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REVIEW ARTICLE

Bone morphogenetic proteins in fracture repair

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Abstract

Bone fractures represent a significant medical morbidity among aged population with osteoporosis. Bone morphogenetic proteins (BMPs) are suggested to have therapeutic potential to enhance fracture healing in such patients. Though BMP-mediated fracture healing has been well-documented in preclinical models, there has been no clinical study that demonstrated unequivocally that indeed a BMP when presented with an appropriate scaffold could provide basis for robust outcome for delayed or non-union diaphyseal bone fractures. This review presents a comprehensive insight towards the existing knowledge on the role of BMP signaling in bone formation and maintenance. Also therapeutic options based on BMP biology are discussed.

A novel osteoinductive autologous bone graft substitute (ABGS) aimed to accelerate bone regeneration was developed and is currently being tested in the clinical setting. It comprises of a biologically compatible autologous carrier made from the patient's peripheral blood (autologous blood coagulum, ABC) and of rhBMP6 as an active ingredient. Such formulation circumvents the use of animal-derived materials, significantly limits inflammatory processes common in commercial bone devices and renders the carrier flexible, malleable, and injectable ensuring the ease of use. The ongoing clinical trials result will provide more detailed insights into the safety, tolerability, pharmacokinetics, and bone healing effects in humans and potentially provide novel and safe therapeutic options for bone repair.

Keywords Bone · Bone fracture · Bone morphogenetic proteins · Regenerative treatment BMP6 containing osteogenic device

Introduction

Bone is one of the few tissues in the adult human body with the ability to repair, regenerate, and restore function spontaneously upon fracture. In the EU, 3.5 million bone fractures were reported in 2010 [1] and it is estimated that more than 50 million men and women will be at bone fracture risk due to osteopenia/osteoporosis in 2050 [2].

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Bone fracture repair

Bone healing process is a prototype for tissue engineering since it involves signal, cells and substratum, and is traditionally divided into three stages: an early inflammatory and cell recruitment stage (callus formation), intermittent cell differentiation and formation of new bone (fracture repair) and late bone remodeling and formation of defined cortices (restoration). Most of the fractures heal in time without any consequences, however, when fractures are compounded or open it can result in indirect or secondary healing due to incomplete mechanical stability of broken fragments in mixed intramembranous and endochondral ossification subsequent to callus formation. Intramembranous ossification produces bone directly under the periosteum within the first days after injury, overleaping chondrogenesis in the centre leading to endochondral ossification. Improper osseous healing has potentially devastating consequences, ranging from disfigurement to the loss of function and eventually loss of limb. In cases where normal bone fracture healing is not achieved, it is advisable to apply BMP containing substratum to induce the formation of new bone locally and assure the bridging [3]. In order to achieve successful bone

union adequate blood supply, aseptic environment, mechanical stability, and appropriate soft tissue management are mandatory as well. There are reports indicating a genetic predisposition for bone non-union, where defective BMP downstream signaling correlated with non-union fractures [4]. In approximately 10% of cases, fractured bones heal slowly (mal-union) or fail to heal (non-union) and they require additional medical interventions to repair the fracture, like in smokers and steroid users [3]. The conditions that predispose fractured bone to impaired healing include old age [5] and smoking [6], as well as other conditions with malfunction in bone metabolism, like osteoporosis, diabetes, and NSAID use [7, 8].

Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are growth and differentiation factors and form a large subfamily of the transforming growth factor-beta (TGF-β) superfamily. They provide morphogenetic signals for skeletal development during embryogenesis and are responsible for adult fracture healing by recapitulating a cascade of cellular events associated with embryonic bone formation. More than 30 different BMPs based on structural similarity have been identified and some of them were suggested to play a role beyond bone [9–11]. Bone-inducing BMPs are divided into several subgroups, based on the homology of their amino acid sequences: BMP2/BMP4, BMP5/BMP6/BMP7, BMP9/BMP10, and BMP12/BMP13/BMP14 groups, while other BMPs do not have proven osteogenic properties [10].

