CHAPTER I

INTRODUCTION

Background and significance

Periodontal disease is one of the chronic inflammatory diseases in humans. International surveys from more than 50 countries demonstrate that periodontal disease is frequently observed. Adults 35 to 44 years of age who had completely healthy periodontal tissues were a small minority. Between 5% and 20% of the populations surveyed had at least one sextant with pocketing of 6 mm or more (Miyazaki et al., 1991). A completed national survey of employed a adults in the United States found the prevalence of pocketing of 4 mm or more was 14% and the prevalence of attachment loss of 3 mm or more was 44% (Brown, Oliver and Loe, 1990). Only 15% of persons in the US in 1981 were free from sign of periodontal disease (Brown, Oliver and Loe, 1989) and it appears that approximately 20% of adults in the United States continue to experience a level of periodontal disease severity that requires professional treatment (Capilouto and Douglass, 1988). Recent epidemiologic studies of periodontal diseases in the United States showed that over 50% of adults had gingivitis and adult periodontitis was found in about 30% of the population (Oliver, Brown and Loe, 1998). It has generally been assumed, based on previous epidemiologic and utilizing study as well as the increasing elderly population, that the 1985 US population required 120 to 153 million hour and $5 to $6 billion annually (Oliver, Brown and Loe, 1989).

Resulting in loss of supporting periodontal tissue with subsequent loss of teeth, periodontitis is considered as one of the major oral disease that cause of oral bone loss in adults (Jeffcoat, 1993). The loss of this osseous tissue is thought to result from local biological active molecule alterations in the resorptive or formative respect of physiologic bone turnover. However, the relationship between alveolar bone resorption and formation is very complex since it is the interactions of multiple tissue with unique anatomical consideration and a variety of unique regulatory factors. The mechanisms regulating the metabolic activity of the bone appear to include a number of growth factors and inflammatory mediators (Oates and Cochran, 1996).

Bone regeneration is one of the chief goals of periodontal therapy. Many procedures are used in order to gain periodontal supporting tissue and regenerate the periodontal attachment structures. Many methods have been designed for enhancing periodontal regeneration including root conditioning agents (Wikesjo et al., 1988), guided tissue regeneration using tissue guiding membranes (Gottlow et al., 1986; Blumenthal, 1988), inert osteoinductive and osteogenic materials (Quintero et al., 1982). These procedures provide the possibility to recruit osteoprogenitor cells, induce cell proliferation and differentiation to form new supporting bone, cementum and periodontal ligament. Research into the osteoinductive and osteogenic molecules have received extensive interest recently because of isolation and characterization of a new class of biological response modifiers termed polypeptide growth factors (PGFs). Polypeptide growth factors are a class of naturally occurring biological mediators which regulate the proliferation, migration and/or matrix synthesis of a variety of cell types. Polypeptide growth factor appear to play an important role as local regulators in the development and growth of osseous tissue since the ability of such growth

factors to recruit mesenchymal cells and induces bone formation.

Bone

Bone is a specialized type of connective tissue of mesenchymal origin. Bone serves as a structural support protecting vital organs and a major ion reserve in the body. Bone is a complex and metabolically active tissue, composed primarily of both organic and mineral phase. The organic phase composed of organic matrix and a number of different cell types or cell families of bone-forming and bone-resorbing cells. Each of these families consists of a number of differentiation stages (Nijweide, Burger and Feyen, 1986). The cells that function in new bone formation which synthesize the organic matrix and direct the events resulting in mineralization are the osteoblasts, the mesenchymal fibroblast-like preosteoblasts and perhaps osteocytes. The osteoblast is widely accepted to be a fully differentiated, non-proliferative cell. The immediate precursor, the preosteoblast, is morphologically recognizable in vivo by its membrane-associated alkaline phosphatase marker, and available evidence suggests that its proliferative potential is probably quite limited (Owen, 1970). The earlier precursors of the osteoblastic line are morphologically unrecognizable in vivo but have been demonstrated by culture techniques to be present among the fibroblastic stromal cells of rodent bone marrow (Friedenstein, 1976). These osteoblasts and osteoblast precursor cells are thought to be important in regenerative potential and bone formation processes. Thus, agents affecting proliferation of osteoprogenitor cells and the activity of mature osteoblasts such as mineralization are of interested and can be used for increase level of bone regeneration.

