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Co-expression of BMPs and BMP-inhibitors in human fractures and non-unions

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ABSTRACT

Bone morphogenetic proteins (BMPs) are increasingly being used clinically to enhance fracture repair and healing of non-unions. However, the potential efficacy of supraphysiological dosing for clinical results warrants further clarification of the BMP signaling pathway in human fracture healing. As BMP signaling can be fine-tuned at numerous levels, the role of BMP-inhibitors has become a major focus. The aim of the present study was to document co-expression of BMPs, pSmad 1/5/8, and BMP-inhibitors in human fracture callus and human non-unions. Using human tissue of fracture callus (n = 14) and non-unions (n = 4) we documented expression of BMPs (BMP2, BMP3 and BMP7), pSmad 1/5/8 and the BMP-inhibitors noggin, gremlin, chordin, Smad-6, Smad-7 and BAMBI. Co-expression of pSmad 1/5/8, BMPs and BMP-inhibitors was noted in the osteoblasts of fracture callus as well as of non-unions. Expression of BMP-inhibitors was generally stronger in non-unions than in fracture callus. The most pertinent differences were noted in the cartilaginous tissue components. Expression of BMP2 in chondrocytes was markedly decreased in non-unions compared to fracture callus and that of BMP7 was almost completely absent. Expression of BMP-inhibitors was almost the same in osteoblasts, chondrocytes and fibroblasts of fracture callus and well as in non-unions. Interestingly, although BMP ligands were present in the chondrocytes and fibroblasts of non-unions, they did not co-express pSmad 1/5/8 suggesting that BMP signaling may have been inhibited at some point before Smad 1/5/8 phosphorylation.

These results suggest co-expression of BMP, pSmad 1/5/8 and BMP-inhibitors occurs in human fracture callus as well as non-unions but the relative expression of BMPs vs. BMP-inhibitors was different between these two tissue types. In contrast to our expectations, the expression of BMP inhibitors was comparable between fracture callus and non-unions, whereas the expression of BMPs was notably lower in the cartilaginous component of the non-unions in comparison to fracture callus. Based on these results, we believe that aberrations in the BMP-signaling pathway in the cartilaginous component of fracture healing could influence clinical fracture healing. An imbalance between the local presence of BMP and BMP-inhibitors may switch the direction towards healing or non-healing of a fracture.

Introduction

Bone healing is a complex regenerative process initiated in response to a fracture; with the final aim of restoring skeletal function. Over the last 2 decades, this well orchestrated cascade of events has become increasingly understood [1]. Interestingly, bone healing seems to recapitulate many events seen in bone development and embryogenesis [1–3]. The key drivers of this process are cytokines, platelets and growth factors, of which bone morphogenetic proteins (BMPs) have emerged as critical players.

BMPs are members of the pleiotropic Transforming Growth Factor-Beta (TGF-β) family [4]. More than 20 BMPs are currently known, and their characteristic feature is the capacity to induce endochondral bone formation [4–12]. Starting after birth, BMPs play a critical role in maintenance of bone mass through inducing commitment of mesenchymal cells towards cells of the osteoblastic lineage, and they also enhance the differentiated function of the osteoblast. Analysis of genetically modified mouse models with various null mutations, dominant-negative or conditional knockouts of BMP ligands, BMP receptors (BMPRs) or Smad proteins, has clearly shown the functional relevance of the BMP signaling cascade in skeletal formation and repair [13]. In addition, naturally occurring mutations of BMPs and BMPR in humans are associated with skeletal abnormalities [14].

The BMP signals are mediated by type I and type II serine/threonine kinase transmembrane receptors (BMPRIA, BMPRIB and BMPRII) [4,11]. These receptors are expressed at different levels in different tissues. BMP binding to BMPRs activates Smad signaling that is translocated to the nucleus. The Smads are intracellular proteins than can be broadly divided in three classes: 1) receptor regulated Smads (R-Smads) such as Smad 1/5/8; 2) co-Smads, such as Smad-4; 3) inhibitory Smads (Smad-6 and Smad-7). It has also been shown that the actions of BMPs are tempered by inhibitors or antagonists, indicating the existence of local

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feedback mechanisms to modulate BMP cellular activities [14–16]. The antagonists function at different levels of the BMP-signaling cascade: extracellular at the BMP-BMPR interaction (e.g. prevention of BMP binding to its receptors by noggin, chordin, and gremlin), by expression of membrane pseudo-receptors (e.g. BAMBI), and at the intracellular level (Smad-6 and Smad-7). Others have also been described (e.g. Ski).

After numerous animal studies showed the presence of BMPs, BMPRs and some of their antagonists [6,17–19] in fracture healing and distraction osteogenesis [20–26], we were the first to show expression of BMPs, BMPRs and intracellular signaling proteins (Smads) in human fracture and non-union tissue [7,8]. Surprisingly, our work showed that expression patterns did not differ between healing and non-healing fractures, suggesting that differences in healing capacity are not directly due to level of expression of BMPs, their receptors, and/or intracellular Smads. The first description of BMP-inhibitors in human fracture tissue was done by Kwong et al. in 2009 [27].