In 1965, Marshall Urist demonstrated for the first time that demineralized bone has capacity to induce new bone when implanted at ectopic sites, which he described as "bone formation by auto induction" [12, 13]. Reddi has shown that implantation of demineralized bone matrix induces a cascade of cellular events involving mesenchymal cell recruitment, proliferation, and differentiation into cartilage-forming cells. With concurrent invasion of blood vessels, cartilage undergoes hypertrophy resulting in formation of new bone and bone marrow elements [14]. It is known that the process of ectopic bone formation stimulated by BMPs is mediated by progenitor cells found around blood vessels and connective tissues without the presence of osteoclasts [15]. Namely, a biological response to BMPs is dependent on the local microenvironment and type of cells present at the site of BMP implantation. BMPs are more effective in abundancy of pluripotential cells. In bone injuries, skeletal progenitor cells originate from multiple tissue compartments including the injured periosteum, endosteum, vascular tissue, and the surrounding musculature, and jointly contribute to skeletal healing [16]. Periosteal bone and the microenvironment outside the bone medullar cavity both lack osteoclasts and, therefore, uncoupled osteoblast precursor cells upon addition of a BMP on an appropriate carrier together form bone [15]. However, during bone remodeling coupling between osteoblasts and osteoclasts occurs in bone shaft, representing a crucial step in bone remodeling that depends on the local milieu of cytokine signaling and systemic hormones.

Currently, effects of BMPs are described after local implantation. It was shown that high levels of circulating BMP9 seem to be associated with faster fracture healing, but results were not significant [17]. Moreover, there was no difference between other measured BMPs in groups with normal and delayed healing. Plasma BMP values were determined in a single time-point, thus disabling the insight into potential difference between healing phases.

Molecular interventions into BMP bone development and growth

It was long thought that BMPs primarily induce new bone at an endosteal bone site. However, recent studies have shown that the inactivation of the BMPR-IA receptor signaling in mouse osteoblasts leads to significantly increased bone volume [18]. Correspondingly, when a conditional deletion of the same receptor in differentiated mouse osteoclasts was introduced, an increase in the osteoblastic bone formation and bone volume was demonstrated [19]. These scientists also showed that BMP4 overexpression in mouse osteoblasts resulted in bone loss [20]. When BMP2 was used intramedullary at an endosteal bone site, a suppression of osteogenesis was observed and explained to be due to downregulation of Runx2 and collagen I synthesis [21]. Additionally, inhibition of Wnt signaling occurs as a consequence of targeting Wnt inhibitors Dkk1 and Sost downstream of BMP signaling through BMPR-IA receptor in osteoblasts [22]. The observed BMP effect on endosteal bone cells is the result of a pronounced activation of osteoclasts and their progenitors and expression of BMPR-IA and BMPR-II receptors on their membranes [15]. The sum effect of osteogenic BMPs seems to be a net bone loss, resulting from their stronger effect on osteoclasts compared to osteoblasts. This was supported by the rat study showing superiority in treating fracture non-union with early endothelial progenitor cells, when compared to late endothelial progenitor cells which release more BMP2 [23]. These unexpected results are inconsistent with the in vitro evidence showing that BMP2 and 7 promote differentiation of various osteoblast-like cells and in vivo induce new bone formation at ectopic sites in experimental animals (Fig. 1). On the other hand, when BMP2 and 7 are applied at orthotopic bone sites bone repair will depend on the presence of coupled bone cells and on the bone microenvironment and the result of therapy will be the loss of the bone volume. However, when osteogenic growth factors are delivered at an ectopic site in an uncoupled cell environment or in the vicinity of the periosteum or muscle, they will support bone formation

