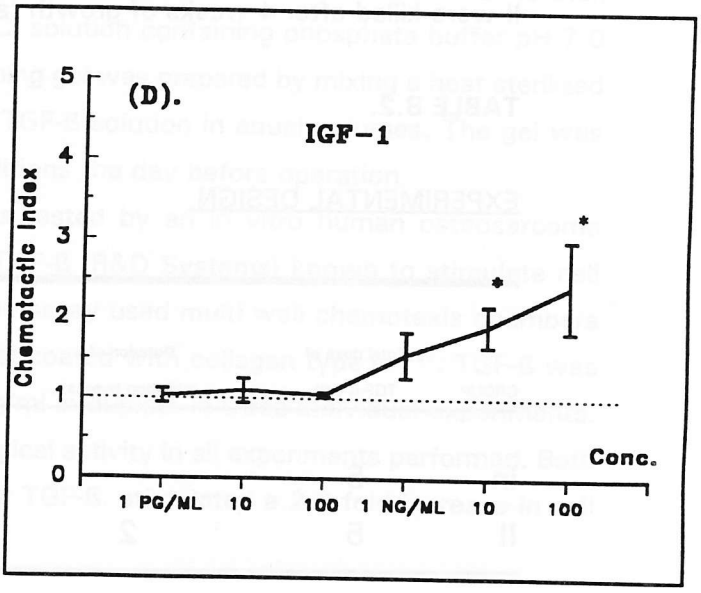
Dose response curve of chemotaxis to TGF-ß **(A)**, PDGF-BB **(B)**, PDGF-AA **(C)**, IGF-1 **(D)**, IGF-2 **(E)** and FGF-acid **(F)**. The chemotactic index (Mean +/- SEM) of duplicate wells from six independent experiments is shown. The one-way ANOVA test showed significant difference between the different concentrations ($P < 0.01$) for all growth factors found to have chemotactic properties. P values are marked * ($P < 0.05$) and ** ($P < 0.01$).

