Current concepts

Growth factors in bone

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Summary. *Bone contains several growth factors, including bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), insulin-like growth factors I and II (IGF-I and IGF-II), platelet derived growth factor (PDGF) and basic and acidic fibroblast growth factor (bFGF and aFGF). Spatial and temporal variations in the expression and secretion of the various growth factors have been demonstrated in osteoblastic cultures and in various experimental and clinical in vivo models, including fracture healing in humans. Local application of various growth factors influences proliferation, differentiation and protein synthesis in osteoblastic cultures and bone formation in different animal models, including experimental fractures and skeletal defects. The BMPs are the only growth factors known to provoke bone formation heterotopically by making undifferentiated mesenchymal cells differentiate into osteoblasts (osteoinduction). BMPs and other growth factors, soon to become commercially available for clinical use, need a delivery system for their sustained release, as the factors are otherwise rapidly absorbed. Some existing systems inhibit bone formation by inducing chronic inflammation or physically by unresorbed carrier obstructing bone formation. New delivery systems are being investigated.*

Résumé. *L'os contient plusieurs facteurs de croissance notamment: la protéine morphogénétique osseuse (BMPs), le facteur de croissance bêta (TGF-B), les facteurs de croissance insuline-like I et II (IGF-I et IGF-II), le facteur de croissance plaquettaire (PDGF) et les facteurs de croissance fibroblastique basique et acide (bFGF et aFGF). Des variations spatiales et temporelles de la secrétion des différents facteurs de croissance ont été démontrées dans les cultures ostéoblastiques et dans différent modèle expérimental et clinique notamment, la consolidation de fracture chez l'homme. L'application locale des différents facteurs de croissance influence la prolifération, la différenciation et la synthèse protéique dans les cultures d'ostéoblastes ainsi que la formation osseuse dans les différents modèles animaux avec notamment fracture expérimentale et perte de substance squelettique. Les BMPs sont les seuls facteurs de croissance connus pour provoquer une formation d'os hétérotopique par différenciation en ostéoblastes des cellules mésenchymateuses indifférenciées (ostéoinduction). Les BMPs et les autres facteurs de croissance seront bientôt commercialement disponibles pour l'usage clinique ce qui nècessitera des systèmes de distribution particuliers pour leur préservation. Il existe déjà des systèmes inhibant la formation osseuse de façon physique ou en induisant une inflammation chronique.*

Introduction

In 1938, Levander found that an acid alcohol extract of bone and callus induced heterotopic cartilage and bone when injected intramuscularly in rabbits and concluded that bone regeneration takes place as the result of some specific bone-forming substance activating the nonspecific mesenchymal tissue [38]. However, the study of growth factors in bone was hampered because these factors are sequestered in abundant extracellular matrix which has to be removed before further purification can be done. Mod-

ern laboratory techniques, including recombinant DNA technology, have made it possible to identify several bone growth factors in recent years, including bone morphogenetic proteins (BMPs), insulin-like growth factors I and II (IGF-I and IGF-II), transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF) and basic and acidic fibroblast growth factor (bFGF and aFGF).

Growth factors are polypeptides that are generally synthesised by specific tissue where, in very low concentrations, they act as local regulators of cell function. Most growth factors are released as high molecular weight precursors which are split by proteolysis and produce active factors which are generally of low molecular weight. Growth factors elicit their action by binding to specific large transmembrane receptors on the cell surface of the target cell. Binding to the extracellular domain of the receptor triggers the intracellular domain, which generally activates a protein kinase. The kinase cascade activates transcription of a gene into mRNA, which is then translated into proteins to be used within the cell or exported [71]. Generally, the number of active receptors of a cell decreases when the growth factor is present in excess (down regulation), whereas it increases when there is a deficiency (up regulation).

Most of the knowledge about the effect of growth factors on osteogenic cells derives from studies on cultures of osteoblast-like cells from embryonic bone tissue of the rat or mouse, or from osteosarcoma cell lines. Less is known about the physiological effect of the different growth factors in vivo, but some studies have been done on immunolocalisation and expression of different growth factors in fracture healing (Table 1).

BMPs

Urist believed that osteoinduction by demineralised bone [72], was caused by a contained factor which he named bone morphogenetic protein (BMP), and in 1979 the group isolated an extract of osteoinductive glycoproteins [73]. In 1988, Wozney et al. extracted 40 μg inductive proteins from 40 kg of bone. Chromatography showed that the extract contained several proteins, but further purification was not possible. The proteins were tryptinized, cDNA was cloned, recombinant human peptides were made in Chinese hamster ovary (CHO) cells and three proteins were demonstrated, BMP-1, BMP-2A and BMP-3 [80]. Later, more BMPs were identified, and there are at present at least 15. The BMPs, except BMP-1, are part of the TGF-β supergene family. They are important in both extraskeletal and skeletal organogenesis, bone generation and regeneration, and are involved in certain disease processes; mutations of their genes may cause developmental anomalies [54].

