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Bone Morphogenetic Proteins: An Update on Basic Biology and Clinical Relevance

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Urist made the key discovery that demineralized bone fragments implanted either subcutaneously or intramuscularly in animals induce bone formation (100,101). The hunt for the factors responsible for this effect has resulted in the identification of a family of bone morphogenetic proteins (BMPs) (115). BMPs play a crucial role in cell growth and differentiation in a variety of cell types including osteoblasts (49,83,84). BMPs trigger cellular effects by way of heterotetrameric serine/threonine kinase receptors and intracellular signaling proteins known as Smads (31,62).

Evidence suggests BMPs and their receptors are required to promote bone regeneration following fracture (91). Shortly after a bone is fractured, BMP is released from cells at the injury site (4). Difficulties with bone regeneration may be linked to abnormal or insufficient endogenous BMPs, their receptors, and other recognized clinical etiologies (91,115). An overwhelming number of preclinical studies have validated the ability of recombinant human BMPs (rhBMPs) to regenerate bone (18,20,22,63,121). Therefore, because BMPs will likely become commonplace therapeutic agents for surgeons, it is timely to survey some of the recent exciting findings about the proteins, their receptors, signal transducers, and preclinical applications, as well as BMP-responsive genes. Moreover, by clearly defining current BMP biology, scientists and clinicians may collectively pursue the answers to questions that will benefit patients.

Update: Review and Discussion of Current Knowledge

Classification and Characterization

BMPs are members of the transforming growth factor-β (TGF-β) superfamily. Major subdivisions within the superfamily include the TGF-βs, BMPs (excluding BMP-1), growth/differentiating factors 1-10 (a subclass of BMPs [71]), inhibins, activins, Vg-related genes, nodal-related genes, *Drosophila* genes (e.g., *Drosophila decapentaplegic* and *Drosophila* 60A), and glial-derived neurotropic factor (32,48,50,60,61,85).

BMPs 1-9 were identified by the screening of human cDNA libraries to derive recombinant clones that encoded human BMPs. BMP-1 is not part of the TGF-β family; it is a proteinase and a member of the tolloid-like proteins associated with the dorsoventral patterning of *Drosophila* embryos, the sea urchin blastula, *Xenopus* and mouse mesoderm, and chick neural tube development. BMPs 2-9 are members of the TGF-β family on the basis of their similar amino-acid sequences (12-14,79,80,115). BMPs 10-13 have been identified by low-stringency hybridization and consensus polymerase chain reaction.

Structural studies of BMPs reveal that they contain a mature domain that is cleaved, allowing monomeric units to become dimers by a cysteine-disulfide bridge. Following intracellular glycosylation, the dimer is expressed in an active form. Protein assembly can produce homodimers, heterodimers, and glycosylation variability, which may influence the activity and effects of BMP (115). In addition, extracellular BMP antagonists regulate the biological effects of BMPs during bone formation (9).

Studies in the *Xenopus* embryo and mice identified five protein regulators of BMPs called noggin, chordin, gremlin, dan, and cerberus. These antagonist proteins bind to BMPs and thus govern cartilage and skeletal morphogenesis (9,40,123).

Osteoblast Differentiation

When bone is injured, such as by fracture, a local population of pluripotent progenitor cells is activated by growth/differentiating factors. The local cells are determined osteoprogenitors that reside in the cambial layers of the periosteum, endosteum, and dura. Another class of cells, the inducible osteoprogenitor cells, such as pericytes, arrive at the injury locus approximately 3-5 days after bone injury by transit in developing capillary sprouts (Fig. 1) (7,77,78,97). Peri-