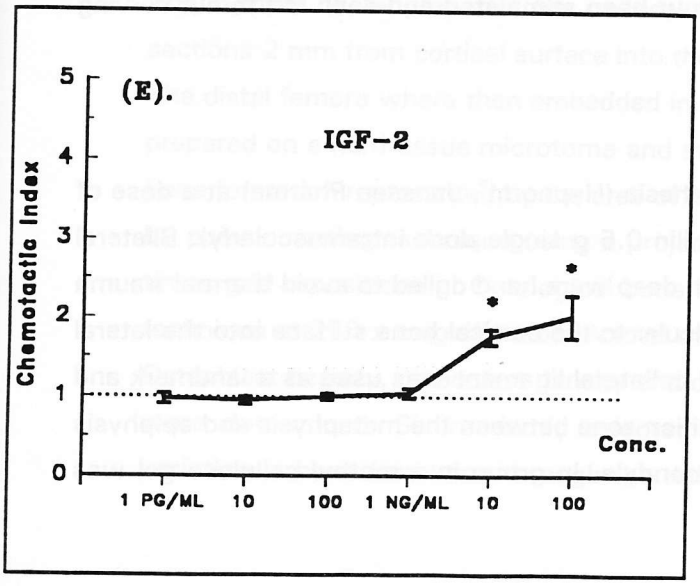
FIGURE 8.1

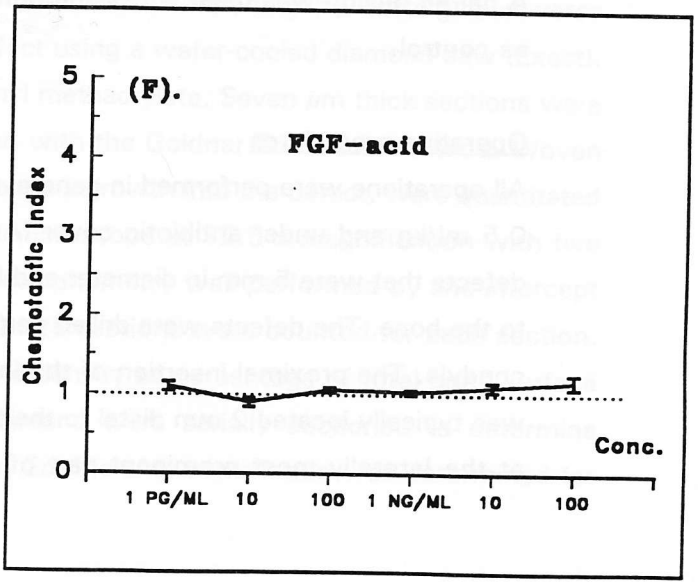

(A).
TGF-beta
Chemotactic Index
Conc.
1 PG/ML
10
100
1 NG/ML
10
100


(B).
PDGF-BB
Chemotactic Index
Conc.
1 PG/ML
10
100
1 NG/ML
10
100


(C).
PDGF-AA
Chemotactic Index
Conc.
1 PG/ML
10
100
1 NG/ML
10
100


(D).
IGF-1
Chemotactic Index
Conc.
1 PG/ML
10
100
1 NG/ML
10
100


(E).
IGF-2
Chemotactic Index
Conc.
1 PG/ML
10
100
1 NG/ML
10
100


(F).
FGF-acid
Chemotactic Index
Conc.
1 PG/ML
10
100
1 NG/ML
10
100

(II) EFFECTS OF LOW DOSE TRANSFORMING GROWTH FACTOR-ß ON BONE HEALING IN RABBIT CONDYLAR DEFECTS.

MATERIAL AND METHODS

Experimental design

Twenty New Zealand white rabbits were randomly allocated into two groups (table 8.2). Both groups were matched with regard to sex and weight. The rabbits were 9 month old and bone mature, weighing between 3.5 - 4.5 kg. All animals were given bilateral condylar defects. In group in fresh defects were filled with 150 μl 3 percent methyl cellulose gel containing 5 μg TGF-ß on the right side. On the left side the gel contained only the solvent thus serving as intra individual control. In group II a press fit titanium cylinder 5 x 8 mm was inserted into the defects in both sides for 14 days, in order to inhibit the endogenous bone healing of the defect. After the 14 days the animals in group II were all reoperated with removal of cylinders and methyl cellulose gel were injected in the same manner as group in. The animals in group in and II were killed after 4 weeks of growth factor stimulation.

TABLE 8.2.

EXPERIMENTAL DESIGN.

GROUP	Total dose of TGF-ß (μg)	Duration of inhibition (weeks)	Duration of stimulation (Weeks)	No. of animals entering the study	No. of animals completing the study
in	5	-	4	10	9
II	5	2	4	10	8

A paired design was used with each right femur been stimulated and each left femur serving as control.

Operative procedures

All operations were performed in general anesthesia (Hypnorm®, Janssen Pharma) at a dose of 0.5 ml/kg and under antibiotic cover (Ampicillin 0.5 g single dose intramuscularly). Bilateral defects that were 5 mm in diameter and 8 mm deep were hand drilled to avoid thermal trauma to the bone. The defects were drilled perpendicular to the cortical bone surface into the lateral condyle. The proximal insertion of the lateral collateral ligament was used as a landmark and was typically located 2 mm distal to the transition-zone between the metaphysis and epiphysis at the laterally most prominent part of the condyle. In group in a methyl cellulose gel was

applied as described in experimental design. In group II similar defects were initially filled with the titanium cylinder in press-fit. Periosteum and fascia were then closed over the defect to prevent the gel from leaking. The skin was closed with nylon. Correct cylinder position was verified by radiographic control. After two weeks the animals in group II were reoperated using the same anesthesia as the first operation. Through a small incision the cylinder was removed by means of a screw that could be fixed into a centrally placed thread of the cylinder. At two week no significant osseointegration had occurred and the cylinders could easily be removed. Animal were killed using an intra-cardial overdose of barbiturate. The femora were dissected free from soft tissue for further histological preparation and bone histomorphometric analysis.

Source and application of TGF-ß.

TGF-ß was extracted from human platelets by gelfiltration methods [8]. The extracted TGF-ß was freeze dried and stored at -80 °C until usage. The extracted TGF-ß was more than 95 percent pure determined by SDS-page silver stained electrophoreses and HPLC reverse face electrophoreses. TGF-ß was initially dissolved in 4 mM HCl and stored at -80 °C. It then redissolved just prior to surgery in isotonic NaCl solution containing phosphate buffer pH 7.0 and 0.1 % bovine serum albumin. TGF-ß containing gel was prepared by mixing a heat sterilized 6 % methyl cellulose gel and a sterile filtered TGF-ß solution in equal volumes. The gel was placed in tuberculin syringes under sterile conditions the day before operation.

