

Bone morphogenetic proteins (BMPs): how do they function and what can they offer the clinician?

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Abstract: Bone Morphogenetic Proteins (BMPs) form a unique group of proteins within the Transforming Growth Factor beta (TGF- β) superfamily of genes and have pivotal roles in the regulation of bone induction, maintenance and repair. They act through an autocrine or paracrine mechanism by binding to cell surface receptors and initiating a sequence of downstream events that have effects on various cell types. Differentiation of osteoprogenitor mesenchymal cells and up-regulation of osteoblastic features occur under the influence of cytokines and growth factors that are expressed with the direct or indirect guidance of BMPs acting at the transcriptional level or higher. The Smads family of proteins has been identified as the downstream propagator of BMP signals, whereas hedgehog genes are possible modulators of BMP expression. The inflammatory response observed during wound repair and fracture healing, results in by-products that interact with BMPs and affect their biologic potential. Additive, negative or synergistic effects are observed when homodimeric or heterodimeric forms of BMPs interact with BMP receptors. Storage within the bone matrix allows for their involvement in the modeling/remodeling process by mediating coupling of osteoblasts and osteoclasts. Micro-environmental conditions, dose, possible carrier materials and geometrical parameters of delivery matrix are critical determinants of the

pharmacokinetics of BMP action and the biologic outcome during wound repair. Because of their osteogenic potential, BMPs are of tremendous interest as therapeutic agents for healing fractures of bones, preventing osteoporosis, treating periodontal defects and enhancing bone formation around alloplastic materials implanted in bone. (J. Oral Sci. 45, 57-73, 2003)

Key words: bone morphogenetic proteins; bone; growth factors; delivery systems; therapeutic regeneration.

Introduction

Bone Morphogenetic Proteins (BMPs) form a unique group of proteins within the Transforming Growth Factor beta (TGF- β) superfamily. BMPs were first identified by Urist in 1965 when demineralized bone matrix implanted in ectopic sites in rats was found to induce bone formation (1). There is extensive evidence supporting their role as regulators of bone induction, maintenance and repair, as well as being critical determinants of the embryological development of mammalian organisms (2,3). During embryogenesis, they regulate dorsal-ventral patterning (4), establishment of embryonic body plan (4), cell apoptosis (5), differentiation of neural cells (6,7), patterning of the limb bud (5) and epithelial-mesenchymal interactions during organogenesis (8,9). The TGF- β superfamily includes the activins/inhibins, BMPs, TGF- β , growth and differentiation factors (GDFs), mullerian inhibiting substance, *Drosophila dpp* and *Xenopus Vg1*, amongst others (10). BMPs play a role in the differentiation, proliferation, growth inhibition and arrest of maturation

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of a wide variety of cells, depending on the cellular micro-environment and the interactions with other regulatory factors (11).

Ligand-receptor interactions and downstream events

At their carboxy-terminal ends, all BMPs possess a region containing seven cysteine residues that is conserved among all the reported members of the TGF- β superfamily (12). BMPs are synthesized inside the cell in a precursor form with a hydrophobic secretory leader and a pro-peptide sequences joined to the mature region. Proteolytic cleavage frees the mature region, which can then dimerize with other BMPs. Mature BMP-2 is a homodimer of two 114-peptides subunits (13). Dimeric molecules can be either homodimers, when both subunits are the same, or heterodimers, consisting of two different subunits. Structural and chemical differences between the homodimeric and heterodimeric forms may be responsible for variations of their biologic potential and binding characteristics.

BMPs initiate signaling from the cell surface when they bind to and bring together type I and type II serine-threonine kinase receptors (14,15), both of which have sub-categories (17). BMP receptors are composed of three parts: a short extracellular domain, a single membrane-spanning domain, and an intracellular domain with the active serine/threonine region (18). The 75kDa type II receptor is the primary binding site of the ligand and upon its activation, phosphorylation of the type I receptor (50-55 kDa) occurs (14,17). It is only after the type I receptor becomes phosphorylated that receptor signals are propagated to downstream substrates (9). It is believed that the type II receptor does not actually bind the ligand but rather stabilizes the type I receptor (19), or accelerates ligand binding to the type I receptor (20).

