Expression and Activation of the BMP-Signaling Components in Human Fracture Nonunions

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Background: The potential use of bone morphogenetic proteins (BMPs) to promote bone-healing is of great interest to orthopaedic surgeons. Although the complex mechanism leading from the local presence of BMP (whether endogenous or exogenous) to bone formation is increasingly understood, limited information is available as to whether endogenous BMPs, their receptors, or other molecules involved in their signal transduction, such as Smad1, are present or disappear during the development of fracture nonunions. The purpose of the present study was to determine, by immunohistochemical analysis, whether BMPs, BMP receptors, or Smad1 disappear from tissues during the development of a fracture nonunion.

Methods: Twenty-one patients (average age, sixty-one years; range, thirty to eighty-five years) with a delayed union (four patients) or a nonunion (seventeen patients) were included. The average duration of the delayed union or nonunion was twenty-two months (range, 3.5 to 120 months). With use of immunohistochemical analysis, we studied the localization of BMP-2, BMP-4, and BMP-7 and their receptors BMPR-IA, BMPR-IB, and BMPR-II as well as pSmad1. With use of a pSmad1 antibody, we also studied whether the BMP receptors that were expressed were activated.

Results: The immunohistochemical localization of all seven BMP-signaling components was demonstrated in seventeen (81%) of the twenty-one patients. The remaining four patients lacked one or more of the components. Areas of newly formed bone had the highest percentage of positively staining cells, with the staining generally decreasing in areas remote from bone formation. However, even in areas of dense fibrous tissue and in specimens that lacked newly formed bone, immunostaining was still present. The staining patterns showed co-localization of the BMP-2, BMP-4, and BMP-7 proteins with the BMP receptors. The presence of pSmad1 signified the activated state of the BMP receptors, which implies that the BMP signal is transduced inside the cell.

Conclusions and Clinical Relevance: In the present study, nonunions of long duration were noted to have evidence of ongoing BMP-signaling. The profiles of BMP, BMP-receptor, and pSmad1 immunostaining were heterogeneous in this group of patients. The concept that the expression and activation of BMP-signaling components are lacking at the site of delayed unions and nonunions was not supported by the results of the present study.

Despite ongoing advances in the treatment of fractures and increasing knowledge about the molecular and cellular mechanisms involved in bone-healing, an estimated 5% to 10% of fractures do not heal properly and go on to delayed union or nonunion. Normal fracture-healing requires a well-orchestrated interplay among different cell types, including chondrocytes, endothelial cells, osteoprogenitor cells, pericytes, and osteoblasts. In addition, bone morphogenetic proteins (BMPs), including BMP-2, BMP-4, and BMP-7, play a pivotal role in skeletal tissue formation. The actions of the BMPs are mediated through a heteromeric receptor complex of type-I receptors (BMPR-IA, BMPR-IB, or ALK-2/ActR-I) and type-II receptors (BMPR-II, ActR-II, or ActR-IIB). Once activated, the receptor complex transduces signals intracellularly through the activation of specific Smad proteins, which form heteromeric complexes that accumulate in the nucleus, where they participate in the regulation of gene expression. The Smad proteins can be subdivided into three groups: the signal-transducing receptor-regulated Smads (R-Smads, such as Smad1, Smad2, Smad3, Smad5, and Smad8), the common mediator Smads (co-Smads, such as Smad4), and the inhibitory Smads (I-Smads, such as Smad6 and Smad7). Whereas Smad2 and Smad3 are involved in the signaling by transforming growth factor-β (TGF-β) and activin, Smad1, Smad5, and...
Smad8 are involved in BMP-signaling\textsuperscript{7,14,15}.

Given their presumed roles in normal fracture-healing and their efficacy in the healing of fractures and segmental defects in animal models\textsuperscript{16-19}, it has been suggested that the administration of exogenous BMPs may elicit the healing of fracture nonunions in humans\textsuperscript{20-23}. Trials of their use in the clinical setting are beginning to appear in the orthopaedic literature\textsuperscript{24,25}.