Biological activity of the produced TGF-ß was tested by an in vitro human osteosarcoma chemotaxis assay together with recombinant TGF-ß (R&D Systems) known to stimulate cell migration at 1-10 ng/ml (Unpublished data). The assay used multi well chemotaxis chambers (Neuroprobe) with 5 μm membranes (Nucleopore) coated with collagen type in [237]. TGF-ß was tested in concentrations from 1 pg/ml to 100 ng/ml in duplets in three individual experiments. The TGF-ß used in the study showed intact biological activity in all experiments performed. Both the TGF-ß produced in our lab. and recombinant TGF-ß. stimulated a 2-3 fold increase in cell migration at concentration of 1-10 ng/ml.

Bone preparation and histomorphometry

The femora were prepared for undecalcified microtome sectioning by cutting transverse sections 2 mm from cortical surface into the defect using a water-cooled diamond saw (Exact). The distal femora where then embedded in methyl methacrylate. Seven μm thick sections were prepared on a hard tissue microtome and stained with the Goldner Trichrome method. Woven bone formation represented by the area of new bone ingrowth into the defect, were quantitated by point counting technique using a projection microscope at 12.5 x magnification with five times grid repositioning. Cytological bone histomorphometry was performed by line-intercept technique at 250 x magnification. A total of 400 intersections were counted for each section. Osteoblast density, osteoclast density and osteoid density as percentage of total bone surface were determined. Six randomly selected specimens were serially sectioned to determine variation in bone formation through the defects. Coefficients of variation was determined for

all histomorphometric parameters from 8 double measurements .

Statistics

All data are presented as mean (SEM). All data sets were tested for approximation to normal distribution by probit analysis. Unpaired two-tailed student T-test were used to compare differences between group in and II. Paired two-tailed students T-test were used to compare differences between control and stimulated specimens within a group. P values lesser than 0.05 were considered significant.

RESULTS

In group in and II, one and two animals, respectively, were excluded due to fracture through the drill holes (Table 8.2). The animals had an average weight loss of 200 g after six weeks of observation. The weight loss did not differ between groups. Bone formation was significantly decreased in the inhibited group II in controls as compared with group in ($P = 0.011$). Within group in and II no significant differences were found between controls and TGF-ß stimulated defects with regard to woven bone formation but there was a trend towards inhibition of bone formation when the defects were stimulated with TGF-ß (Table 8.3). Regarding cytologic parameters no differences were found for osteoblast fraction, osteoclast fraction and osteoid fraction (Table 8.3). Coefficients of variation determined by 8 double measurements were 0.03 for woven bone formation, 0.06 for osteoblast fraction, 0.07 for osteoid fraction and 0.12 for osteoclast fraction measurements.

Table 8.3

BONE MORPHOMETRY.

GROUP	Woven bone formation (mm^2)	Osteoblast density (Percent)	Osteoclast density (Percent)	Osteoid density (Percent)
in (Cont)	8.5 (0.9)*	65.6 (2.0)	2.2 (0.35)	38.9 (4.2)
in (Stim)	5.3 (1.7)	68.4 (1.7)	2.4 (0.37)	35.2 (1.8)
II (Cont)	4.3 (1.1)*	75.4 (3.1)	1.7 (0.30)	45.6 (5.3)
II (Stim)	2.8 (0.7)	69.3 (1.7)	1.2 (0.35)	45.8 (4.0)

Group in : Fresh defect.

Group II: Inhibited defect.

* Significant difference between group in and II controls ($p < 0.02$)

(III) Stimulation of fracture healing in rabbit tibiae with transforming growth factor beta.

MATERIAL AND METHODS

Experimental design

Thirty New Zealand white rabbits were randomly allocated to three groups (table 8.4). All groups were sex and weight matched. The rabbits were 9 month old and bone mature weighing between 3.5 - 4.5 kg. All animals were given unilateral plated mid-tibial osteotomies. Animals in group in, II, III had local application of 0 μg, 1.0 μg and 10 μg TGF-ß/day, respectively. The group receiving only the solvent with 0 μg TGF-ß served as control group. The observation period was 6 weeks.

TABLE 8.4

EXPERIMENTAL DESIGN.