Various signaling pathways have been proposed to be activated by the ligand binding to the receptors. Recently, a novel family of proteins, the Smad family, have been identified as the downstream effectors of the phosphorylated type I receptor (21-23). More specifically, Smads 1 and 5 become phosphorylated at the carboxy-terminal serine residues and then translocate to the nucleus (24) where they interact with DNA binding proteins (25), or exhibit direct transcriptional activity (26), either as monomers or in association with Smad 4 (21). Specific Smads are expressed at different phases of the cell cycle and exhibit either an inhibitory or stimulatory function (27). Smads 6/7 have been shown to antagonize and inhibit phosphorylation of Smads 1/5 by BMP-2 (28,29). Phosphorylation of Smad1 leads to the transcriptional activation of the DPC4 gene

that expresses Smad4 and the subsequent Smad1/4 complex translocates to the nucleus (21). The lack of DNA binding motifs on Smads does not allow their direct association with genomic sequences. It has been shown that small interacting proteins are necessary in bridging molecules and helping to mediate Smads action.

Genetic screens in *Drosophila* revealed that the gene *schurri* becomes up-regulated after BMP binds to their receptors, leading to the formation of an active transcription factor similar to zinc finger proteins in mammals (30). In addition, the protein kinase TAK1 has been isolated and shown to transduce signals downstream following BMP binding and function as a mitogen-activated protein kinase (31). Overall, BMPs regulate cell function at the transcriptional level or higher (32), by increasing the rate of transcription and/or stabilizing the mRNA (33). Moreover, the large number (2,500/cell) of BMP receptors (6) and their variability (16,29) allows heteromeric complexes to be formed with different signaling potential (16) capable of inducing various responsive cascades when binding to the same ligand (29,34).

Another line of evidence indicates the involvement of the Ras/Raf pathway in the downstream response to BMPs (35). According to this model, the signal is transmitted through Ras to Raf and, subsequently, activates other transcription factors such as AP-1 and GATA-2. BMP-2 up-regulates Id (inhibitor of differentiation) gene expression in osteoblastic cells and promotes their specific phenotypic expression (32). BMP-2 has an effect on cell-matrix interactions by suppressing the expression of the α 3 integrin subunit at the mRNA level (36), suggesting that BMP action, in part, may be exerted through altered cell adhesion to the extracellular matrix (ECM), modified cell migration and differentiation (37,38). In another report, cadherin expression in osteoblasts was unaffected by BMP-2 suggesting that the latter does not play a role in cell-cell interactions (39).

The family of Hedgehog genes (*Sonic hedgehog*, (*Shh*), *Desert hedgehog*, (*Dhh*) and *Indian hedgehog*, (*Ihh*)) present a striking correlation with expression of BMP genes in the mouse embryo (40) and act as possible modulators of BMP expression (41). In addition, growth factors such as TGF- β 1 exert a negative regulation on BMP-2 at the transcription level (42) and retinoic acid receptors also affect BMP expression (43). Noggin, chordin, cerberus, dan and gremlin are some of the molecules that have recently been found to regulate BMP expression and modulate their role in eliciting various biologic responses (44). A number of BMP-binding proteins (lipovitellin 1, Ep45) (45) and antagonist molecules (noggin) (46), affect and control the presence of growth

factors thus defining various concentration gradients, which has been shown to play a role in cytodifferentiation. There are several reports on the associated action of BMPs and noggin (47,48) suggesting that transcription factors from the *fos* family are downstream targets of noggin-related calcium regulation (47). These transcription products in turn limit the stimulatory action of BMPs on osteoblasts and thus act as autocrine regulators (49).

In summary, the variety of BMP receptors and the numerous pathways they regulate, together with the fact that BMPs exist in the form of homodimers, suggest that they may evoke synergistic, negative or additive net effects. This draws attention to the complexity of the signaling cascade and the variability of downstream responses to BMP-receptor signaling.