BMP receptors have been identified [50], and upregulation of the expression of BMP receptors during bone formation in embryonic development and fracture healing have been demonstrated [34]. A large

Table 1. Growth factors and fracture healing

Stage of healing	Growth factor	Source, localisation and action	References
Immediate injury response	BMP-2/4	Found in mesenchymal cells in hematoma and cambium layer of periosteum adjacent to fracture. BMP-4 mRNA is present in osteoprogenitor cells of the proliferating periosteum, the medullary cavity and the muscles near the fracture.	[16, 48]
	TGF-β	Released by platelets and inflammatory cells into the fracture hematoma. Stimulates proliferation of mesenchymal cells in cambium layer of periosteum.	[14, 37]
	PDGF	Released by platelets and inflammatory cells into the fracture hematoma. Found in macrophages close to the periosteum 2 days post-fracture, decreases from day 3. Stimulates proliferation of mesenchymal cells in cambium layer of periosteum.	[14, 17]
	aFGF	Found in cells of the expanded cambium layer and associated with rapid increase in mesenchymal cells.	[17]
Intramembranous bone formation	BMP-2/4	Found in osteoblasts lining primitive (woven) bone by 6 days after fracture. Decreases as the bone matures.	[16]
	TGF-β	Found in proliferating mesenchymal cells, in osteoblasts lining new bone and in the matrix.	[37]
	PDGF	Released by platelets and stimulates intramembranous bone formation.	[14]
Chondrogenesis	BMP-2/4	Chondroid precursor cells stain intensely just prior to becoming immature chondrocytes.	[16]
	TGF-β	Found in mesenchymal cells, young chondrocytes and mature chondrocytes.	[37]
	IGF-I	Found in young chondroblasts at the edge of the cartilage mass replacing fibrous tissue.	[17]
	aFGF	Synthesized by chondocytes, their precursors and macrophages. Stimulates chondrocyte proliferation and is probably important for chondrocyte maturation.	[14]
Endochondral ossification	BMP-2/4	Intense intracellular staining of osteoblasts lining the calcified cartilage matrix.	[16]
	TGF-β	Found in the matrix surrounding hypertrophic chondrocytes.	[37]
	TGF-β	Found in chondrocytes at the edge of the ossification front.	[17]
	bFGF	Probably produced by chondrocytes and is important for endochondral bone formation.	[14]

number of binding sites for BMP-2 have been found not only on osteoblastic cells but also on various other normal cells and tumour cells [35].

Recombinant human BMP-2 in rat inactive demineralised bone matrix carrier implanted subcutaneously in rats induces cartilage by 5 days, some new bone by 7 days and an ossicle with marrow at 21 days [75]. When the concentration of BMP-2 is increased, bone formation is observed earlier and cartilage and bone are formed concurrently. The osteoinductive response by BMP-4 and BMP-5 seems to be weaker than that of BMP-2 [79]. Cartilage and bone formation is induced in athymic mice by CHO cells transfected with the murine BMP-6 gene [29] or BMP-4 gene [58].

The ability of implanted BMPs to induce heterotopic osteogenesis in primates is still not clear. Aspenberg et al. found that rhBMP-2 combined with demineralised bone induced heterotopic osteogenesis in squirrel monkeys, whereas demineralised bone alone did not [9]. However, the amount of induced bone was small. In a recent study, rhBMP-2 and bovine type I collagen implanted intramuscularly in squirrel monkeys resulted in an osteoinductive response that was small, inconstant and partly dependent on placing the implants close to the muscle aponeurosis [8].

Several BMPs, including BMP-2, BMP-3, BMP-4, BMP-6 and BMP-7 converted the differentiation pathway of different pluripotent mesenchymal cell lines into that of an osteoblast lineage [2, 3, 81]. BMP-7 (OP-1) can make different pluripotent mesenchymal cell lines differentiate into both chondroblastic and osteoblastic cells, depending on the stage and potential of the target cell [6]. Messenger RNAs for BMP-2 and BMP-4 are expressed in foetal rat calvarial osteoblasts as they differentiate in primary cultures prior to forming mineralised bone nodules coinciding with expression of osteocalcein, osteopontin and alkaline phosphatase [31]. BMPs have been shown to be involved in fracture healing (see Table 1) [16, 48].