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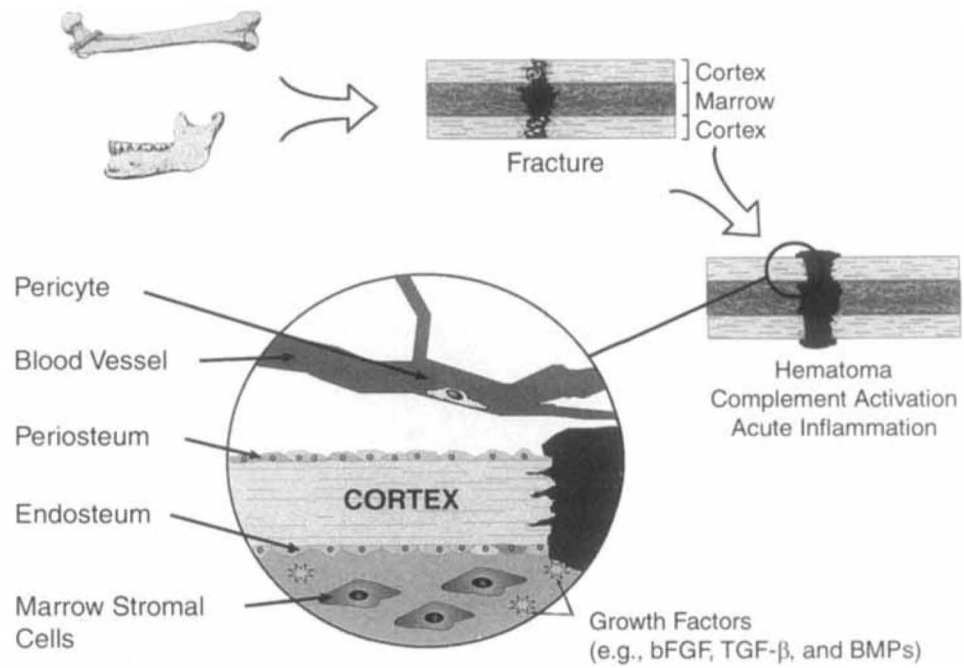

FIG. 1. The 5-day sequence of events following bone fracture. After fracture, a hematoma develops, the immune system is activated, and debris is removed from the wound site. Between 3-5 days after fracture, new blood vessels begin to develop while osteoprogenitor and mesenchymal stem cells localized at the wound site respond to environmental factors, including bone morphogenetic proteins (BMPs), to initiate bone restoration. bFGF = basic fibroblast growth factor, and TGF-β = transforming growth factor-β.

cytes may become osteoblasts following interactions with endogenous BMPs. According to Brighton and Hunt, a population of polymorphic mesenchymal cells can appear as early as 12 hours following fracture and become pre-osteoblasts (6). Moreover, mesenchymal stem cells within the bone marrow contribute to the repair blastema. These cells possess multilineage potential and can convert to either cartilage-forming chondrocytes or bone-forming osteoblasts, depending on the presence of environmental cues such as nutrient supply, BMP concentrations, growth factors, blood vessels, and mechanical stability (8,10,82). For example, marrow-derived inducible osteoprogenitors undergo osteoblastic differentiation in response to BMPs and growth factors (Fig. 1) (1,44,57,86,88). The conversion of osteoprogenitor cells to mineralizing osteoblasts is a key event for bone regeneration. BMPs are molecular cues for osteoprogenitor cells to differentiate into osteoblasts (Fig. 2) (84,115). They also initiate bone formation in a sequential cascade on the basis of concentration-dependent thresholds (84), and they bind specific surface receptors and initiate intracellular responses that result in a mineralizing osteoblast (54,71,91,119). Contemporary work has elucidated some of the intracellular signaling pathways for BMPs and subsequent gene activation leading to osteoblast differentiation. Additional efforts to understand the pathways that BMPs use to activate particular osteoblast genes will provide a scientific basis to develop rational clinical therapies.

Receptors and Activation

BMPs bind and initiate a cell signal through a transmembrane receptor complex formed by types I and II serine/threonine kinase receptor proteins. Type-I (BMPR-IA or BMPR-IB) and type-II (BMPR-II) re-

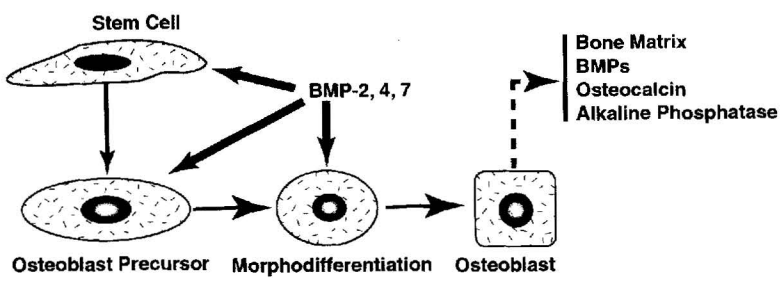

FIG. 2. Osteoprogenitor and mesenchymal stem cells at the fracture can respond to bone morphogenetic proteins (BMPs) 2, 4, and 7 and differentiate into osteoblasts. Osteoblasts typically produce and secrete several proteins, including osteocalcin, osteopontin, and alkaline phosphatase, as well as bone matrix.