BMPs in bone formation

Bone formation can take place *via* a direct (intramembranous) or an indirect (endochondral) process. Intramembranous ossification occurs during embryonic development of the cranial vault bones by the direct transformation of mesenchymal cells into osteoblasts. Endochondral ossification, which is the process by which long bones develop, involves the formation of an intermediate cartilaginous anlagen that eventually becomes ossified and contains all the cellular components of mature bone (50). In both mechanisms, the induction of bone and cartilage occurs through an epithelial-mesenchymal interaction (51) that initiates specific cell differentiation and leads to precursor cells of the osteoblastic or chondroblastic lineage.

The development of cartilage and bone from mesenchyme, is characterized initially by a condensation of mesenchymal cells (52). This condensation can occur in two ways: either by cells moving toward a central focal point or by a localized region of increased proliferation. Direct cell-to-cell contact, diffusible molecules produced by the signaling cells, or matrix mediated interactions can result in a cellular mass of increased proliferative activity (53-55). An early step in the endochondral bone formation process is the condensation of mesenchymal cells into discrete pre-cartilaginous nodules. Chondrogenic cells become hypertrophic and pass into a bio-synthetically active state that involves mineral deposition in the cartilaginous matrix. The hypertrophic chondrocytes will then secrete chemotactic agents that both attract and direct the invasion of the sites by blood vessels. The chondrocytes eventually die and their matrix is partially destroyed during vascular invasion, which is when osteoblasts appear. Initially, osteoid will be deposited and remodeling will finally produce functional bone tissue (56).

Glucocorticoids mediate their action on osteoblasts through BMPs (57) and retinoic acid, a derivative of vitamin A, is a possible modulator of BMP expression (58). Cell adhesion molecules such as laminins (59), neural cell adhesion molecules (N-CAM) (60), and integrins (36) are known to interact with BMPs and other growth factors and have been localized at the areas of initial mesenchymal condensation (60,61). Related to this intra-molecular interaction are the chemotactic properties of BMPs. Depending on their concentration gradient, BMPs can attract various types of cells (19,62) and act as chemotactic, mitogenic or differentiating agents (63,64). BMPs may affect proliferation of cartilage-forming and bone-forming cells and can induce differentiation of mesenchymal progenitor cells into various cell types, including chondroblasts and osteoblasts (19,65). The latter property suggests that BMPs may be able to influence both the endochondral bone induction pathway and direct bone formation. In ectopic bone formation, associated with implantation of BMPs, the sequence of events recapitulates the process of bone formation that is observed during embryonic long bone development and many of the BMP properties can be extrapolated from there.

One of the most difficult subjects to study with regard to the *in vivo* response to BMPs is the characterization of the responding cell population. The wide spectrum of cells that are sensitive to BMP action includes fibroblasts (61), mesenchymal connective tissue cells (66), muscle derived connective tissue cells (67), the astroglial lineage (68) and many more (61). Bone marrow stromal cells form an important source of mesenchymal pluripotential progenitors that are capable of differentiating into various cell lineages under the appropriate conditions. Demineralized bone matrix, dexamethazone, beta glycerophosphate, vitamin D and BMP-1 have been shown to stimulate bone marrow stromal cells to take on an osteoblastic phenotype (69, 70). Bone marrow mesenchymal cells have the potential to differentiate along the osteoblastic and adipocytic lineages. Studies have demonstrated a concentration specific response with lower doses of BMPs inducing the adipocyte lineage and higher doses eliciting a chondrogenic/osteoblastic response (71). Treatment with rhBMP-2 protects and enhances cell commitment towards the osteoblastic phenotype (72,73). Osteoblasts and chondroblasts originate from a common precursor which is a bipotential mesenchymal progenitor called osteo-chondroprogenitor (74) or skeletoblast (75-78). Osteoprogenitors can be classified as either determined or inducible, based on their need for additional signals in order to differentiate (58,79). This difference is important as it reflects the variation between cell commitment, when the fate of cells is

programmed, and cell differentiation, when the fate of cells is expressed due to the permissive signals of the micro-environment. The answer to this debate is not clear yet but it is obvious that some form of control over phenotypic expression must occur to ensure that the tissues develop in a coordinated fashion in the appropriate places and amounts. (80). Evidence supports the hypothesis that BMPs act on the skeletal progenitor cells and induce the differentiation of both the osteoblast (65,71,81-83) and chondroblast (56,60,84,85).