Implanted rhBMP-2 enhances the healing of: (1) defects of the femur of rats [82] and sheep [28]; (2) defects of the mandible of rats (in combination with membranes) [41], dogs [70] and rhesus monkeys [18]; and (3) lumbar intertransverse process fusion in rabbits [55] and dogs [32]. Recombinant human BMP-7 (OP-1) enhances the healing of segmental ulnar defects in rabbits [24], dogs [23] and African green monkeys [25]. Recently, rhBMP-2 has been shown to stimulate the repair of bone and hyalin-like cartilage in experimental osteochondral defects in rabbits [57].

Recombinant human BMP-2 is currently being evaluated in clinical studies in humans for augmentation of the floor of the maxillary sinus to increase the bone height and allow the placement of dental implants. It is expected that in the next few years, rhBMPs in suitably delivery systems will be evaluated in clinical studies for a number of other indications in orthopaedic and cranio-maxillofacial trauma and disease.

TGF-β

The TGF-βs are members of the TGF-β superfamily. So far, five subtypes have been demonstrated. TGF-β4 is found in chickens, TGF-β5 in amphibians. The TGF-βs are 25 kDa homodimers sharing 60 to 80% similarity in their amino acid sequence. TGF-βs influence a variety of tissues, generally stimulating cells of mesenchymal origin and inhibiting cells of ectodermal origin [39]. TGF-β is produced by osteoblasts and is stored in the bone matrix, making bone the largest reservoir of TGF-β in the body [15]. Osteoblasts have the highest number of TGF-β receptors [39].

TGF-βs mediate tissue embryogenesis, normal cellular physiology, inflammation and tissue repair [27]. TGF-β1 is found in the periosteum at an early stage in fractures, both in experimental fractures in animals [14] and clinically in humans [4]. TGF-β1 enhances the proliferation of mesenchymal cells and osteoblasts in fractures as well as in experimental bone defects [10, 14, 65].

Whereas BMPs induce bone in heterotopic sites, stimulation of bone formation by TGF-β depends on orthotopic application, for example, subperiosteal injection [65]. TGF-β1 enhances the healing of experimentally created defects of the skull in rabbits [11, 47] and bone ingrowth in porous titanium rods [64] and tricalcium phosphate coated implants in dogs [40].

PDGF

PDGF is a dimer of two peptides, A and B, sharing 60% amino acid sequence homology. Dependent on the combination of peptides, the molecular weight varies between 28 and 35 kDa. PDGF-BB homodimer is more biologically active than the two other dimers. PDGF-BB and AB act as systemic growth factors, whereas PDGF-AA acts as a local growth factor in bone. Normal human and rat bone cells express only the PDGF-A gene under unstimulated culture conditions, whereas osteosarcoma cells express both PDGF-A and B genes. Alpha2 macroglobulin binds PDGF and may act as a transport protein [21].

PDGF is synthesised by blood platelets, monocytes, macrophages and endothelial cells and influences most mesodermal cells. It increases DNA synthesis, cell replication, collagen and non-collagen protein synthesis in cultures of rat calvarial cells. The mitogenic effect, however, is not specific for cells of the osteoblastic lineage, as PDGF-BB also stimulates the replication of non-skeletal fibroblasts [1, 21]. Two PDGF receptor subunits have been demonstrated, alpha and beta, and the two subunits form dimers in response to PDGF binding as part of the receptor activation process. PDGF-B polypeptide binds to both the alpha and beta subunits, while PDGF-A polypeptide binds primarily to the alpha subunit. Activation of both receptor subunits may be needed for maximal

response. IL-1, TNF-α and TGF-β1 affect the binding of PDGF [21].

PDGF-BB is probably released by platelets at the wound site during platelet aggregation and is important for normal healing of wounds and fractures (see Table 1). Locally applied PDGF enhances demineralised bone-induced heterotopic osteogenesis in rats [33], healing of tibial osteotomies in rabbits [49] and formation of bone and cement in conventional periodontal surgery in dogs (combined with IGF-I) [44].

FGF

The FGF family consists of nine members of structurally related polypeptides. Most abundant and best characterised in man are acidic FGF (FGF-1) of 16 kDa and basic FGF (FGF-2) of 17 kDa [76]. FGF is synthesised by various cells, including monocytes, macrophages, osteoblasts and chondrocytes and affects most mesodermal and neuroectodermal cells. The two FGFs have 55% structural homology and bind to the same receptor. As opposed to TGF-β1, bFGF is stored in matrix in an active form [30]. It probably initiates the normal embryonic limb bud formation [22] and contributes to normal wound [62] and fracture [14] healing (see Table 1).