Bone formation and resorption are the two major processes involved in tissue remodeling. Bone cells secrete a variety of cytokines and growth factors that play an important role in both processes. However, bone formation and resorption *in vivo* are regulated by both systemic (e.g., parathyroid hormone, vitamin D, calcitonin) and local regulation. Local mechanisms are believed to involve the actions of growth factors that act as autocrine or paracrine effects of osteoblastic and osteoclastic proliferation and differentiation. It has been believed that there is a stromal stem cell present in bone and bone marrow that is able to give rise to both osteogenic cells and cells of the bone marrow stroma (reticular cells, fibroblasts and adipocytes). Many *in vivo* and *in vitro* studies have shown that the recruitment of osteoblasts from the pool of precursor cells can be greatly increased by growth factors. In contrast, the biosynthetic activity of the mature osteoblast is limited, as it can not be increased more than approximately 2-fold as evaluated by the mineral apposition rate. Therefore, the optimal therapeutic strategy to increase bone formation would be to increase the proliferation and also differentiation of osteoblast precursors rather than the activity of mature osteoblasts. This will lead to an increased number of osteoblasts and increase in the amount of bone matrix deposited.

Local regulators of bone formation

Although systemic factors such as estrogens, growth hormone, parathyroid hormone, and vitamin D exert control of bone metabolism and remodeling, it is certain that local intercellular signals represent the final common pathway necessary for coupling. These include a group of bone morphogenetic protein (BMPs), and growth factors such as PDGF and IGF (Canalis, McCarthy and Centrella, 1988).

Bone morphogenetic proteins (BMP)

Overview

Bone morphogenetic proteins (BMP) are members of the transforming growth factor-β (TGF-β) superfamily, a large family of secreted signaling molecules. Although the name BMP is descriptive of one particular function, many roles have been assigned to the BMPs. While BMPs may induce ectopic bone or cartilage formation, they also play important roles in the development of the viscera. This includes roles in cell proliferation, apoptosis, differentiation, and morphogenesis.

The alignment of amino acid sequences of BMPs indicates that a significant amino acid sequence identity exists among all the BMPs in the carboxy-terminal region of the protein. Like other members of the TGF- superfamily, BMPs are synthesized within the cell as precursor forms, and then proteolytically processed at the carboxy-terminal regions to yield the mature proteins of approximately 130 amino acids. Most of the BMPs as well as other members in the TGF- superfamily share a conserved pattern of seven cysteine residues in the mature domain. Each mature, active BMP is a disulfide-linked homodimer of the same members or a disulfide-linked heterodimer of two different members (Sampath et al., 1990). BMP dimers are interconnected by seven disulfide bonds and this dimerization is a prerequisite for bone induction.

Fifteen BMPs have currently been identified (Groeneveld and Burger, 2000), and they are further divided into at least four subfamilies according to their amino acid sequence similarities. One group consists of BMP-2 and BMP-4, which

are 92% identical in the seven-cysteine region. BMP-5, BMP-6, and BMP-7 (OP-1) are closely related to one another with an average of 89% amino acid sequence identities in their corresponding regions. The third group is GDF-5 to -7, and the fourth contains BMP-3 and GDF-10 (BMP-3b). Members of each subgroup have shown osteoinductivity (**Table 1**). BMP-1 is not a member of the TGF- superfamily as it lacks the structure conserved in the TGF-β superfamily, and it has recently been shown as a procollagen-C-proteinase that processes procollagen to collagen.

Structure of BMPs

Structure studies of BMPs reveal that they contain a mature domain that is cleaved, allowing monomeric units to become dimers by cysteine-disulfide bridge. Little is known about intracellular assembly, processing, and secretion of BMP proteins and their extracellular localization. All of the evidence suggests that dimerization and proteolytic cleavage of the variable length pro-segment between the pro- and mature region occurs prior to secretion. The core of monomer structure is a cysteine knot involving intramolecular bonding of six of the seven conserved cysteines; the seventh participates in intermolecular disulfide bonding. The presence of N-linked glycosylation sites is variable. Therefore, protein assembly can produce homodimers, heterodimers, and glycosylation variability, which may influence the activity and effects of BMPs. A number of other extracellular proteins probably bind BMPs *in vivo* and so limit their availability. The BMPs/GDFs have been grouped into subsets based on amino acid sequence homology. The groupings are suggested to be 1) BMP-2 and BMP-4; 2) BMP-3 and BMP-3b; 3) BMP-5, BMP-6, BMP-7 and BMP-8; 4) BMP-9 and BMP-10; 5) BMP-12, BMP-13 and BMP-14; and 6) BMP-11 and GDF-8.