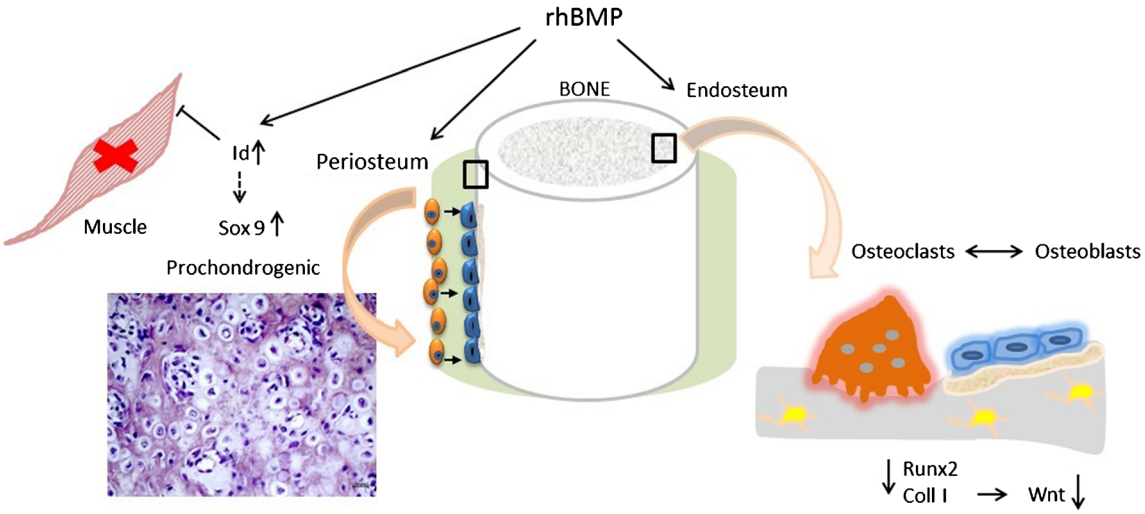

Fig. 1 Different responses of rhBMPs on bone endosteal, periosteal, and muscle compartments. At the endosteal bone surface, BMP affects bone resorption and formation resulting in downregulation of Runx2, collagen type I, and Wnt signaling. At the periosteal surface BMPs upregulate the expression of Id genes in surrounding muscles which results in endochondral bone formation spreading from the bone surface into the medullary canal. They also stimulate differentiation of periosteum progenitor cells into osteoblasts

and bone healing by extending the area of new bone from an uncoupled to a coupled bone surface, which will then incorporate into an endogenous coupled bone microenvironment.

In the presence of calcium phosphate-based biomaterials, BMP, Wnt, and PKC signaling pathways are activated discriminating the bone forming from non-bone-forming constructs [24] which additionally influences the quality of newly formed bone.

BMPs and physiological bone repair

Fracture healing recapitulates events that occur during embryonic bone formation, and indicates that the cells and pattern defining molecules that drive bone formation during embryogenesis are still present in the adult bone. Bone healing process is traditionally divided into three stages: an early inflammatory stage, a repair stage, and a late remodeling [25]. Most of the fractures undergo indirect or secondary healing due to incomplete stability of broken fragments resulting in mixed intramembranous and endochondral ossification and subsequent callus formation. Intramembranous ossification produces bone directly under the periosteum within the first days after injury, overlapping chondrogenesis which is the main step in endochondral ossification. BMP 2, 4, and 7 are highly upregulated in this early stage around the injured periosteum. Role of BMP signaling in both intramembranous and endochondral ossification was suggested two decades ago [26]. This early phase of healing enables temporarily fracture stabilization and further endochondral bone formation. It is characterized by the recruitment of mesenchymal stem cells (MSC) and successive chondrogenesis resulting in soft callus formation [27]. BMPs potentiate differentiation of MSC towards chondroblast and osteoblast lineage. It was demonstrated in the mouse fracture healing studies that BMP2 initiates the repair cascade with its mRNA expression peaking at 24 h after bone injury [28]. In the mouse MSCs differentiation experiments, BMP2 regulated the expression of several other BMPs and was crucial for the successful differentiation of MSCs into osteoblasts [29]. BMP3, 4, 7, and 8 are expressed in the osteogenic stage of bone repair, when the resorption of calcified cartilage, osteoblastic recruitment, and bone formation are most pronounced. BMP5 and BMP6 are constitutively expressed from days three to 21 during fracture healing in mice, suggesting their active role in both intramembranous and endochondral ossification. Although BMP8 has a high osteogenic potential, BMP2, BMP6, and BMP9 are the most potent inducers of MSCs differentiation to osteoblasts, while other BMPs mainly stimulate the maturation of osteoblasts [30]. In the presence of adequate angiogenesis during the late phase of healing, cartilage tissue is replaced by the woven bone, which undergoes remodeling to potentiate normal function of the healed bone.