GROUP	Daily dose of TGF-ß (µg/day)	No. of animals entering the study	No. of animals completing the study
in (Control)	0	10	8
II	1.0	10	9
III	10.0	10	6

Operative procedures

In general anesthesia (Hypnorm®, Janssen Pharma, Holland) at a dose of 0.5 ml/kg and under antibiotic cover (Ampicillin 0.5 g intramuscularly) unilateral midtransverse tibial osteotomies were performed through the tibiofibula junction with an oscillating saw. The osteotomy was stabilized using a four-hole AO DCP plate (52 x 7.5 x 2.0 mm) and four screws (2.7 mm) positioned on the anterolateral surface of the right tibia. A subcutaneuos mini-osmotic pump (Alzet 2ML4 pumping rate 3.0 μL/hour) was connected to the osteotomy line via a 0.5 mm inner diameter polyvinyl catheter. [120]. All pumps were exchanged in general anesthesia after 3 weeks. To minimize variation in stability of the osteotomy, all operations were performed by the same surgeon. The animals were killed after 6 weeks using intra-cardial overdose of barbiturate. In the control group and the 10 μg TGF-ß group one and three animals, respectively, were excluded due to fracture through the distal drill holes. In all three groups one animal

died from unknown reasons. The animals had an average weight loss of 250 g after six weeks of observation. The weight loss did not differ between the groups.

Source and application of TGF-ß.

TGF-ß was extracted from human platelets by gelfiltration methods [8]. The extracted TGF-ß was more than 95 % pure was freeze dried and stored at -80 ° C until usage. TGF-ß was dissolved in isotonic NaCl solution containing phosphate buffer pH 7.4 and 0.1 % bovine serum albumin. Biological activity of the produced TGF-ß was tested by an increase in collagen production in a fibroblast in vitro assay. TGF-ß was delivered by Alzet miniosmotic pumps connected to the osteotomy lines via polyvinyl catheters. Also TGF-ß released from a miniosmotic pump for 3 weeks at 37 ° C was tested in the fibroblast in vitro assay. The freshly produced TGF-ß was able to stimulate collagen production in the in vitro assay. Also TGF-ß, that had been released from a miniosmotic pump at 37° C for three weeks, showed intact biological activity.

Bone densitometry

After termination the AO plate was removed from the tibia and the bone mineral content (BMC) was determined by photon absorptiometry (Gammatec® Osteodensiometer Model GT 30). Bone mineral content (BMC) was determined in a standardized area from 8 mm proximal to 8 mm distal from the osteotomy line. The scanner determined BMC in 4 mm segments. BMC was calculated as the mean of the 4 segmental scans. Coefficient of variation determined by 10 double measurements was 0.03.

Mechanical testing

The tibial shaft was loaded to failure in a three point bending set-up on an universal testing machine (Instron Ltd, England) at a constant deformation rate of 5 mm/min. The tibia was placed on the posterior surface and the load was applied at the osteotomy site on the anterior edge of the bone. The distance between the supporting bars was 4 cm. The load-deflection curve was plotted by a X-Y writer. From the plot maximal bending strength (F-max) and stiffness at the osteotomy was determined.

Bone morphometry

Transverse sections 100 μm in thickness were cut on a water-cooled diamond saw (Exact®) 5 mm distally to the osteotomy line. From these sections histomorphometric evaluation was performed using a computer assisted light microscope. Callus area was determined by computer tracing of the callus tissue laying on the cortical bone. Cortical thickness and Haversian canal diameter were determined at ten randomly selected places on each section using a digitizer connected to the microscope. Coefficients of variation was determined for all histomorphometric parameters from 8 double measurements .

Statistics

All data are presented as mean (SEM). One-way ANOVA followed by Fisher LSD-tests were

used to compare differences between control and stimulation groups. p values less than 0.05 were considered significant.

RESULTS

Mechanical Testing: The mechanical test showed a stronger maximal bending strength in the TGF-ß stimulated groups when compared with the control group (Figure 8.2) P = 0.03. In the group stimulated with 1.0 μg TGF-ß the result was significantly different from control. In the 10 μg TGF-ß/day group a tendency of increased strength of the osteotomy was observed but the result was not statistical significant (p = 0.07). Stiffness were found to be 358 (93), 571 (149), 680 (134) N/mm in group in, II, and III, respectively. Here a clear trend to increased values in the groups stimulated with TGF-ß was found, but the result was not statistical significant.

Histomorphometry: The callus area was increased in both groups given TGF-ß as compared with the control group (Table 8.5) . No differences in cortical thickness and Haversian canal diameter were found (Table 8.5). The histological appearance of the callus tissue in both control and TGF-ß groups was mature woven bone with an woven bone fraction between 50 -70 %. Coefficients of variation determined by 8 double measurements were 0.03 for callus area, 0.026 for cortical thickness and 0.061 for Haversian canal diameter measurements.