Little is known about the microenvironment of nonunions in humans and the biological events that lead to the interposition of fibrous scar tissue rather than normal bone\textsuperscript{26-28}. It has been suggested that the conversion of cells from an osteogenic to a fibroblastic phenotype is influenced by the spatial and temporal presence or absence of signals that lead to osteoblast differentiation, such as those generated by BMPs\textsuperscript{29}. Aberrations in the ideal combination of such signals could theoretically be optimized by the application of exogenous BMP. However, there are no data as to what these presumed aberrations are or whether they are consistently present at the site of all delayed unions and/or nonunions. Whether osteogenic BMPs are absent or deficient at the site of delayed unions and/or nonunions in humans is unknown.

We investigated the expression of components of the BMP-signaling cascade and the activation state of BMP-receptor-regulated Smads at the site of twenty-one delayed unions and nonunions as a first step toward answering some of these questions. Our null hypothesis was that the BMP-signaling components are present at the site of delayed unions and nonunions in humans.

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*The staining for the different BMPs, BMP-receptors, and pSmad1 was found in osteoblasts on the surface of woven bone (OB), in osteoblast-like cells near the woven bone (NB), and in fibroblast-like cells (FB) in the fibrous tissue of the delayed unions and nonunions. In those sections that did not contain bone, only data on the expression in fibroblast-like cells (FB) are shown. − = no staining; + = 1% to 33% of cells were positively staining; ++ = 34% to 66% of cells were positively staining; and +++ = 67% to 100% of cells were positively staining.
Materials and Methods

Tissue Fixation and Embedding

Twenty-one patients (average age, sixty-one years; range, thirty to eighty-five years) with a delayed union (four patients) or a nonunion (seventeen patients) were included in the study (Table I). Approval for the study was obtained from the institutional review board at our institution. The exclusion criteria included malignancy, infection, corticosteroid use, pregnancy, metabolic bone disease, alcohol abuse, or the use of vitamin D, calcium, or hormones. A nonunion was defined as the absence of osseous healing at six months after the operative or nonoperative treatment of a fracture, and a delayed union was defined as the absence of osseous healing at more than three months after the injury on three consecutive radiographs. Nineteen patients had involvement of the appendicular skeleton, and two had involvement of the clavicle. The average interval between the time of the fracture and the time that the specimen was obtained was twenty-two months (range, 3.5 to 120 months). All patients presented with complaints of pain, deformity, and decreased function of the involved extremity. No patient had evidence of an active infection as determined on the basis of radiographic, physical, laboratory, and intraoperative findings.

At the time of surgery at our institution, a representative portion of tissue that was confined to the delayed union or nonunion gap was obtained and frozen at –80°C in O.C.T. Compound (Sakura Finetek USA, Torrance, California). The tissue was subsequently fixed in 10% neutral buffered formalin for forty-eight hours. The samples were washed and then were dehydrated through a graded ethanol treatment. Follow-
ing dehydration, each sample was embedded in paraffin and was sectioned to a thickness of 5 to 6 µm for immunohistochemical analysis. If needed, surface decalcification was performed prior to sectioning. All slides were coded to prevent bias in scoring the immunohistochemical reaction. Hematoxylin and eosin staining was also done to allow for comparative histologic analysis.

**Antibodies**

Polyclonal antibodies for BMP-2, BMP-4, and BMP-7 were used for BMP immunostaining (Santa Cruz Biotechnology, Santa Cruz, California). Polyclonal antibodies for BMPR-IA (ALK-3), BMPR-IB (ALK-6), and BMPR-II were prepared as described previously with use of synthetic peptides corresponding to the intracellular juxtamembrane parts of BMPR-IA, BMPR-IB, and BMPR-II as immunogens. The antisera were affinity purified on Protein-A Sepharose columns (BioSynthesis, Lewisville, Texas). These antibodies have been tested extensively and have been shown to be specific without cross-reactivity between antisera.

The pSmad antibody was made against serine phosphorylated peptide KKK-NPISS(p)VS(p) corresponding to the carboxyterminus of Smad1. This antibody, which detects phosphorylated Smad1, phosphorylated Smad5, and phosphorylated Smad8, has been previously described.

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed with use of the avidin-biotin-peroxidase complex method. A Vectastain Elite ABC Goat IgG Kit (Vector Laboratories, Burlingame, California) was used for the BMP-2, BMP-4, and BMP-7 antibodies, whereas a Vectastain Elite ABC Rabbit IgG Kit (Vector Laboratories) was used for the BMPR-IA, BMPR-IB, BMPR-II, and pSmad antibodies. Preliminary studies were done to determine the appropriate concentration for each antibody.