Factors affecting BMPs bone inductive ability are amounts, qualitative composition, possible presence of inhibitors, correct processing and storage (86). In addition, the dose, concentration and time of BMP action are important parameters of the inductive outcome (64,87). Low concentrations of BMP-2 (50ng/ml) up-regulated the expression of the collagen II gene whereas higher concentrations (100-400ng/ml) inhibited collagen II expression in chondrocyte cell lines and increased osteocalcin (OC) expression (72). These results clearly show that chondrocytes are able to express osteoblastic features. It is more realistic to assume that BMPs induce cytodifferentiation along those lineages when permissive conditions for each cell type exist. Stability or structural integrity that allows blood vessels to grow (88), micro-environmental conditions that affect oxygen tension (89), and geometrical/architectural characteristics of the ECM affecting the cytoskeleton (90) through membrane receptors are critical factors for cytodifferentiation. Differences in the partial pressure oxygen and in the amount of mesenchymal cells present in intramuscular and subcutaneous sites are responsible for the lower dose of BMP needed to induce ectopic bone formation in the former site (91).

Treatment with cytochalasin D disrupts the cytoskeleton, making cells spherical in shape and resulting in high concentrations of endogenous soluble and matrix factors, thus promoting the chondrogenic phenotype (92). Reduced serum, high cell density, and type I collagen have been described as necessary parameters for chondrogenic differentiation (93). Cells at high density become attached to type I collagen leading to cellular condensation that promotes the chondrogenic phenotype. Further differentiation into hypertrophic chondrocytes and mineralization does not require BMP-2 but it is dependent on the presence of ascorbic acid and serum factors (94,95). Depending on the dose and the coordinated action of other cytokines (100,101), BMP-2 plays a regulatory role for the sequential progression of chondrocytes through their maturation (96,97), with development of hemopoietic bone marrow (98,99), and inhibition of myogenic

differentiation (83). Differentiation of mesenchymal cells into pre-chondroblasts is induced by BMPs but the coordinated progression along the chondroblastic and subsequent osteoblastic lineage is regulated by other growth factors that work in an autocrine or paracrine manner (102). BMPs acting through an autocrine mechanism reduce the expression of collagenase-3 and noggin, thereby inhibiting BMP binding and function and, result in increased production of collagenase-3 (103). Although BMPs exert their action on both osteoblasts and chondroblasts, they do not change the fate of the respective progenitors (104). Early exposure of undifferentiated mesenchymal cells to BMPs induces the chondroblastic pathway, whereas later exposure accelerates osteoblastic differentiation (105). BMPs can stimulate osteoblast differentiation independently of cartilage formation (106). This means that in the case of endochondral bone formation, osteoblasts do not form from a transdifferentiation of chondrocytes but rather as a result of a separate induction (1,70,93,107). Endothelial cells invading the cartilage may serve as a homing target of the stem cells that later develop into pre-osteoblasts (98). Numerous reports show an up-regulation of the osteoblastic phenotype by BMPs. Up-regulation of osteocalcin (OC), osteopontin (OP), osteonectin, bone sialoprotein (BSP), alkaline phosphatase (ALP), receptors for parathyroid hormone, collagen I production and the rate of mineralization are all proof of a promotive effect of BMPs on mesenchyme-derived cells (64,82,86,106,108,109). BMPs can act on various cell types and elicit a response that is specific to that stage of cell differentiation (106). There is evidence that BMPs trigger the production of osteopontin in preosteoblasts whereas in osteoblastic cells osteocalcin is upregulated and bone sialoprotein is expressed in differentiated osteoblasts prior to mineralization (110).