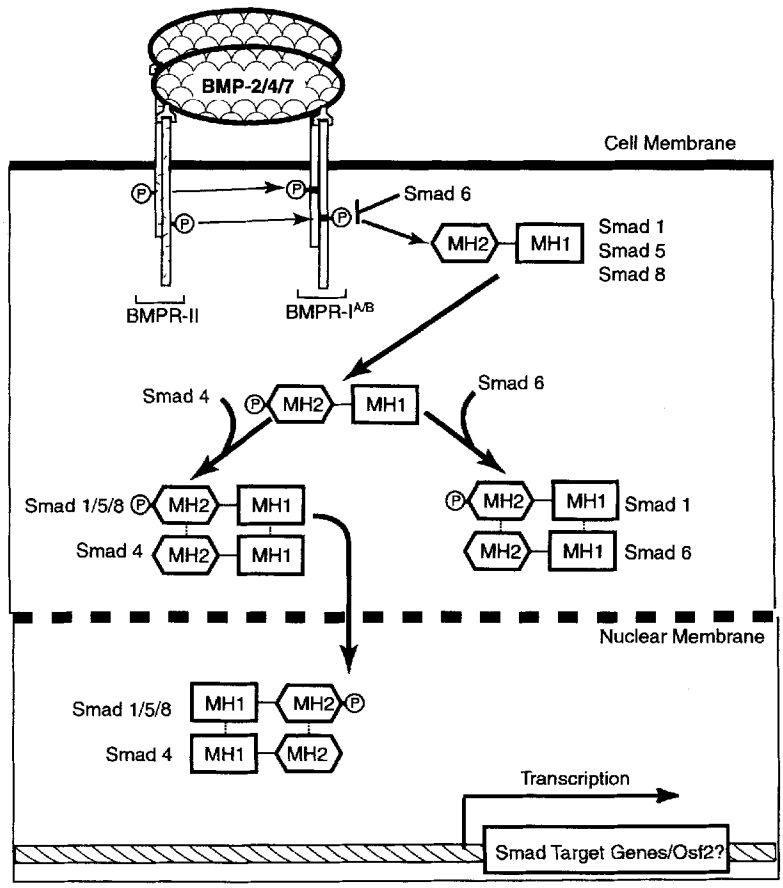

FIG. 3. Bone morphogenetic protein (BMP) receptor binding and intracellular signal transduction. BMPs bind types I and II serine/threonine kinase receptors to form a heterotetramer. Following binding, the type-II receptors phosphorylate the glycine/serine-rich domain of the type-I receptor. The type-I receptors phosphorylate the MH2 domain (Smad homology domain) of Smads 1, 5, and possibly 8. Smad6 may block the phosphorylation cascade by binding the type-I receptor. After phosphorylation, Smads either bind to Smad4 and translocate to the nucleus or bind to Smad6 where the signal is stopped. Once inside the nucleus, Smads activate gene transcription. The Smad complex may directly or indirectly initiate transcription of the osteoblast-specific factor-2 (Osf2) gene (31). P = phosphorylation (phosphorylated or "activated" protein), BMPR-II = type-II bone morphogenetic protein receptor, and BMPR-I$^{A/B}$ = type IA or IB bone morphogenetic protein receptor.

ceptor proteins are distinguished on the basis of their molecular weights, the presence of a glycine/serine-rich domain located on the type-I receptor, and the ability to bind a particular ligand. Individual receptors have low affinity for BMPs; however, as a heterotetrameric complex, high-affinity binding is achieved (Fig. 3) (54,72,90). Evidence suggests that the type-II receptors are active continuously (autophosphorylating) and function upstream of the type-I receptors but cannot independently initiate cell signals (116). On binding BMPs 2, 4, and 7, the type-II receptor kinase transphosphorylates the type-I receptor at the glycine/serine-rich region; this event generates an intracellular response (Fig. 3) (62,116). Specificity in signaling appears to be determined primarily by the type-I receptor (11).