Complex interactions between osteoblasts and osteoclasts result in bone remodeling and take place at particular sites throughout the fracture called bone metabolic unit (BMU). Effects of BMP signaling on osteoblasts mostly depend on the maturation stage: BMPs enhance their early phase, while have little or no influence on mature osteoblasts [31]. BMP2, 4, and 7 were detected by immunostaining in osteoclast-like cells in the newly formed trabecular bone, mostly between days 14 and 28 after fracture [32].

Chemotactic signal for osteoprogenitors is provided by numerous growth factors, among which bone morphogenetic proteins (BMPs) have the central role. Integrin-linked kinase was shown to have huge impact in modeling cytoskeletal

organization and adjusting BMP signaling in osteoprogenitor cells [33]. Mice lacking functional integrin-linked kinase in osterix-expressing cells displayed a significantly reduced trabecular bone mass after five weeks, which persisted into adulthood.

Expression of BMP signaling components in fractures

As BMP signaling pathways emerged as mainstay of successful fracture healing, potential clinical application of BMP antagonists in bone repair was proposed [34]. Kloen and collaborators were the first to report BMP signaling in human callus obtained from patients with complicated fracture who underwent a surgical procedure [35]. Immunohistochemical analyses revealed an increased staining for BMP2 and 4 in the area of endochondral ossification, particularly in the matrix between the newly formed osteoid. In contrast, BMP3 and 7 were greatly expressed in osteoblasts inside the novel osteoid tissue. Osteoclasts did not exhibit significant expression of BMPs, except BMP3, which is generally considered to be an antagonist of regular BMP effects. BMPR-IA and BMPR-IB were present in all cells of interest, mainly fibroblasts, osteoblasts, chondroblasts, and osteoclasts, while BMPR-II staining was less intense in osteoblasts and cartilaginous tissue. Further studies scrutinized expression of BMP inhibitors in fracture callus [36]. Inhibition of Noggin and Chordin increased the osteogenic differentiation of murine and human mesenchymal stem cells [37]. The expression of BMP2, BMP4, Noggin, and Chordin in healing tissue was highlighted in the areas of endochondral ossification, confirming the central role of BMP signaling in this phase of bone repair. Namely, their expression was only moderate in initial and remodeling phase. BMP14 demonstrated the strongest staining in human fractures consistent with its inhibition of long bone healing in mice resulting from the delay of chondrocyte differentiation [38]. Concept of impaired healing and subsequent non-union because of the imbalance between BMPs and their antagonists have been introduced by Reddi and colleagues [39]. However, expression of Noggin and Chordin was similar in patients with non-unions and normal bone healing [40].

Recent study using human tissue confirmed distortion in expression of BMPs and BMP inhibitors in a non-union, when compared to the normal fracture healing [41]. In non-unions, the chondrocyte expression of BMP2 was significantly decreased, and BMP7 was completely absent, while mature osteoblasts exhibited normal expression of BMPs maintaining the expression of BMP inhibitors similar in both osteoblasts and chondrocytes. This imbalance hypothesis would therefore be supported mainly by the lack of BMP expression in cartilage tissue and impossibility to contribute to osteoblastic differentiation and subsequent ossification.

Additional clinical trials and carefully designed animal experiments including knockout mice and specific inhibition of BMP antagonists are necessary for clarification whether a bone non-union is a consequence of increased BMP antagonists' concentration and insufficient BMP levels.

BMP-based therapy for fracture healing

Following FDA approval for specific indications in 2002 and 2004, safety and efficacy of BMP2 and 7 have been extensively investigated in randomized, blinded, and controlled trials (RCT) [42, 43].