BMPs play an important role in the process of bone modeling and remodeling. The morphogenetic activity of bone matrix is apparent only after its demineralization, which occurs with the controlled action of osteoclasts. Insulin-like growth factors (IGF-I, IGF-II), TGF β -1, TGF β -2, PDGF, basic and acidic fibroblast growth factors, BMPs and other molecules are produced and become incorporated into the forming bone matrix that serves as a reservoir (111). BMPs bind to collagen type IV (112) or type I (113) and under these conditions are inactive. A heparin-binding site has been identified at the N-terminal segments of the BMP-2 that may function to localize the growth factor and restrict its diffusion (13). Acid treatment associated with osteoclastic action liberates BMPs from their collagenous substrate rendering them biologically active (114) and able to affect cell proliferation and differentiation (70).

Guided streaming of specific cell types into the appropriate pathways makes BMPs important regulators of bone formation (113), with a pivotal role in bone remodeling (110). “Basic Multicellular Units” (BMUs) refer to the functional units of bone in which osteoblasts and osteoclasts act in coordination (115). This is called coupling. During remodeling, which is a “self-maintenance” process, existing bone is resorbed and new bone is deposited. Resorption during each remodeling cycle is balanced by an equal amount of bone formation since the amounts of BMPs and other growth factors released from bone are proportional to the extent of resorption (116,117). There is evidence that BMP-2 promotes expression of cyclooxygenase-2 and the osteoclast differentiation factor in osteoblast-like cells, thus regulating osteoclastogenesis (118). Based on the above, the mitogenic, chemotactic and differentiating effect of BMPs may help to mediate coupling of bone formation to resorption during the adaptive response of remodeling. It supports the cellular components and amplifies the molecular signals needed for the coordinated interaction of various cell types.

BMPs in fracture healing

Fracture healing involves complex interactions among many local and systemic regulatory factors as well as cell types that cluster at the fracture site. Fracture repair represents a situation in which cell differentiation is re-initiated in an otherwise mature organism (2). Mesenchymal stem cells congregate at the area and form a gap-spanning, highly cellular “repair blastema” (119,120). The principal phases during ectopic bone induction are the migration and attachment of progenitor mesenchymal cells, proliferation, differentiation into cartilage or bone cell lineages, mineralization and remodeling, and marrow tissue formation.

The first demarcation of osteoprogenitor cells during fracture repair, referred to as the “stacked-cell layer” (120), is derived from mesenchymal stem cells (119). They are brought into the fracture area under the influence of paracrine or autocrine mechanisms (121) and then differentiate into pre-osteoblasts. In the case of bone fracture where there is a substantial gap between the originally continuous bone, a sequence of cellular and molecular events is initiated in response to the trauma, including inflammation, repair and remodeling (122). Injury leads to blood-clot formation that results in lysis of platelets, releasing numerous growth factors. The blood clot then begins to organize and the formation of a provisional callus that bridges the fracture site becomes apparent. Increased vascular permeability allows fluid and plasma proteins to leave the blood vessels. Various

cell types then emerge from the vessels in significant numbers. In acute inflammation, neutrophils (PMNs) are the first leukocytes to provide an effective defense in response to the trauma.

PGE₁ has a strong and dose-dependent promotive effect on the osteogenic activity of rhBMP (123), and glucocorticoids exert similar effects (69) whereas binding of BMP to free heparin at the fracture site could help to localize the growth factor by restricting its diffusion (13). Another component of the inflammatory process is the acidic conditions that develop and, together with the proteolytic fragments of the plasminogen system, help activate latent forms of growth factors such as TGF-β1 and regulate a positive feed-back system that amplifies activation of TGF-β1 from platelets, thus stimulating cartilage and bone formation (113). BMPs can also induce chemotaxis of monocytes and stimulate their expression of TGF-β1 mRNA (62). Finally, blood-clot formation following tissue injury results in the lysis of platelets that release numerous growth factors that are involved in wound healing and contribute to bone repair (121).