Receptors

BMPs transduce signals through trans-membrane serine/threonine kinase receptors. Receptors for BMPs are complexes of two different types of membrane-bound serine/threonine kinase: type I BMP receptors, BMPR-IA and BMPR-IB which are also termed the activin receptor-like kinase (ALK)-3 and ALK-6 respectively, and type II receptors. These BMP receptors are closely related in structure and function to transforming growth factor-β receptors (TRs) and activin receptors (ActRs) and these receptors are distinguished on the basis of their molecular weights, the presence of a glycine/serine-rich (GS) box located on the type I receptor, and the ability to bind a particular ligand. Type I receptors have no cytoplasmic C-terminal extension, but do possess a 20-30 aa glycine-serine (GS) rich domain between their transmembrane region and kinase domain. Signal transduction requires the formation of heterodimer complex between type I and type II receptors. After ligand binding, the type II receptor then phosphorylates the type I receptors at the specific region (the GS box) of the cytoplasmic domain. This phosphorylation activates the type I receptor kinase domain and initiates downstream signaling cascade. The activated type I receptor then phosphorylates a member of Smad family of intracellular proteins, which are the functional signal transducers of the TGF-β/BMP family. The Smad superfamily can be subdivided into class I-III. After binding of a BMP to its receptor, Smad 1 and 5 (class I Smads) form heteromeric Smad-Smad complexes with Smad 4 (class II Smads). The complexes regulate molecular transcriptional responses directly. Smad 6 and 7 (class III Smads) are inhibitors of TGF-β/BMP signaling.

Table 1 Overview of identification and cloning of bone morphogenetic proteins (BMPs) and their role in osteoinduction.

BMP	Bone induction
BMP-2 (BMP-2a)	Bone induction in subcutaneous pockets in rats
BMP-3 (osteogenin)	Bone induction in subcutaneous pockets in rats
BMP-4 (BMP-2b)	Bone induction in subcutaneous pockets in rats
BMP-5	No report on osteoinductivity. Role in embryology in the initiation of formation of particular skeletal elements
BMP-6 (Vgr-1)	Bone induction in subcutaneous pockets in nude mice
BMP-7 (OP-1)	Bone induction in subcutaneous pockets in rats
BMP-8 (OP-2)	No report on osteoinductivity
BMP-9 (GDF-2)	No report on osteoinductivity
BMP-10	No report on osteoinductivity
BMP-11	No report on osteoinductivity
BMP-12 (GDF-11, CDMP-3)	No report on osteoinductivity. Inhibition of terminal myoblast differentiation
BMP-13 (GDF-6, CDMP-2)	No report on osteoinductivity. Inhibition of terminal myoblast differentiation. Ectopic induction of tendon and ligament in rats
BMP-14 (GDF-5, CDMP-2)	No report on osteoinductivity. Ectopic induction of tendon and ligament in rats
BMP-15 (CDMP-1)	No report on osteoinductivity

OP, osteogenic protein; GDF, growth and differentiation factor; CDMP, cartilage-derived morphogenetic protein.

Biological Activity

BMPs have been implicated in a variety of functions. The hallmark of BMP activity *in vivo* is the induction of both cartilage and bone (Wozney and Rosen, 1998). It has been shown that endogenous BMPs had important roles in bone formation during distraction osteogenesis (Sato et al., 1999). In vivo, BMPs are expressed in cells in developing bone, and in fracture callus (Bostrom et al., 1995). Therefore, BMPs may play an important role in the development and growth of cartilage and bone. In addition to the general differentiative activity, BMPs also have been reported to have chemotactic and mitogenic activities on bone cells. In addition, BMPs play a role in a number of non-osteogenic developmental processes including dorsal/ventral patterning. BMPs are also expressed in other tissues including heart, kidney and central nervous system (Elima, 1993). Presumably BMPs act as regulators in growth and morphogenesis. However, little is known concerning regulation of BMP expression in osteoblast or other cells.

Biological functions of individual BMP molecules have been reported differently in different cell lines, while some general trend is featured. Multipotent cell types respond to BMPs by increasing or decreasing their proliferation. BMPs have a strong regulatory action to commit primitive mesenchymal progenitors into precursors of various phenotypes, and promote the differentiation of committed cells into the specific tissue. In osteoblastic cell lines, the effects of BMPs on cell proliferation have been reported differently as well, while BMPs stimulate the expression of the osteogenic phenotype. BMPs also strongly promote the differentiation of chondroblasts and chondrocytes, and the cell proliferation was variously reported in different cell lines (Sakou, 1998).