BMC: No differences in bone mineral content were demonstrated between the control and the TGF-ß stimulated groups. The mineral content was 0.515 (0.023), 0.505 (0.026), 0.551 (0.029) g/cm in groups in, II and III respectively.

TABLE 8.5

BONE MORPHOMETRY.

GROUP	Callus area (area unit)	Cortical thickness (μm)	Haversian canal Diameter (μm)	
in (Control)	44.7 (5.5)	1232 (38)	32.9 (3.4)	(n=8)
II	77.5* (14.0)	1225 (41)	33.0 (2.0)	(n=9)
III	99.2** (18.4)	1202 (17)	30.9 (2.1)	(n=6)

*: Significant difference between group in and II ($p < 0.05$)

**: Significant difference between group in and III ($p < 0.01$)

FIGURE 8.2

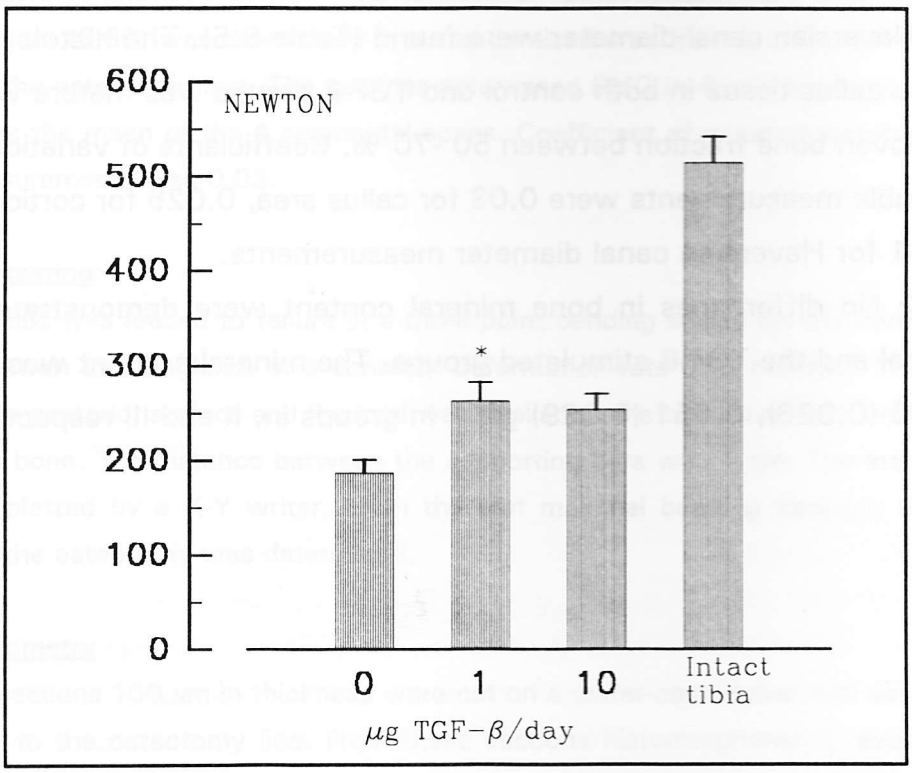


FIGURE 8.2

Effect of TGF-ß on maximal strength in three point bending test after 6 weeks of stimulation. The group receiving only solvent (0 μg TGF-ß/day served as control. The intact tibia group illustrates the strength of the intact tibia on the contralateral side. Values are expressed as mean (SEM). * $P < 0.05$.

CHAPTER 9

DISCUSSION OF EXPERIMENTAL RESULTS

(in) (Chemotactic effects of growth factors) 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 [185,237]. The present study indicates 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. Both showed a four-fold stimulation of migrated cells and these growth factors probably serves as key regulators of osteoblast migration in bone tissue. Many growth factors are incorporated in bone matrix during bone formation [95]. In fact, bone matrix contain the highest concentrations of TGF-ß in the body [44].

In the present study TGF-ß exhibited the most potent osteoblastic chemotactic response. This finding supports the hypothesis that TGF-ß is a key regulator of both bone remodeling and bone healing. A theory of how TGF-ß exerts its chemotactic actions during bone remodeling could be that: TGF-ß is protected against hydrolytic cleaverage by binding to a binding-protein during osteoclastic bone resorption. Subsequently the peptide is released from the binding protein [56,182,183] and may exert inhibitory actions on osteoclasts and enhance osteoblast recruitment. The osteoblast will migrate toward the resorption lacunae where other local factors liberated from bone matrix (e.g. IGF's) will further stimulate osteoblastic proliferation and production of matrix proteins [174,184,239]. Thus, the findings in this study 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 bone physiological importance since migration of osteoblasts are crucial events in both situations.

