Bone Morphogenetic Proteins and Smad Expression in Ovine Tendon-Bone Healing

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Purpose: Bone morphogenetic proteins (BMPs) are being developed to improve tendon-bone healing. To do this, it is essential to understand the endogenous expression of BMPs and their downstream signal transduction factors, Smads, during tendon-bone healing. **Methods:** An extra-articular patellar tendon-bone healing ovine model was set up, and histologic evaluation of the healing progress at the tendon-bone interface at 1, 2, 3, and 6 weeks was performed. Immunohistochemical staining of BMP-2, BMP-7, Smad1, Smad4, and Smad5 was carried out in all sections. **Results:** The model revealed formation of a loose granuloma tissue layer between the tendon and bone at 1 week, remodeling starting at 2 weeks, and Sharpey-like collagen fiber formation at 3 and 6 weeks. All detected factors were elevated at the tendon-bone interface during healing, and the expression peaked at 2 to 3 weeks. The cells involved were osteoblastic-like cells, osteoclastic-like cells, mesenchymal cells, and fibroblasts. BMP-7 staining was mainly at the interface close to the bony side, whereas BMP-2 expression shifted to the tendon side at 6 weeks. The expression pattern of Smad1 and Smad5 was similar to that of BMP-7. Smad1 was also found to be expressed in osteoclastic-like cells at 1 and 2 weeks. Smad4 expression was the highest among all of the factors at all time points. **Conclusions:** The data suggest that endogenous BMP-2 and BMP-7 participate in tendon-bone healing and their functions involve their downstream signal transduction mediators, Smad1, Smad4, and Smad5. **Clinical Relevance:** The temporal expression of BMPs should be considered when setting up therapeutic strategies using BMPs. **Key Words:** Tendon-bone healing—Bone morphogenetic proteins—Smads—Immunohistochemistry—Ovine model.

The healing tendon-bone interface initially involves the organization of the cellular vascular infiltrate into an extracellular collagen-rich matrix. This ultimately progresses to a continuum between the tendon and the adjacent bone, which can withstand applied loads. Although a number of publications have recently reported healing tendon-bone gross morphology in sheep, goat, dog, or rabbit models,[1-7] the molecular events (protein expression) at the healing tendon-bone interface have yet to be reported. An improved understanding of the process of tendon healing to bone has multiple applications especially in the selection of methods of fixation and postoperative rehabilitation, as well as in therapeutic strategy.

A different healing pattern of tendon to bone, direct[4,5,8] or indirect,[1-3,8-10] is often seen in human cases and in animal models. More indirect than direct tendon-to-bone reattachment was seen after repair, and this phenomenon presented in many different animal models including intra-articular anterior cruciate ligament (ACL) reconstruction,[1,2,7] extra-articular patellar tendon-to-bone reattachment,[11] rotator cuff repair,[5] and flexor digitorum profundus reattachment.[6] In one of the human studies of ACL reconstruction using

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patellar tendon graft, a fibrous insertion was found in the tibial tunnel whereas the femoral tunnel showed a chondral enthesis.[8] The mechanisms by which the different healing processes in direct or indirect insertions are initiated are not clear.

In 1965 Urist[12] discovered that the extracellular matrix of bone had the ability to induce new bone formation. This substance was later named bone morphogenetic protein (BMP).[12,13] Over 14 BMPs have been isolated so far, and among them, BMP-2 and BMP-7 are being produced commercially for clinical use with specific indications. Both BMP-2 and BMP-7 have been used to enhance tendon-bone healing in animal models.[14-16] In one of the studies the authors detached the long digital extensor tendon of the dog from its femoral insertion, transplanted it through a bone tunnel into the proximal tibial metaphysis, and applied recombinant human BMP-2, together with an absorbable collagen sponge carrier, to the tendon-bone interface in the bone tunnel.[14] BMP-2–treated groups showed more extensive bone formation around the tendon with closer apposition of the new bone to the tendon in comparison to the control groups. The other BMP-2 study used an intra-articular ACL reconstruction rabbit model and double-bundle semitendinosus tendon grafts.[15] The tendon graft was harvested, transduced with either adenovirus-BMP-2 (AdBMP-2; *BMP-2* gene), adenovirus-LacZ (AdLacZ; vector control), or nothing (nontreatment control) in vitro, and implanted back into the same animal through the femoral and tibial tunnels to replace the ACL. The authors showed that the stiffness and the ultimate load to failure were significantly enhanced in the specimens with an AdBMP-2–transduced graft when compared with the controls. Histologically, the AdBMP-2–treated group presented a transition from bone to mineralized cartilage and nonmineralized fibrocartilage that was not seen in the control groups. In the study of BMP-7 the authors compared allogeneic bone plates saturated with BMP-7 in a collagen putty with plates coated with autologous cancellous bone chips and saturated with autologous bone marrow for the reattachment of supraspinatus tendon to a metallic implant.[16] BMP-7–treated allografts showed results similar to those treated with autologous bone chips and bone marrow. The cellular and molecular mechanisms by which BMPs enhance tendon-bone healing have not been well reported.

BMPs are members of the transforming growth factor (TGF) β superfamily of signaling proteins, and the BMP signaling cascade is activated by binding to TGF-β transmembrane cell surface receptors on mesenchymal cells. The signals are sent via intracellular signaling molecules known as Smads to the cell nucleus, which results in the expression of target genes for specific cell differentiation and tissue formation. Eight mammalian monomeric forms of Smads have been identified, which have been classified in relation to their mode of action: the receptor-regulated Smads (R-Smads) (Smad1, Smad2, Smad3, Smad5, and 8), the inhibitory Smads (I-Smads) (Smad6 and Smad7), and the common mediator Smad (Co-Smad) (Smad4), which forms heteromers with the R-Smads to positively regulate target gene expression.[17-20]

Understanding BMP/TGF-β signal transduction pathways during tendon-bone healing represents an important step in the search for therapeutic strategies designed to augment or control this process. This study investigated the expression of BMPs (BMP-2 and BMP-7) and their downstream regulators, Smad1, Smad4, and Smad5, in a patellar tendon reattachment animal model.[11] We hypothesize that the expression of BMP-2 and BMP-7 would vary with time after repair, as would the expression of their signal transduction mediators.

METHODS

Surgical Procedure

After the approval of the local animal care and ethics committee was obtained, the patella tendon in 8 skeletally mature cross-bred merino wethers was divided and reattached to the tibial tuberosity. The right stifle joint was used in all animals. Once the tibial tuberosity was denuded of all tendons, two 3.2-mm-diameter holes were made 5 mm medial and lateral to the center of the tuberosity. Anchor devices, consisting of a hollow cylinder of glutaraldehyde cross-linked fibrillar purified type I collagen and a polymethyl methacrylate washer at the tip, threaded with 2 braided polyester sutures, were loaded onto a delivery instrument and tapped with a mallet into the drill holes. A whipstitch of alternating 2- and 4-mm bites was applied to each edge of the tendon. Two animals were killed at 1, 2, 3, and 6 weeks after surgery.

The tibial insertion site and patellar tendon were harvested and fixed in buffered formalin for 48 hours. The tissues were decalcified in 10% formic acid–formalin solution. The central third of the patellar tendon–tibia insertion site was isolated and embedded in paraffin to provide a reproducible site for histology. The paraffin blocks were sectioned onto saline-coated