Platelet-derived growth factors (PDGF)

Overview

Early tissue culture work demonstrated the superiority of serum over plasma in stimulating the proliferation of fibroblasts in vitro. These observations suggested that a factor released from platelets during degranulation was probably responsible for this stimulatory activity. Subsequent investigations clearly demonstrated that a factor released from platelets upon clotting was capable of promoting the growth of various types of cells. This factor was subsequently purified from platelets and given the name platelet-derived growth factor (PDGF) (Antoniades, Stathakos and Scher, 1975; Ross and Vogel, 1978). PDGF was initially isolated from platelet and subsequently found to be synthesized by a number of cells besides platelets including fibroblasts, endothelial cells, osteoblasts, activated macrophage, blood monocytes, vascular smooth muscle cells and tumor cell lines (Ross, Raines and Bowen-Pope, 1986). PDGF also stimulates activity in almost all cells of mesenchymal origin that produce supporting connective tissue and skeleton such as fibroblasts, smooth muscle cells, osteoblastic cell and periodontal ligament cells and connective tissue cells (Graves and Cochran, 1990).

Structure of PDGF

Platelet-derived growth factor is a family of cationic homo- and heterodimers of disulfide-bonded A- and B-polypeptide chains. The mature parts of the A- and B-chains of PDGF are ~100 amino acid residues long. Two PDGF genes independently encode two distinct PDGF chains from chromosome 7 and chromosome 22 for PDGF-A and –B respectively, which shared ~60% amino acid sequence homology and can combine to form disulfide-linked dimers of PDGF-AA,

PDGF-BB, or PDGF-AB (Hannink and Donoghue, 1989). Eight cysteine residues are perfectly conserved between the two chains. Two of the cysteine residues (the second and the fourth) are involved in cysteine bonds between the two subunits in the PDGF dimer, and the other six are engaged in intrachain disulfide bonds (the first pairs with the sixth, the third with the seventh, and the fifth with the eighth). Reduction of interchain disulfide bond destroys its mitogenic activity and generates multiple protein species of MW 12,000 to 18,000. The different molecular masses of PDGF are thought to result from proteolytic cleavage and do not appear to effect its biological activity (Antoniades and Hunkapiller, 1983). The three PDGF isoforms are mitogens for a variety of cells with different potency: PDGF-BB>PDGF-AB>PDGF-AA. This has been attributed in part to the ability of PDGF-BB to bind to both the PDGF- α and β receptors, whereas PDGF-AA only occupies the α-receptor (Canalis et al., 1992). PDGF-A and/or B chain is expressed in cells from epithelial tissue, muscle, nervous tissue, and cells of hematopoitic origin. However, the expression of PDGF in normal cells appears to be tightly regulated.

Platelet-derived growth factor-BB has been crystallized for its three-dimensional structure. The two subunits in the dimer are arranged in an antiparallel manner. They consist of a tight knot-like structure (cysteine knot) in which one of the intrachain disulfides goes through the hole formed by the other two and the intervening sequences. The three-dimensional structure of PDGF-BB is not only similar to that of VEGF, which has a related amino acid sequence, but also shows some resemblance to those of nerve growth factor and transforming growth factor-β (TGF-β), despite the fact that the latter factors have no sequence similarity with PDGF. All these factors are dimers and show the characteristic cysteine knot structure.

Binding Proteins

Platelet-derived growth factor does not only interact with matrix molecules but also with soluble proteins. Like many other cytokines, PDGF binds to α_2-macroglobulin. This interaction, which involves the B chain-containing PDGF forms, AB and BB, regulates the amount of PDGF available for interaction with receptors. Significantly, the binding is reversible, and PDGF dissociation is suggested to occur at either low pH or when equilibrium kinetics favor dissociation, such as might be the case when PDGF is removed from the circulation by binding to its own receptors. Another PDGF binding protein was isolated from a rat neural retina cell line and called PDGF-associated protein (PAP). PAP binds PDGF with low affinity and was found to enhance the activity of PDGF-AA but depress the activity of PDGF-BB.

Receptors

Platelet-derived growth factor isoforms exert their effects on target cells by activating two structurally related protein tyrosine kinase receptors. The α– and β–receptors have molecular sizes of ~170 and 180 kDa, respectively (Gronwald et al., 1988; Bonner, 1994). The two receptor proteins are structurally related and consist of an extracellular portion containing five immunoglobulin-like domains, a single transmembrane region, and an intracellular portion with a protein-tyrosine kinase domain. Because PDGF isoforms are dimeric molecules, they bind two receptors simultaneously and thus dimerize receptors upon binding. A functional PDGF receptor is formed when the two chains of a dimeric PDGF molecule each bind one of the above receptor molecules, resulting in their approximation, dimerization and activation. Between the two proteins, there is 44% overall sequence identity. Within

the extracellular domain, 30% of the aa residues are identical. In addition, a 90 kDa soluble form of PDGFR alpha, consisting of the extracellular segment of the alpha-receptor, has been found in cell culture medium and in human plasma (Tiesman and Hart, 1993). High-affinity binding of PDGF involves dimerization of the receptors, forming either homodimers or heterodimers with the alpha and beta receptors/chains. Although it appears that each subunit of dimeric PDGF binds to one receptor monomer, it is unclear if these PDGF subunits need to be covalently linked. The α-receptor binds both the A- and B-chains of PDGF with high affinity, whereas the β-receptor binds only the B-chain with high affinity. Therefore, PDGF-AA induces α-receptor homodimers, PDGF-AB induces α-receptor homodimers or αβ-receptor heterodimers, and PDGF-BB all three dimeric combinations of α- and β-receptors.