Abnormalities Associated with BMPs and their Receptors

Mice deficient in BMPs 2, 4, and 7 die at early embryonic stages or shortly after birth (25,58,113,122). Zhang and Bradley observed that animals lacking BMP-2 possessed a malformed amnion and chorion and had abnormal cardiac development (122). Moreover, mesodermal differentiation was aberrant in mice lacking BMP-4 and no mesoderm developed in those lacking BMPR-IA (65,113). Furthermore, alterations in the kidney, eyes, rib, skull, and hindlimbs resulted when expression of BMP-7 was absent in mice (25). In addition to the developmental abnormalities discovered through mice knockout models, several skeletal disorders have been mapped to mutations in BMP genes. The mouse short-ear mutation, which maps to the BMP-5 gene, results in an abnormally shaped and sized external ear, as well as aberrations in the ribs and vertebral processes (49). Other mutations are associated with BMPs 2-4, suggesting roles for these morphogens in the pathological conditions known as fibrodysplasia ossificans progressiva (46,81) and dentinogenesis imperfecta (96). Fibrodysplasia ossificans progressiva can be characterized by malformation of the great toes and ectopic bone formation (94), and

dentinogenesis imperfecta is characterized by dental abnormalities (96).

Intracellular BMP Signal Transduction

Several intracellular proteins associating with receptors of the TGF-β family have been identified with yeast two-hybrid interactive screens. From these screens, the tryptophan and aspartic acid repeat proteins TRIP-1 (TGF-β-receptor interacting protein), the subunit farnesyl-protein transferase, and FKBP-12 (an abundant immunophilin protein, capable of binding the TGF-β receptor) were observed interacting with the receptors (15,107,108). An important breakthrough in understanding how BMPs transmit intracellular responses came from genetic screening with *Drosophila* proteins. Data revealed enhanced expression of the *Drosophila decapentaplegic* gene, a homolog of vertebrate BMPs 2 and 4, by the gene product termed Mothers against *Drosophila decapentaplegic* (MAD) (93). MAD proteins are required for *Drosophila decapentaplegic* signaling and function downstream of the *Drosophila decapentaplegic* receptor (38,69,110). Several homologs to MAD proteins (i.e., Smads) have been identified in *Caenorhabditis elegans*, *Xenopus* mice, and humans. The current vertebrate proteins related to MAD include MADR1/Smad1, MADR2/Smad2, MADR3/Smad3, and Smads 4-9. *Drosophila* MAD is 81% identical to Smads 1 and 5 and 70% identical to Smads 2 and 3 (2).

Smad proteins interact with BMP receptors by an L3 motif and possess conserved N-terminal (MH1) and C-terminal (MH2) domains separated by less conserved threonine, serine, and proline linker regions (26,38,56,69,92,93). The various TGF-β-superfamily isoforms appear to signal through different Smad isoforms (26,38,62). Human Smad1 is activated and directly phosphorylated on a serine residue by type-I BMP receptors (52). Following activation, Smad1 associates with Smad4 as a hetero-oligomer, rapidly accumulates in the nucleus of the cell, and may play a role in bone formation (Fig. 3) (38,53,55). Interestingly, overexpression of Smads 1 and 5 converts myoblasts to osteoblasts independent of BMP activation (118). This may suggest a functional action for Smads that can be exploited as a clinical therapeutic device to promote osteoblast differentiation.

Smad5 is activated by BMP-2 and associates with Smad4 (70). Recently, it was shown that Smad8 is structurally similar to Smads 1 and 5; however, its function has yet to be elucidated (109). The Smad1 signaling pathway appears to be regulated by Smad6, which inhibits Smad1 signaling through binding to the type-I receptor and by competing with Smad4 for binding to receptor-activated Smad1. This process produces an inactive complex of Smads 1-6 (Fig. 3) (30,42). The C-terminal domain (MH2) of Smad1 is required to activate gene transcription (55). C-terminal binding of Smads to DNA and subsequent transcriptional activation has been demonstrated in *Drosophila* (47).

Parallel pathways for the transduction of specific signals may exist. TGF-β-activated kinase 1 (a member of the mitogen-activated protein kinase kinase kinase family) has been shown to be activated by either TGF-β or BMP-4 (117). In addition, Ras or Rac families of small GTP-binding proteins become activated by TGF-β (29,66). These secondary message pathways may integrate with primary signal-transduction mechanisms and functionally modulate cell activity.