Depending on the mechanical stability of the fracture, mesenchymal stem cells can differentiate into chondroblasts or osteoblasts. If the fracture is mechanically unstable, cartilage will form and the bone fragments will be mechanically joined. If the fracture is mechanically stable, the chondrocytes within the blastema become hypertrophic, and along with their extracellular matrix, become eroded and replaced by osteoblasts and osteoid deposits (119,120). This process is similar to the one observed in embryonic long bone development. If the original break is mechanically stable, the repair blastema can be spanned by vasculature and the mesenchymal cells differentiate directly into secretory osteoblasts (119, 120). Mesenchymal stem cells may originate from the periosteum, the marrow space or are brought to the repair site via the circulatory system. Although the exact origin is not determined, the important fact is that mesenchymal cells will be attracted to the fracture site and play an important role in the repair process (124).

The inflammatory response acts as a multipotential modulator and initiator of the repair process, using mechanisms that will be described later, and as such must be considered a necessary event for the healing of bone wounds. TGF-β1 is an important and multifunctional autocrine regulator of bone formation (125). It has been demonstrated that TGF-β1 downregulates alkaline phosphatase, osteocalcin, osteopontin, collagen I and BMP-2 mRNA expression. This provides evidence that TGF-β1 acts as a powerful bone growth stimulant at the level of pre-osteoblasts (126), which is needed for the

coordinated progression of cell types along their differentiation pathways (127). TGF- β 1 stimulates DNA synthesis and replication of osteoprogenitor cells and is chemotactic for mesenchymal cells and osteoblast-like cells for recruitment of osteogenic cells to sites of bone formation and remodeling (113). EGF and FGFs are other molecules with demonstrated involvement in the complex molecular cascades involved in cellular change (128,129).

Implantation of rhBMP-2 resulted in early cartilage formation that was later replaced by bone tissue with bone marrow elements. Invasion of blood vessels into the cartilaginous anlagen lead to bone formation and bone marrow development (98). When larger doses of rhBMP-2 were used, bone formation was observed concurrently with cartilage formation, suggesting bone induction through both endochondral and intramembranous pathways. BMPs are believed to act through chemotactic, mitogenic or differentiating mechanisms. It is important to understand that BMPs are not the only determinants of cell fates along the above-mentioned lineages. Specific nutrients, growth factors and cytokines at specific concentrations and in a specific sequence of exposures are fundamental for the bone formation process.

During fracture healing, BMP-2/4 affect precursor cells to become chondroblasts and express proteins needed for production of woven bone (121). When lamellar bone replaces woven bone, BMP expression is significantly reduced (130). rhBMP-2 can induce bony trabeculae and bone marrow (99) with concomitant shortening of the time required for osteogenesis and increased amount of bone formation (85). BMP-4 is also expressed in less differentiated cells at fracture healing during distraction osteogenesis (131). rhBMP-2 does not increase the mitotic activity of osteoblasts (132) and does not affect DNA synthesis, but rather initiates sequences of gene expression in these cells (133). rhBMP-2 up-regulates the expression of BMP3/4 mRNA (109) with a mechanism that probably augments the transcriptional rate of the gene rather than stabilizing the mRNA (33). Evidence of BMP-4 activating the transcriptional factors Msx-1/2 and Egr-1 in epithelial-mesenchymal interactions during tooth development make this mechanism a valid working hypothesis (8). BMPs 1/2/4/6 are expressed by osteoblasts before they form mineralized bone nodules and during expression of ALP, OC, OP (134), thereby becoming guiding factors in osteoprogenitor cells (106). Unlike the BMPs, the TGF β s do not induce ectopic bone formation and inhibit chondrogenesis (63,110) but promote bone healing and fracture repair (125,135). However, acting at the level of cell adhesion molecules (36), they may stimulate mesenchymal cell attraction and proliferation (136). Being

part of a BMP-TGF β heterodimer can amplify the BMP effect (137) especially at the early stages of the bone repair process (61, 138). BMP-2 up-regulates the phenotypic expression of osteoblasts (82,83,133,139) and may indeed antagonize the repressing action of TGF- β by cross-reacting with TGF- β receptors (132). Additional studies have demonstrated a promotive effect of rhFGF (140) or prostaglandin E1 (PGE1) (123) on the action of BMPs, reducing the amount of growth factor needed to elicit a specific biologic response (141).