Receptor binding by PDGF is known to activate intracellular tyrosine kinase, leading to autophosphorylation of the cytoplasmic domain of the receptor as well as phosphorylation of other intracellular substrate (Claesson-Welsh, 1994). Specific substrates identified with the β-receptor include Src, GTPase Activating Protein (GAP), phospholypase Cγ (PLCγ) and phosphotidylinositol 3-phosphate (Claesson-Welsh, 1994). Both PLCγ and GAP seem to bind with different affinities to the α- and β-receptors, suggesting that the particular response of a cell depends on the type of receptor it expresses and the type of PDGF dimer to which it is exposed. The three dimeric PDGF receptor combinations transduces overlapping, but not identical, cellular signals. Both α- and β-receptor homodimers transduce potent mitogenic signals. However, there is difference between the receptors regarding their effects on different cell type. Activation of the β-receptor stimulates chemotaxis; in contrast, activation of the α-receptors inhibits chemotaxis of certain cell types including

fibroblasts and smooth muscle cells, whereas chemotaxis of certain other cell types is stimulated (Heldin and Westermark, 1999).

Biological Activity

Because there are differences between cells relative to the amounts of α- and β-receptors that they express, and because of the variability in PDGF isomer binding to receptors, there is a tremendous range of possibilities for biological responses by PDGF. This is reflected in at least four experimental systems where different isoforms of PDGF elicit different results. Vascular smooth muscle cells (SMC) and fibroblasts are both known to express both the α- and β-receptors. On SMC, PDGF-AA initiates cellular hypertrophy (increased protein synthesis), while BB induces hyperplasia (mitosis) (Inui et al., 1994). On fibroblasts, the BB isoform initiates chemotaxis, while AA inhibits chemotaxis (Siegbahn et al., 1990).

In general, PDGF isoforms are potent mitogens for connective tissue cells, and some epithelial and endothelial cells. In addition to its activity as a mitogen, PDGF is chemotactic for fibroblasts, smooth muscle cells, neutrophils and mononuclear cells. PDGF also appears to be ubiquitous in neurons throughout the CNS, where it is suggested to play an important role in neuron survival and regeneration, and in mediation of glial cell proliferation, differentiation and migration.

PDGF is likely to play an important role in bone metabolism and remodeling. PDGF is one of a number of growth factors that stimulates DNA and collagen synthesis and also cell replication in the calvarial cultures and in primary cultures of osteoblast-enriched cells from rat parietal bone (Centrella, McCarthy and

Canalis, 1989). PDGF-BB could induce both the chemotactic activity and the DNA synthesis in a normal osteoblast-like cell line (MC3T3-E1) and in cultures of intact fetal rat calvariae (Centrella, McCarthy et al., 1989). The effect was also observed in fibroblast and precursor cell-rich periosteum. PDGF is also able to regulate gene expression, presumably at the level of transcription. At least two cellular proto-oncogenes, c-myc and c-fos are transcriptionally activated soon after PDGF treatment of quiescent cells and these early PDGF-inductive genes may be involved in progression of the cell from the resting G_0 into G_1 and S phase (Hannink and Donoghue, 1989). PDGF also enhanced [^{3}H] proline incorporation into collagen which was observed primarily in the osteoblast-rich central bone, and also enhanced non-collagen protein synthesis.

Insulin-like growth factors (IGF)

Overview

Insulin-like growth factors (IGF-I and IGF-II) are single chain polypeptides consisting of 70 and 67 amino acids respectively, with a reciprocal 70% homology in humans. Both proteins share an approximate 50% homology with insulin, which is in line with concomitant insulin-like properties of the IGFs. Systemic IGF-1 and IGF-2 levels are mainly determined by production in the liver, however, most cells throughout the body can synthesize these growth factors (Daughaday and Rotwein, 1989). Insulin circulates at picomolar concentrations and has half-life of minutes. IGFs on the other hand circulate at nanomolar levels and have a half-life of minutes which can be extended up to 15 h when complexed with one of the 7 known IGF-binding proteins (IGFBPs).