Jingushi et al. found that repeated injections of aFGF into the fracture-site in rats induced cartilage enlargement and decreased mRNA expression for type II procollagen and proteoglycan core protein [36]. In rats, bFGF enhances demineralised bone-induced heterotopic osteogenesis [7] and the incorporation of bone grafts and porous hydroxyapatite implants [76–78]. Human recombinant aFGF in agarose promotes the healing of rat parietal critical size defects [26].

IGF I–II

IGF-I (somatomedin-C; 7.5 kDa) and IGF-II (skeletal growth factor; 8.7 kDa) are produced by different types of cells, including osteoblasts. IGF-II is the growth factor found in the highest concentration in bone matrix. IGF-I is 4 to 7 times more potent than IGF-II [39]. Cortisol inhibits IGF-I production, whereas PTH, GH, prostaglandin E2 and BMP-2 stimulate IGF-I production in cultured osteoblast-like cells. The biological actions of the IGFs are modulated in a cell-specific manner by six IGF-binding proteins (IGPBP) that are all expressed by osteoblasts. The expression and secretion of both IGFBPs [13] and IGF-I and IGF-II [12] changes during the development sequence of rat osteoblast cultures. IGF-I stimulates bone DNA, collagen and non-collagen protein synthesis in cultures of foetal rat calvaria [20]. IGF-I and IGF-II increase bone collagen synthesis and decrease collagen degradation in intact rat calvaria in vitro [45] and stimulate proliferation of osteoblasts in serum-free cultures of human bone cells [46].

Endothelial and mesenchymal cells expressed IGF-II mRNA at the granulation tissue stage of healing fractures in humans [5]. At the stage of bone and cartilage formation, osteoblasts and non-hypertrophic chondrocytes expressed mRNA for both IGF-I and II. The findings support a role for IGFs in the local cellular regulation in human fracture healing.

IGF is important in demineralised bone-induced heteropic osteogenesis. Prisell et al. found that IGF-I mRNA was expressed in correlation with the recruitment and proliferation of mesenchymal cells, whereas IGF-II mRNA was activated later, at the start of the calcifying process during endochondral bone formation [53]. Yu and co-workers found that an IGF-II (M-6-P) receptor was present in implants from day 1 to day 21; the highest levels were expressed on day 11 during bone differentiation [83].

IGF-I enhanced the healing of experimental defects of the skull [66] and the zygomatic arch [68], the longitudinal growth of diaphyseal bone [69] and induced premature closure of the frontal suture [67] in rats. We found enhanced healing of experimental calvarial defects in rats by polyorthoester membranes for guided tissue regeneration containing IGF-I [19].

Delivery systems

A system for the delivery of osteo-inducing agents should preserve the shape and bulk of reconstructions and assure local, sustained release of the factors which may otherwise be rapidly absorbed before instituting their effect. Advanced systems (polymers) may allow accurate dosage and combining factors to be delivered concurrently or at different points of time. The ideal material should be biocompatible, be resorbed and replaced by bone within 6 weeks [74] and not inhibit bone formation by inducing a chronic inflammation or physically obstructing bone formation by incomplete resorption.

Several biodegradable materials have been investigated, including (1) organic materials such as inactive demineralized bone proteins, collagen, fibrin sealant, fibrin-collagen paste, squalene and glycerol; (2) ceramics, including β-tricalcium phosphate and plaster of Paris (calcium sulphate); and (3) synthetic polymers such as polylactic acid [42], polyactide-polyglycolide co-polymer [56], polyanhydride [43] and polyorthoester [51, 61].

Some existing delivery systems do not fulfil the above criteria. Unresorbed β-tricalcium phosphate [74], co-polymer of polylactide-polyglycolide [56] and polylactic acid [42] were detected at 4 months, 24 weeks and 6 months, respectively. Fibrin-collagen paste and fibrin sealant were incompletely absorbed, induced a chronic inflammation and inhibited heterotopic osteoinduction [52, 61]. We have evaluated the effect of a bioerodible polyorthoester made for sustained delivery on heterotopic [51, 61] and orthotopic [59, 60, 63] osteogenesis. The polyorthoester does not inhibit bone formation, it induces

little tissue reaction and it is mostly resorbed by 4 weeks.

In the near future we will probably be able to treat skeletal defects and pseudarthrosis by implants of growth factors in a resorbable sustained delivery system. However, the prerequisite to such treatment is more knowledge about the importance of various growth factors in the different steps of the osteoinduction cascade and the different stages of impaired bone healing, the relative importance of lack of growth factors as opposed to the lack of osteoprogenitor cells in impaired bone healing, and the characteristics of the ideal biodegradable systems for sustained delivery of osteogenic growth factors.

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