The samples were prepared for the immunohistochemical reaction by first removing the paraffin from the sections and hydrating the tissue samples. Endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for thirty minutes at room temperature, followed by washing with phosphate-buffered saline solution. The samples were subjected to antigen retrieval by placing them in citrate buffer and heating to boiling in a microwave, after which they were allowed to cool to room temperature in a humid chamber. Immunostaining was then carried out by applying the appropriate concentration of antibodies to each sample and incubating overnight at 4°C. For negative controls, phosphate-buffered saline solution, normal rabbit IgG, or normal goat IgG (Sigma Chemical, St. Louis, Missouri) was used in place of the primary antibodies. The following day, the samples were washed in phosphate-buffered saline solution and were incubated with the biotinylated secondary antibody for one hour at room temperature. The avidin-biotin-peroxidase complex was then added for thirty minutes at room temperature. The color reaction was developed with use of 3,3' diaminobenzidine tetrachloride (Sigma Chemical). Counterstaining was performed with use of hematoxylin, and a coverslip was applied with Permount (Fisher Chemical, Fairlawn, New Jersey). In addition, one slide per patient was prepared with hematoxylin and eosin for routine evaluation.

**Quantification**

The slides were evaluated with use of the MetaMorph Imaging System (Universal Imaging, Downingtown, Pennsylvania). The number of cells staining positively for each antibody was assessed by observing a total of five fields of at least ten cells each. To prevent bias, a grid was superimposed on the field of interest, and only cells that were at the intersection of the horizontal and vertical gridlines were scored. This was done for cell types in three different representative tissue types: bone, areas adjacent to the bone (that is, 20 to 50 µm away from the bone), and dense fibrous tissue. No attempt was made to quantify the intensity of staining. Rather, cells were evaluated simply for the presence or absence of staining. The results from all five fields were used to calculate an average percentage of staining for each tissue type.

**Results**

**Histological Findings**

The delayed unions and nonunions revealed a mixture of different tissue types. Eleven (52%) of the twenty-one specimens had foci of woven bone surrounded by large areas of fibrous tissue that was interspersed with areas of numerous blood vessels. The other ten specimens had similar areas of fibrous tissue but lacked woven bone. The woven bone had cuboid-shaped osteoblasts lining the osteoid, suggesting active bone formation.

Within the samples that contained woven bone, two patterns of bone formation were observed. In some samples, bone appeared to be forming directly from fibrous tissues while in others, bone seemed to be forming from cartilage. Other observations included scattered lamellar bone fragments surrounded by osteoclasts and a paucity of lining osteoblasts.

Some specimens also showed villous projections resembling synovial pseudarthroses with lining cells resembling synoviocytes.

**Immunohistochemical Findings**

**Expression of BMP-2, BMP-4, and BMP-7**

Overall, there was a trend toward increased staining with increasing proximity to areas of new-bone formation (Table I). The most consistent expression was that of BMP-2, BMP-4, and BMP-7 in the osteoblasts lining the newly formed osteoid (Fig. 1, A, B, and C; Fig. 2-A; and Fig. 2-B, B.2). The staining was cytoplasmic and, in certain specimens, was specifically located in the Golgi apparatus, illustrating local production of BMP (Fig. 2-B, B.2). The numbers of cells that stained positively for these antibodies in the area close to bone remained fairly consistent in all specimens (Table I).

There was no apparent correlation between the location of the delayedunion or nonunion (e.g., the humerus or tibia)
and the presence of immunostaining.

Other areas revealed a fairly wide range of staining patterns. In the areas of dense fibrous tissue (farthest away from any new-bone formation), the presence of staining for all BMP isoforms tested was the same as or less than that in the areas close to bone at all time-points after the fracture. BMP-4 appeared to be expressed less ubiquitously than BMP-2 and BMP-7 in the fibrous tissue, although no attempt was made to quantify this finding statistically. A variety of different cell types were noted to stain positively in the fibrous tissue, including endothelial cells, smooth muscle cells around small blood vessels, spindle-shaped fibroblast-like cells, and cells resembling cartilage cells going through ossification. There did not seem to be a difference in the immunostaining of the fibrous tissue between specimens that had newly formed bone and those that did not.