RhBMP2 has been approved for treatment of open tibial fractures following a large RCT involving 450 patients with different types of fractures according to Gustilo-Anderson classification [44]. During 1-year follow-up period BMP2 in the higher dose (1.5 mg/mL) enhanced bone healing and reduced the number of secondary interventions when compared to patients treated with the standard of care. Major disadvantage of this study was the fact that surgeons were not blinded for patients receiving rhBMP2. In another RCT, the efficacy of rhBMP7 in tibial non-unions of 124 patients who received autologous bone graft or device containing rhBMP7 has been tested [45]. Emphasized benefit was mostly related to the lack of morbidity at the autologous bone iliac crest harvesting site and subsequently reduced intra-operative blood loss. However, rhBMP7 was less effective than the autograft bone in the tibial non-union repair.

FDA and EMA approved rhBMP2 (Infuse) for open tibial fractures and rhBMP7 as so-called humanitarian device exemption (Osigraft) for tibial non-unions [46]. In addition, rhBMP2 and 7 were used off-label in various indications including scaphoid fractures, distal radius fractures, and cervical and thoracic ALIF [47–49]. A small RCT demonstrated a successful outcome of the proximal pole scaphoid non-union by administering rhBMP7 alone or in combination with an autograft [49]. Two small sample studies with a total of 30 patients enrolled showed a complete restoration of humeral non-unions in all patients when rhBMP7 was used with an autograft [50]. RhBMP2 was slightly less effective in the same indication, and the union was accomplished in eight out of nine patients [51]. In a retrospective study, rhBMP2 and 7 failed to show advantage in the treatment of aseptic clavicle non-union. Radical resection of the non-union tissue from the clavicle was a major step in the healing process [52]. Many off-label clinical studies were performed during the last 15 years, including various skeletal sites [53].

RhBMP2 was approved by the FDA in 2002 for anterior lumbar interbody fusion (ALIF) surgeries in indications including one-level degenerative disc disease [54]. Following

its approval, the use of BMP increased and had 25% share in spinal fusion procedures in US in 2006 [55]. It is important to emphasize that high proportion of these surgeries deviated from the originally FDA-approved indications, including posterior and transforaminal lumbar interbody fusion and cervical fusions. In patients treated for spinal fusion with BMP devices, it has been observed that application in unstable thoracolumbar fractures resulted in severe bone resorption, loss of reduction, and segmental collapse [56]. This effect could be explained with pronounced effect of large amount of rhBMP 2 and 7 on osteoclasts, at trabecular surfaces which form majority of vertebrae. However, the retrospective analyses revealed that the initial rhBMP-induced resorption was transient and that bone formation and repair subsequently occurred [42]. In conclusion, BMP application for spinal fusion should be restricted to approaches with safety and efficacy proven in RCTs, and those in which vertebral canal remains intact, to avoid neurological complications.

In 2007, FDA approved the use of rhBMP2 for maxillary sinus and alveolar ridge augmentation to fulfill tooth extraction sockets and intradental defects and enable installation of dental implants [57]. A randomized controlled clinical trial evaluated rhBMP2 attached to absorbable collagen sponge (mean rhBMP2 dose 1.9 mg/site) for alveolar ridge augmentation following tooth extraction, and demonstrated that extraction socket treated with rhBMP2 maintained the alveolar crestal height, while control sites without treatment showed loss in the crestal bone. Expanded RCT evaluated these findings by testing rhBMP2 at extraction sites with large bone defects. RhBMP2 achieved greater bone formation than collagen carrier alone, thus enabling formation of alveolar ridge more suitable to receive a dental implant [58]. Importantly, quality of newly formed bone after rhBMP2 treatment was the same as a regular healthy bone. Notably, when rhBMP2 was used for maxillary sinus floor augmentation, performance of newly formed bone was comparable to standard of care repair with autologous bone, with height extension ranging from 7.8 to 10.2 mm [59]. Few case series studies demonstrated the potential of both rhBMP2 and 7 in mandibular defect filling [60, 61].