The chemotactic effects found in this study cannot be ascribed to toxic effects of the growth factors since none of these were found to be cytotoxic at the concentrations tested in the chemotactic assays. The stimulation of migration found for TGF-ß, PDGF-AA, PDGF-BB, IGF-in and IGF-II were all due to chemotaxis since elimination of the concentration gradient during the control analysis was able to

lower migration to control levels. Human osteoblastic cell lines are known to be of partly heterogenic phenotype [198]. We found that only small fractions (2-5%) of the seeded cells migrated in our assay. It could therefore be speculated that cells with a fibroblast like phenotype were the cells migrating since fibroblasts are found to be very chemotactic responsive to growth factors. We found, however that a majority of the migrated cells stained positive for osteocalcin which is one of the most specific osteoblast markers.

Osteosarcoma and prostate adenocarcinoma are known to produce both osteosclerotic and osteolytic bone lesions. In an osteosclerotic bone lesion the invading cells have produced an intense stimulation of bone formation which includes recruitment of osteoblasts. It could therefore be speculated that the cancer cells produce high amounts of one or more of the growth factors shown to have chemotactic properties in the present study. In the cancer cell environment the concentration of the growth factors reaches superphysiologically levels thus inducing massive bone cell recruitment, cell proliferation and matrix protein synthesis [125]. Uncontrolled production of growth factors by cancer cells may result in pathological lesions in the bone. In an osteolytic lesion an extensive recruitment and stimulation of osteoclasts must have taken place. However, the mechanisms controlling osteoclast migration are still largely unknown [168].
The chemotactic properties of TGF-ß 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 [19,112]. The chemotactic abilities of TGF-ß combined with the anti-inflammatory properties of TGF-ß [30] makes this growth factor a possible inhibitor of inflammatory bone resorption as seen in rheumatoid arthritis. Clinical usage of growth factors could be speculated in situations where improved bone formation is needed such as healing of large bone defects in orthopedic oncology, healing of pseudarthrosis and in noncemented prosthetic surgery in patients with osteoporosis and rheumatoid arthritis.

(II) (Bone defects in rabbits and effects of TGF-ß) A major problem when investigation effects of bone healing promoting agents in in vivo model is to separate effects of the applied agent and the animals own bone healing ability. In this study we tried to overcome this problem by using a rabbit defect model initially

inhibited for two weeks by a titanium cylinder. Our data shows that it was possible to significantly reduce the bone formation in control defects inhibited by titanium cylinders. A newly traumatized bone surface is known to present and release numerous growth factors activating mesenchymal stem cells and resting osteoblasts to initiate a healing response [101,213,258,259]. The effects of growth factor application after inhibition may therefore be examined with reduced interactions from endogenous growth factors. TGF-ß is found in high concentration in bone matrix only surpassed by IGF-II, and osteoblasts are the celltype bearing the highest concentration of TGF-ß receptors [53,227]. This correlates with studies showing that osteoblasts are very responsive to TGF-ß both regarding proliferation, differentiation and matrix protein synthesis [227]. An ability to promote bone healing when applied exogenous would be anticipated for TGF-ß. Growth factors have previously been applied in polysaccharide carriers in bone healing models with success [19,256]. In the latter study a three percent methyl cellulose gel carrier containing 2 μg TGF-ß induced bone healing of large (12 mm) calvarial defects in rabbits. We adapted this method of using methyl cellulose as carrier for TGF-ß.

Results from the present study did not demonstrate any enhanced healing of trabecular bone as a result of the 5 μg natural TGF-ß stimulation. The effects of TGF-ß was generally inhibitory for woven bone formation although the inhibition was not significant. This trend was seen for both inhibited and fresh defects. In the study by Beck et al. membranous bone healing was successfully stimulated be an equivalent dose of TGF-ß [19]. So our finding of a trend towards an inhibitory effect of TGF-ß on bone formation is controversial compared to previous studies. It could be speculated that a different sensitivity and response to TGF-ß stimulation exist between membranous bone in the calvaria and trabecular bone of the femur. Also the methyl cellulose carrier could have produced an inflammatory reaction with increased local concentration of interleukins and other inflammatory mediators. Previous studies have shown that TGF-ß interacts with interleukin-1 to create a bone resorption response probably by increasing cellular production of prostaglandins [131,146]. Such interactions could explain the trend towards inhibitory effects of TGF-ß in this model. We did, however, not find any signs of inflammation neither during dissection of femora nor at microscopy of the defects.