In the Osteoblast Nucleus

It has been postulated that Smads may function as inducible transcriptional activators associated with a DNA binding component when they enter the osteoblast nucleus (55). For example, Smad2 forms a complex with the DNA binding-component forkhead activin signal transducer-1 in an activin-dependent fashion to generate an activated complex that binds to the activin-responsive gene element (16). Purportedly, once Smad2 interacts with forkhead activin signal transducer-1 inside the nucleus, the complex directs transcription of the Mix.2 gene. We hypothesize that Smads 1, 5, and possibly 8 may bind nuclear elements or proteins and activate gene transcription (Fig. 3). An alternative hypothesis could be that phosphorylated Smads represent a novel class of transcription factors that directly bind DNA and activate transcription (38,47).

We postulate that phosphorylated Smads may activate, directly or indirectly, the osteoblast-specific factor-2/core-binding factor-1 gene, which translates into the osteoblast-specific factor-2 protein. The gene encoding the osteoblast-specific factor-2 protein is closely related to transcriptional activators conserved between *Drosophila* and humans (74). The protein and its homologs possess a conserved runt domain with a Val-Trp-Arg-Pro-Tyr (VWRPY) motif within the C-terminal and an alpha subunit with a conserved 128-amino-acid peptide region (43,45,74). The runt domain allows osteoblast-specific factor-2 to become a heterodimer and bind DNA (73,74). The component that recognizes DNA binds to a sequence-specific gene-enhancer core motif, TGTGGT, found in viral and eukaryotic genes (64,95). Transcription factors (trans-acting) that bind to the gene core sequence have been termed core-binding factors.

The osteoblast-specific factor-2 protein, which has recently been cloned, binds directly to and activates the osteocalcin transcriptional promoter region (23, 24,27). The osteocalcin gene is a molecular marker found solely in osteoblasts, and its promoter contains three cis-acting elements capable of binding trans-

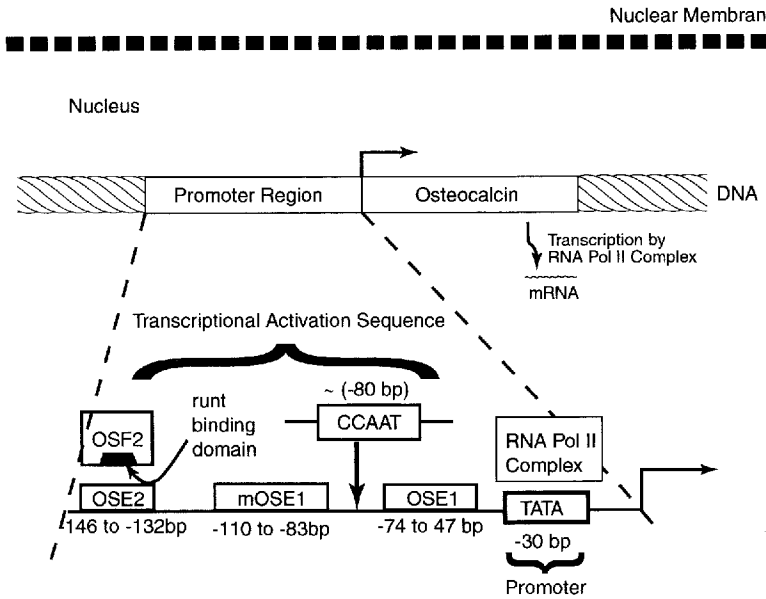

FIG. 4. Osteocalcin gene regulation. Inside the osteoblast nucleus, the osteocalcin gene is controlled by the promoter region, binding several proteins that activate gene transcription. Osteoblast-specific factor-2 (OSF2) binds the osteoblast-specific element-2 (OSE2) by way of its runt domain. Following the binding by osteoblast-specific factor-2, the TATA (a nucleotide sequence with T [thymine nucleotide] and A [adenine nucleotide]) box binds the RNA polymerase II (Pol II) complex, which transcribes the osteocalcin genetic sequence into mRNA. The mRNA is translated into the osteocalcin protein on the ribosomes. Also shown within the osteocalcin promoter region are the genetic sequences mouse osteocalcin E-box sequence-1 (mOSE1) and osteoblast-specific element-1(OSE1).

acting factors (e.g., osteoblast-specific factor-2) (23, 28,99). The three osteocalcin-gene control points have been designated osteoblast-specific element-1, mouse osteocalcin E-box sequence-1, and osteoblast-specific element-2 (23). Geoffrey et al. have shown that the osteoblast-specific element-2 sequence is a transcriptional control point for the osteocalcin gene (Fig. 4) (27). Osteoblast-specific factor-2, a protein that is present only in osteoblastic cell lines and primary osteoblasts, binds osteoblast-specific element-2 (23,27). Genetic sequence elements similar to the osteocalcin osteoblast-specific element-2 sequence have also been found in the promoters of α1(I)collagen, bone sialoprotein, and osteopontin.