IGF-I belongs to a family of peptide hormone that shares a high degree of structure similarity with proinsulin. IGF-I is a basic 70 amino acids polypeptides. The human IGF-I gene consists of six exons, number 1 though 6, that are located within a region of over 90 kb on the chromosome 12. The gene is transcribed into a large mRNA precursor, which is alternatively spliced to yield two different classes mRNA: IGF-IA and IGF-IB.

Binding proteins

Because IGFs are small proteins, the half-life of systemically administered IGFs is relatively short. The action of IGF on cellular metabolism is governed at several levels, including the presence of extracellular, high affinity IGFBPs. IGFBPs are a family of six proteins, Some are membrane-associated and other are soluble, that bind to IGF-I and –II with very high affinity. Because their affinity constants are between two- and 50-fold greater than the IGF-I receptor, they control the distribution of the IGFs. IGFBP-1 and IGFBP-3 are the most prominent IGFBPs. In adults 90% of the circulating IGFs are complexed with IGFBP-3 and this complex is considered a circulating store for IGFs. Moreover, IGFBP-1 modulates the extent of tissue penetration by IGF-I, because the complex of IGFBP-1 and IGF-I is able to translocate from the vascular compartment into the tissues. The affinity of IGFBPs for IGF-I and IGF-II, which is higher or at least equal to the affinity of the IGF-1R for both IGFs, is regulated by specific IGFBP-degrading proteinase. These enzymes cleave IGFBPs and consequently liberate IGF for interaction with its receptor. The affinity of IGFBPs for IGFs can also be lowered when IGFBPs associate with certain receptors on the cell surface or extracellular matrix (Jones and Clemmons, 1995). The actions of the IGFBPs can enhance or inhibit the action of IGFs; generally membrane

bound forms enhance IGF action by attracting IGFs to the region of the receptor and the soluble forms are inhibitory. IGFBPs may also serve to target IGFs to specific targets or cell types.

Receptors

IGFs interact with specific IGF membrane receptors (IGF-R) as well as with the insulin receptor. The IGF-R is similar to, but distinct from, the insulin receptor. The type I IGF-R is a glycoprotein consists of two extracellular α-subunits and two β-subunits joined by disulfide bridges linked in an $\alpha_2\beta_2$. The β-subunits traverses the cell membrane and contains a tyrosine kinase domain in their cytoplasmic protein. The type I IGF-R mediates the action of both IGF-I and IGF-II, as these growth factors bind to and activate this receptor. The IGF-IR can also bind IGF-2 and insulin, although with lower affinity than IGF-1. Studies suggest type I IGF-R mediates most of the known effects of IGF-I and IGF-II (Czech, 1989). In contrast, the type II IGF-R is a single polypeptide chain with a large extracellular domain and short cytoplasmic tail and is identical to mannose 6-phosphate (M6-P) receptor, which involved in targeting of lysosomal enzymes from the Golgi apparatus to the lysosomes, as well as internalization and degradation of IGF. This receptor exhibits no intrinsic kinase activity and has a higher affinity for IGF-II than for IGF-I. It has been suggested that this IGF-R also function as a reservoir and/or clearance for IGF.

Biological Activity

The relative role of the IGFs in bone varies among species and during development. This makes it difficult to directly relate results in rodents to human.

IGFs, along with other growth factors, contribute to processes of bone development, growth and repair by stimulating proliferation and differentiation of osteoblast precursors. Indeed, IGF-I is mitogen for fetal rat calvaria, and IGF-I and IGF-II have mitogenic activities for rat calvaria osteoblasts *in vitro*. IGFs produced by bone cells not only act as acute autocrine and paracrine regulators, but also become incorporated into bone matrix and may be released later during resorption (Linkhart, Mohan and Baylink, 1996).

Cyclopentenone prostaglandin

Prostaglandins (PGs) are formed by the oxidative cyclization of the central 5 carbons, forming the cyclopentane ring, within 20 carbon polyunsaturated fatty acids. The key regulatory enzyme of this pathway is cyclooxygenase (COX or PGH synthase) which catalyzes the conversion of arachidonic acid (or other 20 carbon fatty acids), which is released from membrane phospholipids by the action of phospholipase A_2, to prostaglandin (PG) G_2 and. PGH_2. PGH_2 is subsequently converted to a variety of stable eicosanoids that include PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane (TX) A_2 (Smith, 1992). These PGs described by letter and numeric subscripts indicate the number of double bonds in a side chain. The array of PGs produced and widely distributed in almost every mammalian tissue varies depending on the downstream enzyme present in particular cell type. For example, endothelial cells primarily produce PGI_2, whereas platelets mainly produce TXA_2. These groups of biologically active fatty acids regulate a wide variety of local biological processes (Samuelsson et al., 1975).