Although many questions remain for a complete understanding, scientists and clinicians are keen to leverage what is already known for clinical application. Preclinical studies have led to the clinical use of BMP2 and BMP7 [11,28,29]. So far, however, efficacy seems to be no better than autologous bone graft, with a key disadvantage being exogenous application is more costly [30]. Also, the clinical dosage needed is 100–1000 times higher than endogenous BMPs [28], and complications mostly related to the off-label use of BMPs have been reported [11,29].

To improve the effectiveness of BMPs as treatment, there are many aspects that still need clarification. What is well known is that BMP signaling can be fine-tuned at numerous levels at almost any step along the pathway [13–16,31]. Recently, the role of BMP-inhibitors (e.g. noggin, gremlin, chordin) and the extent to which they can be used as a control mechanism have received much attention [13–16,31]. Therefore, it seems possible that abnormal BMP signaling caused by increased expression of BMP-inhibitors could be related to unsuccessful bone healing. We and others have speculated on focusing on the BMP-inhibitors in bone healing, which is now emerging as a therapeutic target [32].

The objective of the current study was to document naturally occurring levels of BMPs and their inhibitors in human fractures and non-unions. Our hypothesis was that the balance between BMP and BMP-inhibitors differs between healing and non-healing human fractures, which would imply an interventional opportunity. In addition, we also set out to study their co-expression using double and triple immunohistochemistry staining. Fundamental to our hypothesis is a better understanding at the molecular level of why certain fractures heal and others do not.

Materials and methods

Specimens

Fracture callus and non-union tissue was obtained during surgery of 16 different patients at the time of operative repair or revision surgery of the fracture (n = 12) or hypertrophic non-union (n = 4). Three fractures involved the acetabulum (n = 2) or pelvis (n = 1). All other fractures and non-unions pertained to the appendicular skeleton. Although more patients were treated during this period, representative tissue availability was limited. The definition of a non-union was a fracture that had not healed within 6 months. All patients were treated by the senior author (PK) between 2001 and 2010. Patient characteristics are listed in Table 1.

Fracture patients were between 10 and 70 years of age and otherwise in good health. There were 10 males and 2 females. Time to callus harvest ranged from 2 to 10 weeks. Non-union patients were between 37 and 69 years of age and otherwise in good health. There were 3 males and 1 female. Approval of the Institutional Review Board (IRB) was obtained where appropriate. Oral consent for removal of the tissue and its storage in the tissue bank for research purposes was obtained from each patient. Individual consent for this specific project was waivered by the ethics committee of the remaining two hospitals since the research was performed on "waste" material, stored in a coded fashion. Indications for surgery were nascent (impending) malunion, non-union, and failure of fixation or fractures that were operated on in a delayed fashion. All fractures and non-unions have subsequently successfully healed.

Table 1
Summary of patient data.

Specimen	Age/sex	Location	Time since fracture (weeks)
Fracture callus	21 M	Acetabulum	2
	15 M	Acetabulum	5
	34 M	Distal radius	4
	39 M	Proximal femur	8
	43 F	Distal femur	6
	44 M	Humerus shaft	10
	28 M	Distal humerus	3
	35 M	Femur shaft	3
	10 F	Foot	2
	43 M	Pelvis	3
	33 M	Distal fibula	5
	70 M	Humerus shaft	6
Non-union	69 M	Tibia shaft	56
	38 M	Ulna shaft	24
	37 M	Tibia shaft	24
	51 F	Femur shaft	272

After removal from patients, specimens were placed in 10% neutral buffered formalin for 24 h and subsequently decalcified – if needed – in 10% ethylenediamine tetra acetic acid (EDTA), pH 7.2. The tissue was then routinely processed and embedded in paraffin wax. Sequential sections of 5–7 μm thick were prepared for haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC).

Immunohistochemistry

For immunohistochemistry, samples were fixed in 4% paraformaldehyde overnight, decalcified in 20% ethylene diamine tetra-acetic acid for 3 weeks, embedded in MMA (methylmethacrylate), and sectioned using a Leica RM 2255 microtome (Leica Microsystems, Richmond Hill, ON, Canada). Following deparaffinization and hydration, endogenous peroxidase activity was blocked using 10% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubating samples in phosphate-buffered saline containing 10% normal horse serum for 20 min. Commercially available polyclonal goat antibodies were used for the detection of the following proteins: BMP2, BMP3, BMP7, noggin, gremlin, chordin, pSmad 1/5/8, Smad-6, Smad-7, and BAMBI (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA, 1:100 dilution in 1% goat serum).

Tissue sections were probed with the polyclonal goat antibody overnight at 4 °C in a humidified chamber. For negative controls, we omitted the primary antibody. We then incubated the sections with a biotinylated mouse anti-goat secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA, 1:400 dilutions in 1% normal goat serum) for 30 min at room temperature in a humidified chamber. Sections were stained using the avidin–biotin complex method for 30 min, followed by DAB-peroxidase staining. Finally, we counterstained sections with hematoxylin and mounted with Permount (Fisher Scientific, Montreal, Canada). Photomicrographs of the tissues were taken under 10× and 40× magnifications using a Leica microscope (Leica Microsystems, Richmond Hill, ON, Canada) attached to a Q-Imaging camera (Olympus DP70, Japan). For each frame tested, we also performed a negative control where the same procedure was performed but omitting the primary antibodies.