BMP-treated cells express osteoblast-specific factor-2 before expressing other osteoblast-specific genes (24). Furthermore, osteoblast-specific factor-2 can promote osteoblast differentiation from nonosteoblastic cells (24). MC3T3-E1 cells transiently transfected with cDNA for osteoblast-specific factor-2 forced the expression of bone sialoprotein, osteocalcin, and α1(I)collagen. In addition, transfection of C3H10T1/2 cells and mouse skin fibroblasts with this osteoblast-specific factor led to the expression of bone sialoprotein and osteocalcin.

It was recently postulated that osteoblast-specific factor-2 may trigger mesenchymal stem cells to differentiate into osteoblasts during the developmental process (24,89). For example, mice deficient in this osteoblast-specific factor lacked osteoblasts and bone, were smaller in size than those not deficient, and died due to respiratory failure (51,76). Osteoblast-specific factor-2-heterozygous (±) mice, however, exhibited skeletal abnormalities characteristic of the human heritable skeletal disorder cleidocranial dysplasia (67,76).

Osteoblast-specific factor-2 plays an important role in the formation of bone and is activated in response to exogenous BMPs, leading to the formation of osteoblasts and the production of new bone. Further research is required to examine the roles of this osteoblast-specific factor in skeletal development and repair and human heritable disorders and how these anomalies may be treated with therapy based on the factor.

Fracture Repair

Fracture repair is a close recapitulation of embryonic events and includes a complex interaction of growth-regulatory factors and responding cell populations. Fracture repair results in the regeneration of an osseous structure that is physiologically and biomechanically indistinguishable from the original. The regeneration cycle is coupled with and dependent on BMPs (37).

Immediately after a bone is fractured, an inflammatory response is elicited, activation of complement cascade ensues, and vascular damage at the injury site causes extravasation and cell signaling. Proteolytic degradation of the extracellular matrix produces chemotactic remnants luring monocytes and macrophages to the wound bed; activated macrophages release basic fibroblast growth factor (bFGF), stimulating endothelial cells to express plasminogen activator and procollagenase (17). Growth factors released from the

alpha granules of degranulating platelets are signals for polymorphonuclear leukocytes, lymphocytes, monocytes, and macrophages.

The extravasated, localized collection of blood will clot, form a hematoma, establish a hemostatic plug, and prevent blood loss (Fig. 1). Orchestrating the clotting cascade are platelets, which have the dual function of hemostasis control and mediator signaling through expression of platelet-derived growth factor (PDGF), TGF-β, and bFGF (33).

The early fracture environment is characterized by a decrease in oxygen tension and pH, conditions that facilitate the operational activities of polymorphonuclear leukocytes and macrophages (33). Polymorphonuclear leukocytes remove microorganisms and microdebris, whereas larger-sized materials are handled by macrophages that may develop into polykaryon, multinucleated giant cells. Macrophages provide a formidable synthesis capability to the wound site by manufacturing growth factors to fortify cell activity, recruit cells, and provoke mitogenesis and chemotaxis throughout the injury repair cascade until abatement.

By days 3-5 following fracture, a repair blastema develops that consists of new blood vessels, cells (e.g., fibroblasts and macrophages), and collagen isotypes. Selective binding of growth factors to collagens may localize, protect, and temporally position growth factors to optimize cell interaction (83). Therefore, the collagenous component of the repairing wound is a key instructional substratum to present TGF-β, bFGF, PDGF, and the BMPs to receptive cells (Fig. 1). Undifferentiated cells traversing neovasculature and osteoprogenitor cells localized to periosteum and endosteum anchor to the granulation tissue collagen and differentiate into chondrocytes and osteoblasts under the aegis of signaling molecules, namely, the BMPs (83,85). The biological influence of the BMPs on cell differentiation is of particular interest with respect to bone formation. The combinatorial activities of cell anchorage, transduction, and cell-factor interaction promote cell differentiation to specific phenotypes to repair the osseous wound. Teams of cells, as well as growth/differentiating factors (e.g., TGF-β, fibroblast growth factors [FGFs], vascular endothelial growth factor, BMPs, and PDGF), ensure fracture healing by approximately 6-8 weeks after injury (33). The reconstruction of a bone structure indistinguishable from the tissue before injury is carefully crafted by osteoblasts and osteoclasts (104). However, if sufficient quantities of cells are not resident at the fracture site, they must be recruited, expanded in number, and acted on by the proper combination of growth conversion factors. At the injury site, fragments of fibronectin (a ubiquitous attachment factor) and degradation products from the extracellular matrix stimulate the conversion of monocytes to osteoclasts (33). Moreover, macrophages at the wound site release bFGF and vascular endothelial growth factor, prompting neoangiogenesis and vessel formation to provide transit for additional cells to replenish those lost to injury (41).