Prostaglandins are not stored in cells or tissue but are released immediately after synthesis from membrane-associated arachidonic acid by cyclooxygenase enzyme. Two cyclooxygenase isoforms have been identified and are referred to as COX-1 and COX-2. Under many circumstances the COX-1 enzyme is produced constitutively whereas COX-2 is inducible. Nearly all normal tissues were found to express COX-1 with low to undetectable levels of COX-2. Other differences between COX-1 and COX-2 include differences in utilization of arachidonic acid substrate pools as well as in mRNA stability. The regions regulating gene expression of COX-1 and COX-2 show little similarity. For example, the promoter and enhancer regions regulating COX-2 contain a variety of response elements that have been show to explain its inducibility by hormones, growth factors, phorbol esters, cAMP, and cytokines. Much less is known about the elements involved in regulating COX-1 gene expression.

Cyclopentenone, prostaglandins of A and J series, have been shown to be produced *in vitro* by dehydration of the cyclopentane ring of PGE_2 and PGD_2, respectively. Δ^{12}-PGJ_2 is the ultimate metabolite of PGD_2. PGD_2 is an unstable molecule and undergoes dehydration to form PGJ_2 in aqueous solution, and is then converted to Δ^{12}-PGJ_2 in the presence of serum albumin or plasma. These cyclopentenone compounds have attracted considerable attention because they exert unique biological actions. Conventional PGs, such as PGE_2 and PGD_2 act on surface receptors to exert their action. In contrast to PGs acting on a cell surface receptor, cyclopentenone PGs, such as Δ^{12}-PGJ_2 and PGA_2, have no cell surface receptor, are actively incorporated into cells through a temperature-dependent and transporter on plasma membranes and then accumulate in the nucleus (Narumiya et al., 1986;

Narumiya et al., 1987). They have been shown to inhibit cellular proliferation with a G_1 cell cycle arrest and to induce differentiation, an effect that may be related to their ability to modulate a variety of growth-related and stress-induced genes (Fukushima, 1990; Fukushima, 1992). Cyclopentenone PGs can also activate nuclear peroxisome proliferator-activated receptor (PPAR) and suppress macrophage activation and inflammatory responses (Forman et al., 1995; Kliewer et al., 1995). The common feature in these compounds is the presence of a reactive α,β-unsaturated carbonyl group, which is very susceptible to nucleophilic addition reactions and seems to be essential for many of their biological effects.

Cyclopentenone and bone regeneration

The role of PGs in bone metabolism is not only complex, but also is apparently contradictory. For example, while PGs were initially characterized by stimulating bone resorption in culture, human and animal responses to PGs often include stimulation of bone formation (Kawaguchi et al., 1995). Most of the early works on prostaglandins in bone was focused on their ability to stimulate bone resorption and a number of arachidonic acid metabolites were defined as potent, locally produced, osteoclast-activating bone resorptive factors. PGE_2, one of these compounds, has potent biological activities in proinflammatory properties and can trigger bone resorption (Raisz, Pilbeam and Fall, 1993). The results of the early studies showed that PGE_2 is the most abundant prostaglandins in bone and that prostaglandin of E series are the most potent bone resorbers (Katz et al., 1981; Conaway, Diez and Raisz, 1986; Kawaguchi, Pilbeam et al., 1995). However, there are now a large number of studies showing that administration of PGs can stimulate

bone formation both *in vivo* and *in vitro*. A number of *in vivo* studies indicated that prostaglandins could promote bone formation (Nijweide, Burger et al., 1986). Some evidences have been shown that oral administration of prostaglandins has a systemic effect on bone formation (High, 1987). Moreover, prostaglandin-induced new bone mass decreased after PGE_2 withdrawal (Ke, Li and Jee, 1991). Though the effects of PGs on bone resorption have been studied to a much greater extent from those on bone formation, it seems likely that latter effects will be of at least equal importance in regulation of bone metabolism. The concept that certain prostaglandins may enhance bone formation is consistent with the fact that arachidonic acid cascade has evolved as an important cell-to-cell autocrine and paracrine regulatory system which has diverse effects on cell function and often exerts both positive and negative feedback control.