Carriers

The majority of studies investigating the role and action of exogenous BMPs use a matrix to deliver the growth factor to the implantation site. Although the matrix may not contribute any additional factors necessary for bone induction (107), it is a fundamental and very important component of the growth process. Collagenous or synthetic matrices have been used as delivery vehicles and their physicochemical properties, together with the microenvironment they create, play a role in the inductive outcome. Carriers can be solid xenogenic (HA) (89,142), solid alloplastic (polyethylene polymers) materials (143,144), or gels of autogenous (88,145), allogenic (146,147), or alloplastic origin (148), and combinations of the above (149).

One of the carrier functions is to maintain the factor at the site of implantation and thus enhance its local concentration. However, BMPs also help to stabilize the carrier by accelerating bone growth in its mass (150) due to the stabilization brought about by the BMPs absorption to the surface of the carrier matrix particles. As a result, 0.15 μ g of rhBMP-2 with matrix induced bone formation subcutaneously in rats, while a minimum of 75 μ g of rhBMP-2 was required in the absence of matrix (11). The isoelectric point and the structural features of the protein are important determinants of the implant-retained dose but the pharmacokinetics of the growth factor are not affected by carrier properties (151). Collagen matrix retains ~65% of the BMPs during initial impregnation and releases it in two phases: an initial phase within hours of implantation and a second phase that depends on the nature of the carrier and its geometrical characteristics (152).

It is believed that BMPs do not bind to the carrier (152), but rather become physically entrapped in its structure which makes certain designs more favorable for bone induction over some others (153). In the case of collagen sponge carriers, the mass, collagen cross-linking and sterilization methods affect BMP precipitation and subsequent resistance of sponge degradation by collagenase (154). Properties of the best carrier may vary depending

on the specific implantation site and the intended therapeutic outcome. Considerations include biodegradability, structural integrity, absence of immunogenicity, absorption and rate of release of BMP (155). The latter characteristic of the carrier serves its second function, which is controlled release of the BMP. This allows for a more constant and prolonged application. This renders BMPs more efficient and helps to create the chemotactic gradient, necessary for the cells to respond (11). BMP-2 is retained in a hydrogel carrier for more than 30 days whereas direct injection results in its complete elimination within 3 days (156). Collagen carrier also resulted in increased bone density of the regenerate when compared to polymeric matrix (157), emphasizing the importance of the structural properties of the carrier.

Recently, a novel approach has been suggested. This involves implanting matrices that actively concentrate native BMPs at the implantation site instead of passively storing and delivering rhBMPs which are a thousand times less potent than the native BMP complex (158). The matrix also serves as an environment in which bone can form and therefore helps to define the region in which new bone can be formed (159). Delivery vehicles with adequate structural consistency can function as primary scaffolds on which cells can attach and ECM, with subsequent mineralization, can be deposited (152,160). If the delivery matrix can act as a scaffold, then the cartilaginous intermediate may not be necessary. Many investigators agree that it has not been proved definitively that the chondrogenic process is essential for bone formation by BMP (89,161). The type of matrix used may also influence and determine the mechanism of bone formation that is appropriate for the implantation site (146). The material of the matrix and its geometrical parameters (pore size, and %volume) are factors that directly (size of cells able to attach) or indirectly (effect on blood or oxygen supply) determine the micro-environment and influence the mechanism of bone formation (endochondral or intramembranous) (59,89,90). BMPs combined with porous particles of hydroxyapatite or fibrous collagen membrane lead to intramembranous ossification (89,142,161), whereas fibrous glass membrane or insoluble bone matrix support indirect bone formation *via* a cartilaginous intermediate (89,90,142).

In examining the action of BMPs, it is also important to consider dose-related effects. It is evident that various doses elicit different responses on specific cell types at different time intervals (162). The dose of the growth factor determines its chemotactic, proliferative or mitogenic signal and should therefore be well regulated. Increased BMP concentrations result in faster bone growth (11), with cartilage being more rapidly replaced by mineralized

osteoid (163). rhBMPs in the form of monomers, homodimers or heterodimers need to be evaluated and standardized because they exhibit different biological potencies (11,121).