Also of note was the strong staining for these BMP antibodies in the lining cells of the villous projections that were seen in some specimens. Osteoclasts were also shown to have positive staining for these BMP isoforms. There was no BMP staining in the extracellular matrix of the bone or in the fibrous matrix.

Expression of Type-IA, Type-IB, and Type-II BMP Receptors

Positive staining for the receptor antibodies was observed in the osteoblasts lining the ossified tissue, in the areas near the ossification sites, and in the fibrous tissue (Fig. 1, D, E, and F; and Fig. 2-B, B.3 and B.4). There was no clear difference between the expression of the two different BMP type-I receptors (BMPR-IA and BMPR-IB) in the cells located at or near the ossification sites. However, there was a trend toward decreased expression of the BMPR-IB receptor as compared with the BMPR-IA and BMPR-II receptors in the fibrous tissue (Table I). The staining pattern for the various BMP receptors seemed to be identical to the BMP-2, BMP-4, and BMP-7 protein localization described above (Table I). As observed for the BMP antibodies, there was a trend toward decreased staining...
in areas remote from bone formation. There was no clear trend between a decreased percentage of positive staining and an increased duration of the nonunion. Cells that were noted to have positive staining were osteoblasts, osteoclasts, fibroblast-like cells, and chondrocyte-like cells. Staining was also noted in the perivascular structures and the lining cells of the villous projections seen in certain samples. These last cells resembled synoviocytes and were thought to be consistent with a synovial pseudarthrosis.

Expression of pSmad1
pSmad1 staining was observed in the osteoblasts lining the areas of reactive bone formation as well as in osteoclasts, fibroblast-like cells, and chondroblast-type cells (Table I and Fig. 1, G; Fig. 2-B, B.1; and Figs. 3-A and 3-B). This finding indicates that the BMP receptors expressed in these cells were activated, which is consistent with the BMP-2, BMP-4, and BMP-7 immunostaining that was observed in or surrounding these cells. In the areas of new-bone formation and their immediate adjacent areas, this staining remained intense even in the nonunions of longer duration (Fig. 3-B) (Table I).

The absence of staining in control specimens treated with phosphate-buffered saline solution, rabbit IgG, or goat IgG instead of primary antibodies confirmed specificity of the immunostaining (Fig. 1, H; and Fig. 2-B, B.3).

Discussion
The events following the fracture of a long bone have been described in detail both histologically and ultrastructurally. It is well established that BMPs and their receptors play key roles in the healing cascade of bone. Their exact mechanism of action and the source of the osteoprogenitor cells remain unclear. Studies have shown that BMP-4 mRNA is upregulated at the fracture site as early as twelve hours following a fracture and that BMP-2 and BMP-4 proteins are upregulated at two days. Similarly, BMP type-I receptors have been shown to be upregulated at three days after a fracture. Expression of BMPs also has been documented during distraction-lengthening osteogenesis. All of those reports were based on animal studies.

In the present study, key components of the BMP-signaling pathway were present in the specimens from the vast majority (81%) of twenty-one human patients with a delayed union or nonunion. Consistent with the notion that the expression of BMP-2, BMP-4, and BMP-7 proteins was co-localized with the proteins’ receptors, we observed the presence of activated Smad1 in these cells. These results do not exclude the possibility that BMP-signaling components may be limiting for optimal bone-healing. The staining patterns for key components of the BMP-signaling pathway in the human specimens in the present study seem comparable with those in studies involving fracture models in animals.

The present study demonstrated that all components of the BMP-signaling pathway are present in the microenvironment of delayed unions and nonunions. This raises the question as to why certain fractures do not unite. One explanation is that these fractures simply need a better mechanical environment. Pauwels suggested that pseudarthroses result from an unfavorable mechanical environment rather than from an unfavorable biological environment. His theories were proven indirectly by the successful introduction of stable osteosynthesis by the founders of AO. The sites of pseudoarthroses were shown not to be biologically inferior but were able to ossify without bone graft under the right mechanical conditions. Furthermore, studies have shown that the intrinsic capacity of bone to go through a stepwise cascade of regeneration does not appear to diminish with longer duration or with the number of previous operative attempts to obtain healing. Other studies have demonstrated more directly that multipotent cells remain present in the tissues at the site of a nonunion.