Recently, some evidences have been shown that PGD_2 and its derivative, Δ^{12}-PGJ_2 (Δ^{12}-13,14-dihydro-9-deoxy-Δ^9-PGD_2) stimulate calcification in human osteoblastic cells (Koshihara, Amano and Takamori, 1991). The D series of prostaglandins was first identified in the late 1960's. This isomer of prostaglandin E_2 was initially considered to be either a minor and relatively inactive prostanoid (Giles and Leff, 1988; Rangachari, 1992). PGD_2 is spontaneous degradation in plasma to at least four dehydration products including 9-hydroxy PGD_2 (PGJ_2) and Δ^{12}-PGJ_2 (Giles and Leff, 1988). PGJ_2 was produced as a major product from PGD_2 after 24 h incubation at 37 °C in aqueous solution (Fukushima et al., 1982). PGD_2 is slowly dehydrated to PGJ_2 in aqueous media and PGJ_2 is rapidly converted to Δ^{12}-PGJ_2 in plasma (Fukushima, Kato et al., 1982; Narumiya and Fukushima, 1985). When serum albumin, at concentrations found in plasma, was added to PGD_2, it was found that

serum albumin catalyzes the conversion of PGD_2 to Δ^{12}-PGJ_2 with efficiency equal to that of dialyzed plasma. These results suggest that the formation of Δ^{12}-PGJ_2 in dialyzed plasma be catalyzed by serum albumin itself. In addition, when PGJ_2 is added to dialyzed plasma, it is quickly converted to Δ^{12}- PGJ_2. Δ^{12}- PGJ_2 has different biological activities from PGD_2. It lacks antiaggregatory activity for human blood platelet and also is inactive for relaxation of some smooth muscle (Fukushima, Kato et al., 1982), in both of which PGD_2 is quite active.

Δ^{12}-PGJ_2 is a stimulant for osteogenesis *in vitro*. Δ^{12}-PGJ_2 has been show to stimulate alkaline phosphatase activity and calcification in human osteoblastic cells (Koshihara and Kawamura, 1989; Koshihara, Amano et al., 1991). It shown that Δ^{12} PGJ_2 strongly stimulated calcification with an efficacy almost equal to that of 1,25 $(OH)_2D_3$ at 10^{-8} M. In addition, other derivatives of PGD_2, PGJ_1, PGJ_2, and 12-metyl-PGJ_2, had no effect (Koshihara, Amano et al., 1991). Osteocalcin accumulated on cell layer during calcification, calcium and phosphorus were remarkably increased by Δ^{12}-PGJ_2 and PGD_2 at 10^{-5} M in human osteoblastic cells (Koshihara and Kawamura, 1989). Furthermore, Δ^{12}-PGJ_2 enhanced type I collagen synthesis in human osteoblasts during calcification (Tasaki, Takamori and Koshihara, 1991). In ovariectomized rat, subcutaneous administration of a slow-release preparation of PGD_2 partially prevented the ovariectomy-induced decrease of femoral bone mineral density in osteopenic femur *in vivo* (Takagi et al., 1993). Because PGD_2 and PGJ_2 are converted to Δ^{12} PGJ_2 in the present of plasma, most biological activity of the former compounds would be exerted by their conversion to the latter. Thus, it is likely that Δ^{12}-PGJ_2 is the ultimate compound that exerts biological activity of PGD_2. Considering that PGD_2 is a major arachidonate metabolite in bone marrow (Ujihara et al., 1988), Δ

^{12}-PGJ_2 may be involved in the modulation of osteogenesis.

Problems and hypothesis

There is no study regarding the temporal expression of these growth factors or prostaglandin in bone formation using guided tissue regeneration. Therefore, it is of interest to study the cellular dynamics and temporal expression of growth factors or COX-2 as biochemical signals that promote *de novo* bone regeneration *in vivo*. We hypothesized that COX-2 and some growth factors have important roles in bone formation *in vivo* and the effect of COX-2 inhibitor may inhibit bone formation and some growth factor expression. Furthermore, since Δ^{12}-PGJ_2 appeared to promote osteogenesis *in vitro*, we hypothesized that Δ^{12}-PGJ_2 may capable of inducing bone formation *in vivo* and specifically this effect may accompanied by an induction of the expression of growth factor such as PDGF, IGF or BMP. Δ^{12}-PGJ_2 may also have a potential use of for bone regeneration in an experimental implant model.

Specific aims

(i) To establish and study a new model of bone regeneration using principle of guided tissue regeneration in rat.

(ii) To study a temporal expression of COX-2, PDGF-B, IGF and BMP-6 during bone regeneration in rat model.

(iii) To study the effect of COX-2 inhibitor in bone regeneration.

(iv) To study the *in vivo* effect of single application of Δ^{12}-PGJ_2 on bone formation in the rat model.

(v) To study the expression of PDGF, IGF, BMP-2, and BMP-6 in new bone regeneration induced by Δ^{12}-PGJ_2

(vi) To study the effect of single application of Δ^{12}-PGJ_2 on bone regeneration of titanium implant placement in the rat model.