The clinical relevance of BMPs and a responding cell population at the wound site represent the final common pathway of the elements that contribute to the regeneration of bone (85). Cells must be competent to respond to the BMP signal or signals, and sufficient quantities and types of biologically active BMPs must be present to produce the desired outcome, e.g., to regenerate the form and function of bone. BMPs and their receptors are stewards in this marvelous process (91).

Recent studies have revealed increased expression of BMPs 2, 4, and 7 in primitive mesenchymal and osteoprogenitor cells, fibroblasts, and proliferating chondrocytes present at the fracture site (4,68,75). Expression of BMPs 2 and 4 was upregulated in mesenchymal cells that had migrated into a fracture opening and begun to proliferate (4). In addition, BMPs 2, 4, and 7 were present in newly formed trabecular bone and osteoclast-like cells (75). Taken together, these findings suggest that BMPs 2, 4, and 7 work cooperatively and synergistically to promote fracture healing and bone regeneration (91).

Clinical Applications of BMPs

A flurry of research has focused on the application of BMPs in clinically relevant animal models (18,19,22,34,59,63,102,103,105,106,120,121). In animal model systems, rhBMPs promoted fusion of vertebral bodies and regeneration of skull, mandibular, and long-bone defects (3,18-22,59,63,87,98,120,121).

Two published clinical reports used milligram doses of rhBMP-2 (5,39). In these reports, the magnitude of the protein required for effect underscores a penetrating clinical challenge and invokes several compelling questions. Do milligram doses of the protein portend daunting obstacles caused by manufacturing costs that will have to be absorbed by patients? If milligram quantities are needed, can rhBMPs be manufactured to meet these needs? Moreover, does the administration of milligrams of BMPs to a patient unleash sinister, unpredictable, or unexpected biological sequelae? Furthermore, what may be the outcome of multiple dosing?

The format used to administer rhBMP to a patient could have a striking impact on dosing needs. Moreover, the availability of a locally responsive cell population will impact on dosing and outcome (37). In terms of format, the quantity of the protein necessary for a clinical effect may be modulated with a carrier/delivery system (35,36,112). A carrier/delivery

system could titrate BMP release kinetics and bioavailability at the application sites as well as provide a haven for exogenous BMP-responsive cells (35,37). The clinical studies reported for rhBMP-2 used a collagen delivery system (5,39). Perhaps a more suitable carrier/delivery system could economically package and deliver a physiological judicious dose of the protein for clinical applications.

Summary and Perspectives

The regeneration of bone is a remarkable, complex physiological process, and BMPs are a formidable clinical tool to promote its regeneration. By defining roles played by BMPs in developmental biology and bone regeneration, significant progress has been made to identify cell-signaling molecules and their regulators. For example, the regulators of BMPs that include noggin, chordin, cerberus, dan, and gremlin may be harnessed as therapies to offset calcification encountered after total hip arthroplasties. Furthermore, exploiting BMPs and Smads may generate new therapeutic options for bone repair. Another compelling clinical consideration is the trans-acting factor osteoblast-specific factor-2, which can promote osteoblast differentiation. Moreover, the affiliation of osteoblast-specific factor-2 with heritable disorders merits exploration. A recognized daunting challenge includes a carrier/delivery system for the powerful morphogenetic therapeutic tools, as well as osteoprogenitor cells and intracellular transduction and transcriptional factors. In addition, the long-term effects of administering superphysiological doses of rhBMPs to patients must be assessed systematically. A new generation carrier/delivery system may be the answer to offset dosing liabilities as well as to provide residence for exogenous, BMP-receptive osteoprogenitor cells (111,112).

The areas highlighted in this review offer fertile territory for thought and research to develop rational clinical treatments to promote bone regeneration and to understand some of the biological roles of BMPs.

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