However, following clinical testing for both rhBMP2 and 7 bone devices major side-effects have been reported and their therapeutic use have been recently revisited [15, 42, 43]. Local transient swelling, inflammation, and heterotopic ossification as well as early osteolysis were among serious complications following long bone implantation and spinal fusion, particularly in the cervical spine. The inflammation was pronounced in patients with distal radius fractures [62]. The average amount of rhBMP incorporated into the collagen carrier was between 3.5 and 12 mg, depending on the site and size of the fracture gap, while the entire human body normally contains only around 2 mg of BMPs. BMPs are not soluble at neutral pH and only 75 μg of the protein binds to 1 g of bovine collagen, while the rest precipitates and is locally released representing a potential source for local and systemic side effects. According to previous pharmacokinetic and bioavailability studies with commercial rhBMP devices it is expected that only 1–2% of locally administered rhBMP will be present in the patient's circulation in the period of two weeks following implantation. It was recently suggested that the impact of potentially systemically released rhBMP2 and 7 might rather have a positive effect on the skeleton via increasing the skeletal bone volume [63].

Another serious side effect of rhBMP2 device comprises early osteolysis causing the implant to shift and result in the subsequent fracture instability, especially if the periosteum was damaged [62]. In patients with unstable thoracolumbar fractures, rhBMP7 use resulted in a substantial bone resorption, loss of reduction, and segmental collapse [64]. Upon retrospective analyses of several clinical studies, it was suggested that the observed initial bone resorption was of a transient nature and that bone formation subsequently occurred [42, 43]. This was initially overlooked due to insufficient understanding of the BMPs mechanism of action on endosteal surfaces [15].

RhBMP2 faces major drawbacks since current knowledge on bone physiology does not support its current dosing and administration using animal-derived carrier. New solutions for bone healing are therefore needed, taking into account the complexity of BMP signaling and different cellular and tissue effects [65, 66]. Tissues surrounding the injury like periosteum, endosteum, bone marrow, vascular tissue, and muscles provide progenitor cells that initiate formation of bone callus and subsequently new bone by BMPs.

BMP6 as novel therapy for bone repair

A novel rhBMP6 containing osteogenic device (osteoinductive autologous bone graft substitute, ABGS) aimed to accelerate bone regeneration was developed and is currently being tested in clinical trials [66]. It comprises of a biologically compatible autologous carrier made from the patient's peripheral blood (autologous blood coagulum, ABC) and of rhBMP6 as an active ingredient. Such formulation circumvents the use of animal-derived materials, significantly limits inflammatory processes common in commercial bone devices and renders the carrier flexible, malleable, and injectable ensuring the ease of use.

BMP6 and BMP7 are paralogs with 87% similarity in the amino acid sequence. However, at position 60 of the mature BMP domain BMP6 contains lysine instead of aspartic acid (BMP7) or prolin (BMP2). This lysine allows for a reversible binding of BMP6 to Noggin, major BMP antagonist in tissues, so unlike BMP7 or BMP2, BMP6 may dissociate from Noggin and escape from Noggin inhibition [67]. This explains why BMP6 is more potent in promoting osteoblast differentiation in vitro and inducing bone regeneration in vivo when compared with its closely related BMP7 paralog.

ABGS successfully re-bridges critical size defects in animal models as well as enables physiological retention of rhBMP6 in the carrier upon binding to its extracellular matrix molecules and to cell membrane receptors constituting the ABC [3, 16]. This is supported by negligible absolute bioavailability following local implantation in animal models. Overall, non-clinical safety evaluation demonstrates a high safety margin for the use of ABGS in human bone defect indications. The ongoing clinical trials will provide detailed insights into the safety, tolerability, pharmacokinetics, and bone healing effects in humans and potentially provide novel and safe therapeutic options for bone repair.

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Compliance with ethical standards

Conflict of interest IDC, LK, and Marko Pecina declare no conflict of interest. Mihaela Peric is employed by School of Medicine, University of Zagreb and is actively involved in OSTEOproSPINE program and clinical testing in patients with posterolateral spinal fusion. LG is employed by School of Medicine, University of Zagreb and actively leads the development of the final Osteogrow drug product in patients with distal radius fracture, high tibial osteotomy, and lumbar back pain. SV is employed by School of Medicine, University of Zagreb and is founder of Genera Research, a Croatian biotechnology company conducting clinical trials with Osteogrow.

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