In the osteotomy healing study included in this thesis (III) we found a dose dependent increase in callus formation in a tibial osteotomy model using TGF-ß doses from 1 - 10 μg/day [130]. These doses were considerably higher than the 5 μg

total dose used in the present study and a higher dose might be needed to stimulate healing of long bones in rabbits.

A key problem when investigating effects of growth factors is to ensure an intact biological activity of the agent used. Growth factor peptides are known to be labile to different chemical environmental conditions. In the present study the TGF-ß batch used, was tested for osteoblast related biological activity in an osteosarcoma in vitro chemotactic assay [104,237]. Here we found equivalent activity of the used TGF-ß compared to recombinant TGF-ß. It could be speculated that TGF-ß activity decreased with time but we have previously shown that TGF-ß maintain its activity for at least three weeks in physiological liquid at 37 °C [130].

The results from the model suggest that it is possible to inactivate endogenous bone healing in rabbit femoral bone defects. This may provide basis for a more isolated study of effects of bone healing promoting agents. We could not demonstrate any stimulative effects of 5 μg TGF-ß application in this defect model.

(III) (Stimulation of fracture healing with TGF-ß) Sixfold increase in calvarial bone thickness has been demonstrated in rats that were given local daily calvarial injections of 1 μg TGF-ß [113], and a considerable stimulation of new bone and cartilage formation was found when 0.2 μg TGF-ß was injected subperiosteally in femurs of newborn rats [172].

We were able to demonstrate that TGF-ß could enhance the mechanical strength and stiffness of a tibial osteotomy after six weeks of stimulation of 1 and 10 μg TGF-ß daily [130]. The general stimulatory effect of TGF-ß on fracture healing might be ascribed to the stimulation of a larger callus formation around the osteotomy. The increased callus envelope around the diaphyseal bone ends causes increased mechanical strength of the osteotomy (Mark Bolander, Mayo Clinic, personal communication). We found an increased callus formation with increased doses of exogenous TGF-ß. 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). Stimulation of callus formation found in the present study is in agreement with previous in vitro studies on callus tissue, that have shown stimulatory effect of several growth factors [112,114]. 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 [112,204].

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 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. Six weeks growth factor application might therefore not be long enough to see 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.

The doses used in the present study were chosen from previous in vivo studies using injectional application [113,140,172]. In these studies doses from 0.2 μg to 5.0 μg TGF-ß/day were found to stimulate new bone formation in young rats. These studies however, used rats as experimental animal and since rabbits are considerably bigger animals doses of 1 and 10 μg pr day were chosen for the present study.

Most previous in vivo studies with TGF-ß have been performed in immature rodents with a greater growth potential than the mature rabbits used in the present study. Also a species related difference in reaction to TGF-ß stimulation could be speculated. However a study by Beck et al has shown that low doses of recombinant human TGF-ß1 (70 ng/day) released from a polysaccharide matrix was able to stimulate bone formation in calvarial defects in mature rabbits [19]. Other growth factors present in callus tissue may influence bone healing and experimental evidence suggest that TGF-ß and PDGF are able to stimulate cell growth in callus tissue [112]. Combinations of growth factors might be able to enhance bone formation even further.

This study demonstrates that local application of exogenous TGF-ß can enhance

fracture healing in adult experimental animals. This finding indicates that TGF-ß might develop into an important clinical tool that can enhance bone healing if delayed fracture healing is expected.

Conclusions

1) Several growth factors (TGF-ß, PDGF-BB, PDGF-AA, IGF-in and IGF-II) were shown to stimulate chemotactic migration of human osteoblasts.
2) It was possible to inhibit endogenous bone healing in rabbit femoral bone defects. We could not demonstrate any stimulative effect of 5 μg TGF-ß in methyl cellulose gel on bone formation or woven bone cytology in femoral defects. A tendency to decreased woven bone formation in the defects that received TGF-ß was demonstrated.
3) Continuous local application of 1 and 10 μg TGF-ß for six weeks enhanced callus formation and mechanical strength of a stable osteotomy. A tendency to increased stiffness of the osteotomy was demonstrated.

Future Research

The 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. In vitro studies that investigate stimulation of bone cells with combinations of growth factors are desired because growth factors possibly act in concert in vivo. Future in vivo research should also focus on models with clinical relevance.

