L-WNT3a Enhances Bone Regeneration In A Murine Model Of Osteonecrosis

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Introduction: Osteonecrosis of the hip (ONH) has a complex etiology. Contributing factors including microembolic events; vascular impediments; adipocytic hypertrophy, and lipid-incurred osteocytic death secondary to steroid use. The most rigorously validated documented ONH animal models induce ONH either via surgical compromise of the blood supply to a bone, or via cryoablation. Both techniques obliterate the cellular component but leave behind a relatively intact collagen extracellular matrix and maintain in over the short-term the general architecture of the bone. In both scenarios, microscopic signs of necrosis are usually apparent within 48-96h of surgery, and overt ONH can be diagnosed within 7 days. These models allow testing of potential regimens for the treatment of ONH in humans.

Here, we modified an established cryoablation model in mice of ONH to test whether treatment of bone graft material with a liposomal formulation of WNT3A protein was capable of improving the osteogenic response observed following treatment.

Methods: All procedures were approved by Stanford’s Committee on Animal Research. Following anesthesia a 1mm mono-cortical defect was generated in the murine tibia. A 1mm drill bit was positioned in the defect and dry ice was applied to the drill bit for 10 or 60 sec, taking care to avoid contact with any soft tissues. Animals in Group 1 received no therapy after cryoablation.

In Groups 2 and 3, animals received bone grafts from syngeneic host mice. Because syngeneic animals are so closely related, their tissues are immunologically compatible and transplantation of tissues does not provoke an immune response; thus these grafts function as autografts. Group 2 animals received a syngeneic autograft while Group 3 animals received a syngeneic autograft that had been briefly treated with a liposomal formulation of WNT3A protein (L-WNT3A). To treat bone grafts with L-WNT3A the following procedure was carried out: bone graft material was harvested from the tibiae and femurs using a modified reamer-irrigator-aspirator technique. To ensure that bone grafts were equivalent in terms of cellular content, bone graft material from 3 mice (littermates) was pooled then divided into 20 µL aliquots. DNA content was extracted with the DNeasy Tissue Kit (QIAGEN) and relative DNA concentration was measured using the Quant-iT PicoGreen dsDNA Kit (Invitrogen) and microplate fluorescence reader (BERTHOLD, Bad Wildbad, Germany). The percent variation in DNA content was <20%. Freshly harvested bone graft aliquots were then placed into 20µL of culture medium containing a liposomal formulation of either phosphate-buffered saline (L-PBS) or WNT3A (L-WNT3A, effective L-WNT3A concentration = 0.15µg/mL) and maintained at 23°C for 1 hour. Following cryoablation, the treated bone grafts were positioned in the defect site. The soft tissues and skin were then closed.

Animals were monitored by micro-CT imaging and tissues were evaluated by histology at multiple time points after treatment.

Results: Two weeks after cryoablation had been performed, tissues were collected, embedded, sectioned, and analyzed for evidence of cell death using DAPI (to detect viable osteocyte nuclei) and TUNEL staining (to detect fragmented DNA). Animals in which cryoablation was performed for 10 sec
showed evidence of dead and dying osteocytes in the cortical bone (~170µm), coupled with a strong reparative response that resulted in new bone formation at the site of the mono-cortical defect. Animals in which cryoablation was performed for 60 sec also showed evidence of dead and dying osteocytes, but the zone of osteonecrosis was significantly larger (>1000µm). Quantification of the necrotic zone revealed that the percent necrotic bone occupied <5% of the bone surface in the 10-sec cryoablation group versus ~25% of the bone surface in the 60-sec cryoablation group. In addition, histological assessments demonstrated that bone regeneration was absent at the 2-week time point in the 60-sec cryoablation group and in its place was fibrous tissue that bridged the gap created in the cortical bone. We therefore used a 60-sec duration of cryoablation and the 2-week time point to assess the efficacy of a L-WNT3A treatment for bone grafts used to stimulate bone healing in cases of osteonecrosis.

Micro-CT imaging was performed between post-surgery days 1-5, and post-surgery days day 14 to assess the amount of regenerated bone in defect sites treated with autograft alone (Group 2) or autografts pre-incubated with L-WNT3A (Group 3). In addition, histological assessments were made of the injury site. The amount of new bone generated from L-WNT3A treated bone grafts was significantly greater than the amount of new bone generated from autografts alone.

Discussion: In most animal models of osteonecrosis there is early evidence of osteolysis followed rapidly by remodeling of the dead bone by osteoclasts. We observed a similar response following 60 sec of cryoablation using a mono-cortical defect model in the mouse tibia. Within two weeks, the osteonecrotic site was filled with fibrous tissue; whether this tissue ultimately becomes replaced by new bone tissue is not yet clear. In other larger animal models, new bone is evident within two months of creating the osteonecrotic zone but the biomechanical properties and volume of the new regenerate is typically inferior to native bone.

Treatment with bone grafting is a common method for preventing collapse of the femoral head in humans. We tested whether the efficacy of a bone graft could be further improved by a brief incubation with L-WNT3A. Micro-CT imaging suggests that bone formation was enhanced by treatment with this stem cell activator. Histologic and histomorphometric analyses are planned to confirm this observation.

Significance: A modified cryoablation model is presented, along with a strategy designed to improve the osteogenic capacity of bone grafts that are used to stimulate osteogenesis in cases of osteonecrosis.
A murine model of cryoablation produces osteonecrosis. (A,B) DAPI staining identifies viable nuclei in the marrow cavity and the cortical bone (dotted white line); the zone of necrosis is indicated with a dotted red line. (C,D) High magnification of the zone of necrosis and zone of viable osteocytes. (E) Alcian blue staining demonstrates new bone formation in defects when cryoablation lasts 10 sec (histology at the 2-week time point); (F) and no new bone formation in the mono-cortical defects when cryoablation lasts 60 sec.

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