Early Histological and Molecular Characterization of the Local Tissue Microenvironment Following Blast-Related Post-Traumatic Injury in a Rat Model of Heterotopic Ossification

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Introduction: Heterotopic ossification (HO) is the ectopic formation of bone in non-osseous tissue, most commonly occurring in the setting of orthopedic trauma, severe burns, neurotrauma or major surgery. However, the majority of combat wounded personnel have been affected. In fact, the prevalence of HO in the residual limbs of returning service members with combat-related amputations is reported to be as high 65%. The pathogenesis of ectopic bone deposition requires a microenvironment conducive to the formation of bone, appropriate osteogenic progenitor cells and an inducing agent or event and is thought to resemble the process by which endochondral and/or intramembranous bone develops. The cellular and molecular biological mechanisms underlying HO development remain elusive. Our group recently developed a physiologic HO model in rats wherein all animals develop radiographic evidence of HO within 2 months post-injury. Using this model as a platform to characterize the formation of HO, we intend to (1) describe the early histological changes in ectopic bone development, and (2) assess the expression and production of early mRNA gene transcripts, micro RNAs (miRNA) and key signaling proteins that play critical roles in osteogenic, chondrogenic, angiogenic, and adipogenic lineage differentiation signaling networks.

Methods: A rat HO model was established consisting of blast exposure (120kPa ± 7kPa) immediately (< 1 hr) followed by a controlled femur fracture, crush injury, and subsequent transfemoral amputation. Rats were euthanized 3, 5, 7, 10, 14, 21, and 28 days post-injury (D) for analysis. Micro computed tomography (mCT) imaging was used to visualize and quantify ectopic bone formation. Histological examination of tissue samples at the site of injury was conducted. Quantitative RT-PCR (qRT-PCR) used to analyze tissue samples for the expression of 83 rat osteogenic, chondrogenic, adipogenic, and angiogenic gene transcripts. We also analyzed 8 key osteogenic proteins and 700 miRNAs. Muscle tissue from contralateral non-injured femurs was used as control.

Results: All rats developed HO evident by the mCT, and the ectopic bone volume increased until D-28 (Figure 1). Histological analysis at D-3 showed edema, degenerative necrosis, and marked cellular infiltration consistent with an acute inflammatory response. By D 7-14, intense foci of chondrogenesis within the injured/healing soft tissue were observed often resulting in a “hook like” area of active chondrogenesis. This developed immediately off of the amputation site wherein it was surrounded by thick fibrosis and necrotic connective tissue (Figure: 2A-D). Using qRT-PCR performed on muscle tissue
isolated from the injured limb, we detected significance differences, in comparison to naïve non-injured muscle control samples in the regulation of 34 gene mRNA transcripts that are critical in events involving extracellular matrix remodeling, cartilage deposition and bone mineralization. Specifically, increases in gene transcripts involved in tissue remodeling (Mmp9, Has1), synthesis of a cartilaginous matrix (Col1a1, Col10a1, Col11a1, Comp, and Acan), bone formation (BMP2, RunX2) and PPARγ which is expressed by macrophages in response to inflammation. We also identified, using miRNA profiling, a few miRNA expression patterns that may facilitate and hinder the development of the osteogenic phenotype, specifically miR-181a, miR-340, miR-29a, miR-140 and miR-423. These miRNAs are known to regulate endochondral bone development and promote osteoblastogenesis.

**Discussion:** Collectively the findings from this study demonstrate that the injury pattern used in our blast-related post-traumatic rat model of HO induces sequences of histological changes and local osteogenic (bone-related) gene regulation/signaling which are consistent with early endochondral bone formation. These data present the first temporal gene expression profiling analysis of HO development in a blast-related trauma-induced injury model. This model holds promise both in the further study of the pathogenesis resulting in ectopic bone formation and in the development of prophylactic and therapeutic strategies.

**Significance:** This model holds promise both in the further study of the pathogenesis resulting in ectopic bone formation and in the development of prophylactic and therapeutic strategies.
Figure 1: Representative micro-CT image of rats euthanized at post-injury day 3 (A), day 5 (B), day 7 (C), day 10 (D), day 14 (E), day 21 (F) and day 28 (G). The amount of ectopic bone (nm³) was quantified using volumetric analysis in (H). The red arrows points to the ectopic bone formation. * denotes statistically significant (P < 0.05; one sample t-test)
Figure 2: Histological evidence of early HO formation at the site of amputation D7-14. (A-B) Foci of cartilage matrix present in the soft tissue and (C-D) “hook” of active chondrogenesis developing off the amputation site.