Especially local enhancement of non-cemented implant fixation by growth factors is an important area in orthopedic surgery, that could benefit significantly by improving bone healing response, since traditional non-cemented techniques have largely failed. Also studies that investigate dose response effects of growth factor stimulation would be of significant interest. Clinically favorable application methods is crucial for the use of growth factors as stimulators of bone healing in patients. Studies that solve problems with applications are needed before clinical use is possible.

CHAPTER 10

DANSK RESUMÉ

Ph.D. afhandlingen omhandler effekten af vækstfaktorer på knogleheling undersøgt i in vitro og in vivo studier. Projektet har været udført på Biomekanisk Lab, Ortopædisk Hospital, Århus samt Institut for Eksperimentel Klinisk Forskning, Skejby Sygehus.

Vækstfaktorer er peptid signal molekyler som lokalt regulerer celle metabolisme i alle væv. Knoglematrix er reservoir for en række vækstfaktorer som initierer knogleheling og regulerer knogle remodelering. Specielt vækstfaktoren Transforming Growth Factor-ß (TGF-ß) har en høj koncentration i knoglevæv og har meget potente stimulative effekter på knogleceller. Dette har ført til ønsket om at anvende TGF-ß som in vivo stimulator af knoglenydannelse. Anvendt i kliniske situationer med behov for øget knogledannelse vil en sådan stimulation kunne medføre forbedringer af bl.a. ucementeret endoprotese kirurgi og fraktur kirurgi.

In vitro studie: Vækstfaktores effekter på celledeling af osteoblaster og matrix protein syntese er velbeskrevet. Cellemigration (kemotaksi) er en vigtig cellulær proces ved knogleheling men er mindre velkarakteriseret for osteoblasten. Derfor blev syv vækstfaktorer med kendte metabolisk stimulative effekter på osteoblaster undersøgt for kemotaktiske effekter overfor humane osteoblaster. Microwell chemotaxis chambers blev anvendt som kemotaksi assay. Analysen viste at TGF-ß og Platelet Derived Growth factor-BB (PDGF-BB) stimulerede cellemigrationen med 200-300 %, men også vækstfaktorerne Insulin Growth factor I og II (IGF I og II) samt Platelet Derived Growth Factor-AA PDGF-AA kunne stimulere cellemigrationen med 100 %.

Knogle defekt studie: Eksperimentelle studier af eksogen stimulation af heling af defekter i knogle er ofte kompromitteret af stor endogen knoglehelings evne hos forsøgsdyr. Vi forsøgte derfor at konstruerer en dyre eksperimentel model med hæmmet endogen healing af femur kondyl defekter hos voksne kaniner. Vi fandt at var muligt at hæmme endogen knoglenydannelse i borehuls defekter i femur kondylerne ved hjælp af en titanium cylinder. Heling af den trabekulære knogle i femur kondylerne blev forsøgt stimuleret med 5 μg TGF-ß i en methylcellulose gel. Der blev stimuleret med TGF-ß i både friske og inhiberede defekter. TGF-ß applikation medførte ikke øget knogle dannelse eller forandringer i knoglecelle aktivitet efter 4 uger.

Fraktur studie: Hos voksne kaniner blev heling af osteosynteret tibia osteotomi

stimuleret med kontinuerlig tilførsel af 1 og 10 µg TGF-ß dagligt i seks uger. Den mekaniske styrke og stivhed øgedes med 40-50 % ved TGF-ß stimuleringen og kallus dannelsen øgedes med ca. 100 %. Knogle remodelering i tibias diafyse ændredes ikke som følge af stimulationen.

Det konkluderes at vækstfaktorer specielt TGF-ß og PDGF-BB virker kemotaktiske på humane osteoblaster. Disse vækstfaktorer kan derfor være vigtige for rekruttering af osteoblaster under knogleheling. Det lykkedes at udforme en defekt helings model hvor endogen knogleheling blev hæmmet v.h.a. en titanium cylinder. I denne model fandtes ingen stimulativ effekt af 5 µg TGF-ß i methylcellulose gel. Derimod kunne vi påvise en stimulativ effekt af TGF-ß på en stabil osteotomi. Den forøgede mekaniske styrke opstår sandsynligvis p.g.a. en kraftig stimulation af kallus dannelse som danner en øget knoglebro over osteotomien. Disse resultater indikerer at vækstfaktorer specielt TGF-ß kan anvendes til klinisk relateret stimulation af knogleheling ved anvendelse af den rette applikations form og dosering.

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