A second explanation is that the levels of the BMPs and/or their receptors at the sites of delayed unions and non-
unions are suboptimal to obtain normal healing. Indeed, the activation of nonunion cells in vitro by BMP has been reported. Other studies have confirmed the biologic (inducible) activity of nonunion cells. Interestingly, recent data have indicated that one of the modulating effects on the expression of BMPs as well as their receptors and antagonists is the application of mechanical loads.

The documentation of the components of the BMP-signaling cascade in our study of delayed unions and nonunions does not allow us to reject the null hypothesis. This implies that BMP expression and signaling is normal in human delayed unions and nonunions. Although the present study provides some insight into the role or roles of BMP in these problem fractures, it raises many more questions. First, despite a clear trend, we found a relatively wide range of immunostaining patterns for the different antibodies among our patients. The multitude of many additional factors known to play a role in delayed unions and nonunions, including age, smoking, medications, and so on, made it difficult to decipher precisely what was responsible for the variability among our samples. Second, there is no way of knowing which cells in these histological sections were the osteoprogenitor cells that are the sine qua non for bone-healing. In other words, if exogenous BMP were to be added, we would not know which cells are the target cells that need triggering. If the target cells express BMP receptors but not BMP, then the addition of exogenous BMP would make sense. On the other hand, if they do not express BMP receptors or Smads, then the addition of exogenous BMP would depend on receptor or Smad upregulation. Finally, if we assume that the osteoprogenitor cells are among the cells that stained in our specimens, we still need to understand why their mere presence does not lead to healing. Perhaps the amount of local endogenous BMP demonstrated by our staining is suboptimal. Perhaps there is an increased expression of BMP inhibitors such as noggin,
which binds to BMP and prevents binding to its receptors\textsuperscript{11,12}. The lack of effects of an intact BMP-receptor-Smad pathway can also be explained by low expression or defective components with which Smads cooperate in inducing bone, such as Cbfa1 (Runx2)\textsuperscript{57}.

Regardless of the presence or absence of the components of the BMP-signaling cascade, a presumed role of exogenous BMP in delayed unions and nonunions could be to provide a boost to osteogenesis or chondrogenesis, even if the defect involves a factor or signaling pathway other than BMP. It is well

Fig. 3-A
Anteroposterior radiograph of a clavicular nonunion of five-years’ duration in a thirty-seven-year-old man (Case 19). The superimposed line delineates the resected tissue used for immunohistochemical analysis and Masson’s trichrome staining.

Fig. 3-B
Masson’s trichrome staining of a specimen of the nonunion shown in Fig. 3-A, showing an area of ossified matrix (o), calcified matrix (ca), and surrounding fibrous tissue (f) (original magnification, ×6). The superimposed boxes delineate the sections magnified in the insets. B.1, Image showing immunohistochemical staining (brown) for pSmad1 in the fibroblast-like cells in the fibrous tissue (original magnification, ×315). B.2, Image showing immunohistochemical staining (brown) for pSmad1 in the chondrocyte-type cells in the area of fibrocartilage (original magnification, ×315). B.3, Image showing immunohistochemical staining (brown) for pSmad1 in newly formed bone (original magnification, ×315). There was no brown staining when phosphate-buffered saline solution, normal rabbit IgG, or normal goat IgG was substituted for the primary antibodies.
established that exogenous BMPs can drive bone formation, and they will undoubtedly have a role in the healing of segmental defects or spinal fusions1,12 when a large amount of new bone is needed. However, for the routine application of BMP in the treatment of delayed unions and nonunions when bone stock per se is often not a limiting factor (e.g., in hypertrophic nonunions), important questions remain to be answered, such as which BMP isoform(s) should be administered, in what concentration, and at what time? If there is a relative paucity of target cells, can we rely on chemotaxis to recruit these cells or do we need exogenous osteoprogenitor cells?

As we continue to understand more fully the roles of BMP in normal fracture-healing, we hope to be able to address the question of how BMPs may be applied successfully to treat abnormal fracture-healing. Future applications may involve direct apposition or, more elegantly, the use of promising new techniques such as gene transfer13-16.

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References


