Expression of Bone Morphogenetic Protein Antagonists during Fracture Healing

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INTRODUCTION

One of the most significant advances in bone biology has been the discovery of bone morphogenetic protein (BMP) family of growth factors, a subset of the transforming growth factor-β superfamily of growth and differentiation factors. BMP is critical for normal growth and development for a myriad of tissues within a wide spectrum of vertebrate species. During the past three decades, BMP function within the context of fracture healing has progressed to the point at which these growth factors are now being regularly used as adjunct therapy for fracture nonunions and severe acute fractures.

Antagonists to BMP are a group of proteins or transcription factors that interfere with BMP signaling pathway at various levels. They modulate a diverse array of BMP activities, including neural development, prostate development, inflammation of cardiac tissues, and bone development. However, to date few studies have investigated BMP antagonists during fracture healing. By defining the specific roles played by those paracrine and autocrine factors that antagonize BMP activity, potential exists to manipulate or create an environment in which BMP therapy is even more efficacious. In the present study, the temporal and spatial dynamics of gene and protein expression of several BMPs, BMP receptor antagonists were investigated in a mouse femoral fracture model using quantitative polymerase chain reaction (qPCR) and immunohistochemistry.

METHODS

Fracture model and PCR: The animal study protocol was approved by the Saint Louis University Animal Care and Use Committee. Forty-eight mice were anesthetized and a transverse femoral fracture was surgically created in each mouse. Plain film radiographs were taken weekly after surgery. Animals were sacrificed by cervical dislocation at 1, 3, 7, 14, and 21 days after fracture. Tissue blocks were centered at the mid-shaft fracture line and included both fracture ends as well as any surrounding hematoma, soft callus, or hard callus. Femoral bone segments from the mid-shaft of the contralateral, uninjured side were harvested for experimental controls. RNA was extracted and qPCR was performed for gene expression of BMP-2, -4, -6, -7; BMPR-1A, -2; and BMP antagonists: noggin, DAN, CHRD, BAMBI, PRODC, SOST, SMAD6, SMAD7 CERBERUS, and Grem1.

Histology and immunohistochemistry: Selected tissue sections from each time-point were stained with haematoxylin and eosin (H & E) for tissue structure. The primary antibodies for immunohistochemistry were rabbit anti-mouse noggin, BAMBI, and DAN.

Statistical Analysis: One way ANOVA was performed for multiple comparisons of gene expression (ΔΔCt) among different time-points, followed by post-hoc t test.

RESULTS

Radiographs demonstrated normal fracture healing in all animals, although there was significant displacement between the distal and proximal femoral shaft fragments. Early evidence of bony callus formation was seen in day 7 and complete healing of the fractures was visible at day 21.

Gene expression during fracture healing was compared with non-fractured, control bone. The expression of BMP-2 and 7 demonstrated a gradual increase after the fracture with a subsequent descent after day 14. BMP-4 was expressed in a different pattern, which was slightly increased over the first week after fracture and reached a 2-fold increase at weeks 2 and 3, which was statistically significant. The expression of BMPR-1A was not significantly altered in the first 2 weeks of fracture but was downregulated from week 2. For BMPR-2, its expression in the fractured tissue blocks was comparable to the normal or non-fractured bone.

BMP antagonists PRDC, SOST, Smad7, GREM1 and CERBERUS as a group had their expression downregulated during the 3-week course of fracture healing.

Among the upregulated BMP antagonists, noggin was expressed with a unique pattern that was upregulated earlier, from day 1 through day 7, and substantially downregulated at weeks 2 and 3. DAN, CHRD, BAMBI and Smad6 shared a common pattern that was a gradual increase in expression, which reached a peak in the period from day 7 to week 3, followed by lesser upregulation. A steady increase of DAN expression lasted to the final time-point of the study, up to a 50-fold increase compared to the control at week 3. CHRD and BAMBI, had increased expression only after day 7 and CHRD was downregulated at week 3.

DISCUSSION

BMP antagonists are a diverse array of molecules that interfere with BMP activities through various mechanisms, and may function extracellularly or intracellularly. We hypothesized that, while BMP and BMP antagonists are in concert in molecular regulation, an individual BMP antagonist may have specialized functionality in a particular biological event. In this study, a group of BMP antagonists has been investigated, in coordination with BMP and BMP receptor expression, for their roles in fracture healing.

The upregulation of BMP-2 and -7 in this mouse model has shown a consistent pattern as in other animal models. BMP-4 expression was increased compared to controls throughout the time-course of fracture healing, though not to a significant level until week 2. BMPR-2 was steadily expressed by cells in the fracture callus, but BMPR-1A was decreased in the last two weeks of fracture healing. This may be because BMP receptors are expressed by multiple cell types, including fibroblasts, endothelial cells, chondrocytes and osteoblasts. The expression of these receptors is influenced by the progressive cell differentiation in the callus.

The BMP signaling pathways are redefined by BMP antagonists at all levels of the signaling. PRDC, SOST, Smad7, GREM1 and CERBERUS are all BMP antagonists with diverse roles in development and bone formation. During the 3-week period of fracture healing, all of these antagonists were expressed at levels below that seen in the non-fractured bone, suggesting that they may have contributed to bone formation by reducing the levels of signaling which antagonizes BMP activity.

Noggin, one of the most studied BMP antagonists, directly blocks BMP extracellularly and blocks the interaction of BMP with its receptors. Similar to previous findings, noggin was upregulated in the early stage of fracture healing in the current study. BAMBI is a kinase-deficient decoy pseudoreceptor of BMP and inhibits the activation of the BMP signaling pathway. The continuous increase of BAMBI expression during fracture healing possibly functions as a negative feedback to BMP activities during fracture healing. Chordin is particularly important for skeletogenesis, during which it is predominately expressed in the epiphysis. In the current study, chordin expression was increased after fracture and peaked at day 14, which is a period of active bone formation and associated with up-regulation of BMPs. DAN is important for growth and development and is one of the most upregulated BMP antagonists in the study. Like BAMBI, the expression of DAN steadily rose throughout the fracture healing course.

BMP antagonists are dynamically involved in the initiation and modulation of fracture healing in mouse femoral fractures. Moreover, the temporal relationship of BMP antagonist expression to fracture histology indicates the different roles each BMP antagonist may have in influencing fracture healing. The increase of BMP antagonists during fracture healing is a physiological response to the increased BMP expression induced by fracture. For the purpose of enhancing bone formation they could be targets to suppress, in order to amplify BMP signaling for bone formation.

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