Expression of bone morphogenetic proteins activation pathway and its antagonists during tibial fracture healing

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INTRODUCTION:
Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta super family of molecules and play important roles in various cellular functions. BMPs are potent inducers of bone that can be used clinically to treat non-unions and to enhance spinal fusion. BMP signal transduction occurs via activation of type I and type II serine-threonine kinase receptors and their intracellular targets Smads. Ligand-bound BMP receptors phosphorylate and activate Smads 1/5/8 which then move to the nucleus to regulate gene transcription. BMP signaling can also be modulated by antagonists such as noggin and BMP-3, which bind to BMPs and interfere with their ability to interact with receptors. Studies have reported expression of BMPs in fracture tissues, but information about the BMP signaling pathway during fracture repair at the cellular level is limited. The aim of this study was to examine the spatiotemporal pattern of activation of the BMP pathway during fracture repair using immunohistochemistry.

MATERIALS AND METHODS:
Non-stabilized fractures: All procedures followed protocols approved by the Institutional Animal Care and Use Committee. Twelve week old C57/B6 male mice were anesthetized. Closed, standardized non-stable fractures were produced in the mid-diaphysis of the right tibia. Callus tissues were collected at 3, 5, 7, 10, 14 and 21 days post fracture (n=3 per time point). Specimens were fixed in 4% paraformaldehyde overnight, decalciﬁed in 19% EDTA for 2 weeks, and embedded in paraffin.

Immunohistochemistry: Goat polyclonal antibodies against BMP-2, -4, -5, -6, -7 and -8, BMP receptors (BMPR-IA, BMPR-IB, BMPR-II), and antagonists (noggin) were purchased from Santa Cruz Biotechnology (CA, USA); a rabbit polyclonal antibody against p-Smad 1/5/8 was purchased from Chemicon (Millipore). Sections (10μm thick) were incubated with primary antibodies in PBS (1:100 dilution) in a humidified chamber at 4°C overnight. For negative controls, normal goat or rabbit IgG was used. After three washes with PBS, tissue sections were incubated with biotinylated secondary antibodies (Vector Labs) for 30 min in a humidified chamber followed by staining with Vectastain ABC Reagent (Vector Labs). Sections were developed with DAB and counterstained with fast green. Adjacent slides were stained with Safranin O/ Fast Green or Trichrome to detect cartilage or bone.

RESULTS:
Expression of BMPs: During the inflammatory phase (days 3 and 5) of fracture healing, BMP-5 and BMP-6 were detected in osteocytes within trabecular bone around the fracture site. BMP-5 was the only BMP expressed in fibrous tissues within the callus at day 3 post fracture. By day 5, BMP-2, -5, and -8 were moderately detected in periosteal cells near the fracture site as early as day 3 post fracture and persisted until day 5. From day 5 to day 7, p-Smad 1/5/8 was moderately expressed in fibrous tissues within the callus. Consistent expression of p-Smad 1/5/8 was detected in chondrocytes and hypertrophic chondrocytes from day 5 to day 21 post-fracture. Expression of noggin was detected in osteoblasts/osteocytes within the new bone by days 7, 14 and 21, but not by day 10 post-fracture; whereas BMP-3 was only detected in osteoblasts/osteocytes within the new bone by day 10.

Expression of BMP effectors: p-Smad 1/5/8 staining was detected in periosteal cells near the fracture site as early as day 3 post-fracture and persisted until day 5. From day 5 to day 7, p-Smad 1/5/8 was moderately expressed in fibrous tissues within the callus. Consistent expression of p-Smad 1/5/8 was detected in chondrocytes and hypertrophic chondrocytes from day 5 to day 21 post-fracture, and in osteoblasts/osteocytes within the new bone from day 7 to 14 post-fracture.

DISCUSSION:
Bone morphogenetic proteins play an important role during bone development and repair. Yet their mechanism of action and specific targets during tissue regeneration are not completely elucidated. In the present study, we show that BMPs and their receptors are expressed in various cell types throughout the bone healing process. Each BMP has a unique but overlapping expression pattern. BMP-4, -5, -6, -7, and -8 are expressed as early as day 3 post-fracture in periosteal cells or fibrous tissue suggesting their participation in the initiation of bone repair, and possibly in the recruitment and specification of skeletal progenitor cells. Expression of BMP-2, -5, -8 and their receptors in immature chondrocytes suggests the involvement of these BMPs in early stages of cartilage formation. Expression of all BMPs in hypertrophic chondrocytes during the soft callus phase suggests that BMPs can control both remodelling and osteoblastic maturation. The expression of BMP-5, -6, -7, and -8, their receptors and effectors in periosteal cells near the fracture site indicates they may play roles in initiating osteogenesis during fracture healing. Based on their expression, BMPs and their receptors are also implicated in osteoblastic differentiation during new bone deposition. A consistent positive staining of p-Smad 1/5/8 in chondrocytes and osteoblasts/osteocytes within the callus further reveals that the BMP pathway is activated prior to and throughout the processes of chondrogenesis and osteogenesis. Concurrently, BMP signaling appears to be tightly regulated as indicated by the co-expression of BMP antagonists in hypertrophic chondrocytes and osteoblasts/osteocytes during the entire healing process. In conclusion, this study provides a clear picture of the BMP signaling pathway during fracture repair and will serve as valuable information for the analyses of BMPs functions in bone regeneration.

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