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ORIGINAL ARTICLE

Involvement of Indian hedgehog signaling in mesenchymal stem cell–augmented rotator cuff tendon repair in an athymic rat model

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Background: Bone marrow aspirate has been used in recent years to augment tendon-to-bone healing, including in rotator cuff repair. However, the healing mechanism in cell-based therapy has not been elucidated in detail.

Methods: Sixteen athymic nude rats were randomly allocated to 2 groups: experimental (human mesenchymal stem cells in fibrin glue carrier) and control (fibrin glue only). Animals were sacrificed at 2 and 4 weeks. Immunohistochemical staining was performed to evaluate Indian hedgehog (Ihh) signaling and SOX9 signaling in the healing enthesis. Macrophages were identified using CD68 and CD163 staining, and proliferating cells were identified using proliferating cell nuclear antigen staining.

Results: More organized and stronger staining for collagen II and a higher abundance of $SOX9^+$ cells were observed at the enthesis in the experimental group at 2 weeks. There was significantly higher Gli1 and Patched1 expression in the experimental group at the enthesis at 2 weeks and higher numbers of Ihh^+ cells in the enthesis of the experimental group vs control at both 2 weeks and 4 weeks postoperatively. There were more $CD68^+$ cells localized to the tendon midsubstance at 2 weeks compared with 4 weeks, and there was a higher level of CD163 staining in the tendon midsubstance in the experimental group than in the control group at 4 weeks.

Conclusion: Stem cell application had a positive effect on fibrocartilage formation at the healing rotator cuff repair site. Both SOX9 and Ihh signaling appear to play an important role in the healing process.

Level of evidence: Basic Science Study; Molecular and Cell Biology; Animal Model

Keywords: Indian hedgehog; sox9; stem cell; rotator cuff; enthesis; repair

Tendon attaches to bone through a fibrocartilaginous transition zone, functioning to transfer load between 2 very different materials. The native enthesis is not regenerated during tendon-to-bone healing, which contributes to a relatively high incidence of failure after surgery.[1,33]

This study was performed at the Tissue Engineering, Repair and Regeneration Program, Hospital for Special Surgery, New York, NY.

This study was approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery: HSS project #08-14-04R.

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Bone marrow mesenchymal stem cells (MSCs) are made up of a heterogeneous mixture of cells that have various functions, including osteogenic activity, acceleration of bone formation and regenerative repair,[19,22] and an immunomodulatory function.[24] Bone marrow aspirate has been used in recent years to augment tendon-to-bone healing after rotator cuff repair and has demonstrated beneficial results.[25] However, there are very few true "stem cells" (by formal criteria) in bone marrow.[25] Current systems in clinical use for application of bone marrow–derived MSCs do not allow cell sorting and subsequent culture expansion of the stem cells.[6,8,13] Furthermore, current US Food and Drug Administration regulations do not allow "manipulation" of bone marrow aspirate, which prevents the ability to isolate the small number of true stem cells in the aspirate.

We have examined the process of stem cell augmentation of rotator cuff repair using a rat rotator cuff repair model. We found that autologous bone marrow–derived MSCs did not have a significant effect on healing,[10-12] suggesting the limitations of standard autologous marrow-derived cells. We then used this same model to test cells genetically modified to express scleraxis and membrane type 1 matrix metalloproteinase, 2 factors that play a role in insertion site formation during development, and found significant improvements in healing.[10-12] To supply a greater number of undifferentiated cells, we then used purified human bone marrow–derived stem cells in an athymic rat rotator cuff repair model and found superior healing of the rotator cuff based on biomechanical testing and histologic analysis.[7] However, the healing mechanism in cell-based therapy has not been elucidated in detail.

The Hedgehog (Hh) signal transduction pathway has a crucial role in regulating embryonic developmental processes, such as cell differentiation, proliferation, and cell death. Recent studies indicate that Indian hedgehog (Ihh) signaling is linked to fibrocartilage development and mineralization of the tendon-bone insertion site.[21,29] Breidenbach et al confirmed that ablating Ihh signaling reduced mineralized fibrocartilage in the enthesis of patellar tendon, resulting in decreased biomechanical function .[4] However, there has been limited investigation of the role of Ihh in healing after rotator cuff repair.

To address the mechanism of cell-based therapy in rotator cuff repair, we used immunohistochemistry to evaluate healing in animals treated with MSC-based therapy and to evaluate the involvement of Ihh signaling in the formation of fibrocartilage in this process. Our hypothesis was that Ihh signaling would be increased in repairs treated with MSCs.

Materials and methods

Study design

Sixteen mature, male athymic rats underwent right supraspinatus tendon resection and transosseous repair. Animals were randomly allocated to 2 groups: experimental (human MSCs in fibrin glue carrier, n = 8) and control (fibrin glue alone, n = 8). At 2 weeks and 4 weeks postoperatively, the rats were euthanized and the tissue was harvested for immunohistochemistry, providing 4 animals per group at each time point.

Cell culture and preparation for surgery

Human bone marrow–derived MSCs were obtained in the second passage from American Type Culture Collection (ATCC; Manassas, VA, USA; Cat No. PCS-500-012) and cultured following the manufacturer's protocol. Briefly, the cells were seeded at an initial density of 5000 cells/cm,[2] then cultured in Mesenchymal Stem Cell Basal Medium (ATCC, Cat No. PCS 500-030) provided by ATCC containing a Growth Kit for Bone Marrow–Derived MSCs (ATCC, Cat No. PCS-500-041). Cultures were maintained at 37°C in a humid atmosphere with 5% CO_2. The medium was changed every 2 days until 80% confluence. Cells were expanded until the fifth passage. Flow cytometry (FACSCalibur instrument; Becton Dickinson AG, Allschwil, Switzerland) was used to confirm their MSC phenotype, which expressed CD73-Pe-Cy7, CD90-Violet 421, and CD105-APC while lacking expression of CD14-APC-Cy7, CD34-PE, and CD45-PerCP (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA, USA).

Surgical technique

All surgical procedures were performed as previously described .[14,20] Briefly, after anesthesia, a longitudinal incision was made on the anterolateral aspect of the right shoulder, and the supraspinatus tendon insertion site was exposed by splitting the deltoid and acromioclavicular joint. After detachment of the tendon from the humeral tuberosity and decortication of the enthesis of the tuberosity, a modified locking Mason-Allen suture was used to reattach the tendon by pulling the repair sutures through 2 crossed bone tunnels (anterior and posterior) that were drilled using a 23-gauge needle.

In the experimental group, 10^6 MSCs carried by 50 μL of fibrin sealant (Evicel fibrin sealant; Ethicon, Somerville, NJ, USA) were implanted at the tendon-bone insertion site. The control group received only the fibrin sealant carrier.

Histology

At 2 weeks and 4 weeks postoperatively, the rats were euthanized. Both shoulders were harvested for histologic analysis and the left nonsurgical shoulder was used as a normal control. Specimens were fixed in 10% neutral buffered formalin for 48 hours, then decalcified with Immunocal (Decal, Congers, NY, USA) at room temperature for 3 to 4 days. After dehydration, samples were embedded in paraffin. Supraspinatus tendon–greater tuberosity constructs were cut in the coronal plane in 5-μm-thick sections. Hematoxylin and eosin staining and immunohistochemistry were used to quantify the cellularity and protein expression in 5-μm paraffin sections.

After quenching endogenous peroxidase activity treated by 3% H_2O_2, the sections were incubated with primary antibody specific to collagen II (1:150; Novocastra, Buffalo Grove, IL, USA), proliferating cell nuclear antigen (1:1000; Abcam, Cambridge, MA,