The carrier may also act synergistically by serving as a reservoir of the inducible cell population. Bone marrow can be combined with BMPs (164) and, when providing its cellular component, can result in bone formation of superior performance. Recently, investigators attempted the direct (*in vivo*) or indirect (using viral vectors) delivery of BMP genomic sequences to the implantation site (165,166), demonstrating active BMP expression for 2-6 weeks and bone formation with trabeculae and bone marrow. Cost of manufacturing and handling, in addition to ease of clinical application, are equally important factors to consider when deciding on a specific type of delivery vehicle.

Clinical applications

BMPs are of tremendous interest as therapeutic agents for healing bone fractures, preventing osteoporosis, treating periodontal bone defects and enhancing bone response around alloplastic materials implanted in bone (3). rhBMP-2 delivered with an absorbable collagen sponge (ACS) has been used for the augmentation of the maxillary sinus floor in humans (167). An rhBMP-2 dose ranging from 1.77 to 3.4mg per patient generated an average of 8.51mm of vertical bone height in four months providing a promising alternative to traditional grafting procedures (167). Similar results were also achieved in sub-antral augmentation of non-human primates with 6 mm of vertical bone gain and increased density that allowed placement of titanium implants (168). BMP-2 regenerated bone in irradiated tissues also provides the clinical potential to treat patients who have undergone radiation therapy and need bone reconstruction (169).

Periodontal regeneration was achieved when rhBMP-2 was applied to the defect site with a collagen membrane or a collagen gel. However, better results were obtained using the slower dissolving collagen membrane that allowed delivery of the growth factor for a prolonged period of time (170). The clinical outcome was a decreased depth of the defect site brought about by stimulating vertical bone growth and regenerating the periodontal attachment, provided that adequate space is maintained (171-175). The type of carrier, the time of treatment and the use of a barrier membrane are critical factors influencing the therapeutic outcome in cases of bone regeneration around dental implants (157) and have been shown to produce accelerated healing time as well as improved bone-implant contact levels (175-179). Moreover, alveolar ridge

preservation or localized augmentation have been documented in humans (180).

Animal studies also suggest that rhBMP-2/ACS may be an effective treatment for the restoration of segmental bone defects (181,182) and could lead to increased callus volume (183), strength and stiffness (184). A bioerodible polymeric carrier was used to deliver rhBMP-2 in a large segmental defect that was stabilized with stainless steel plates (185). Stabilization was necessary because of the large size of the animals (sheep) but it could have also helped to provide a stable environment for bone bridging since the carrier was reported to fragment easily. In a similar study in rabbits, porous poly-lactic acid carrier combined with rhBMP-2 was found to restore cortical bone with marrow elements in a twenty-millimeter long segmental defect (186). Skull defects were also filled with regenerated bone when BMPs were delivered in combination with hydroxyapatite (2,187), a biodegradable gelatin hydrogel or an aqueous solution (188). Spinal fusion was significantly enhanced when rhBMP-2 was administered with a hydroxyapatite graft or a collagen gel, and demineralized bone matrix revealed improved biomechanical properties and enhanced radiographic and histologic appearance (189).

Although purification and characterization of rhBMP-2 has been described in the Chinese hamster ovary (CHO) cell line (64) rendering BMPs available in large quantities, the fact that their inductive activity is ten times less than that of purified BMPs may present a limitation for their clinical application (190). Combinations of BMPs with other growth factors or biologic molecules forming heterodimers with twenty times higher potency in some cases (191) than homodimeric forms, hold a promising future in the field of bioengineering.

The parameter of host age further affects the biologic potential of many growth factors (161). The bone inductive ability of BMP-2 is diminished in older organisms and higher doses are required to induce the bone formation effect (192). Reduced migration of mesenchymal cells, lower levels of local anabolic agents, age associated reduction of receptor levels and compromised vascularization are some of the aspects to take into consideration (193-195). In the future, delivery of biological agents that control the regulators of BMPs may be of clinical significance in cases where BMP action needs to be halted to prevent pathological or hazardous ossification, such as after total hip or temporomandibular arthroplasties (44). Production of natural autogenous bone in moulds may allow a more efficient reconstruction of defects and deformities.

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