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INTRODUCTION

In this project we study Heterotopic Ossification (HO) and are a potential novel therapy to cure it. HO consists of formation of extra bone within the muscles, near tendons and ligaments, inside the blood vessels and other places in the body. HO is triggered by trauma, burns, nerve damage, immobilization and other conditions and can also occur in patients undergoing large surgeries such as hip or knee replacement. Because trauma, burns and other severe wounds are regrettably common in our soldiers in the current war theaters and conflicts, HO often affects and afflicts many of them. The consequences of having HO are not minor. Patients with HO can experience loss of normal posture and movement, chronic pain, prosthesis fitting problems, formation of pressure ulcers, deep venous thrombosis and other health problems. Indeed, HO has emerged as the single most important barrier to functional activity and return-to-duty in a recent analysis of wounded active duty service- members. Subsequent infection remains one of the common and significant complications following blast-related severe fracture and amputation with *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) being the most common isolate from combat wounds. To more precisely identify the cellular and molecular changes associated trauma-induced HO formation and to test the potential in vivo inhibitor effects of an retinoic acid receptor- γ agonist called palovarotene, we will use an established rat model of combat-related extremity injury/amputation that incorporates the critical elements commonly associated with combat injury namely blast injury, femur fracture and amputations, soft tissue injury and bioburden.

KEYWORDS

heterotopic ossification, traumatic injury, ectopic bone, palovarotene, amputation, combat wounds, crush injury, bioburden, osteogenesis, chondrogenesis, gene expression, extremity injuries, and blast overpressure exposure.

OVERALL PROJECT SUMMARY

NMRC-Regenerative Medicine Department Role (SOW) in the Partnership Award

Proposal Specific Aim 3: To determine whether the retinoid agonists block blast- and combat-related HO (months 1-36):

Task 3a. Implement the rat blast-injury model to include bacterial infection and HO (months 1-12).

Current objective: To determine the effects of *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) infection on the rate development and severity of HO formation.

Results, Progress and Accomplishments with Discussion: We exposed forty-three adult male Sprague-Dawley rats (350-400g) to 120 ± 7 kPa blast over pressure using a shock tube followed by femur fracture, thigh crush injury and transfemoral amputation done

within the zone of injury. Each wound was inoculated beneath the myodesis with 1.0×10^8 CFUs of a highly virulent strain of either *Acinetobacter Baumannii* or *Methicillin Resistant Staphylococcus Aureus* (MRSA) which were isolated from combat wounds. A control group was injured as described above, but did not undergo inoculation. Rats were followed for visual evidence of wound infection requiring irrigation and debridement and euthanized if three serial debridements performed at least 24 hours apart did not result in clinical improvement. We performed microCT (mCT) imaging weekly for the first month and at 8 and 12 weeks post-operatively in order to measure HO volume. Samples of muscle tissue adjacent to the amputation site and bone marrow from the residual femur were harvested to determine persistence of infection. Representative animals in each treatment group underwent en bloc resection of the femur and surrounding musculature for histologic analysis.

All rats in both experimental groups as well as the control group developed HO. Survival rate among treatment groups was less in the MRSA experimental group (14/20, 70%) in comparison to the *Acinetobacter* experimental (16/18, 89%) and control (5/5, 100%) groups. Specifically, 6 of 20 rats were euthanized in the MRSA group, chiefly for overwhelming infection persistent after three debridements, which occurred between the 4th and 5th week postoperatively. Whereas two rats inoculated with *Acinetobacter* were euthanized during week 2 and week 4 for weight loss of greater than 10% of preoperative weight. At 12 weeks, we observed significantly more robust HO on mCT volumetric analysis in animals infected with MRSA ($122.32 \text{ mm}^3 \pm 29.22$) when compared to *Acinetobacter* ($15.04 \text{ mm}^3 \pm 2.43$; $p < 0.05$) and controls ($11.22 \text{ mm}^3 \pm 2.77$; $p < 0.05$). There was no significant difference shown in this measure when comparing *Acinetobacter* with surgical controls not inoculated with bacteria. Sample tissue from a cohort of five rats inoculated with MRSA during initial surgery still had culture positive results in the tissue of two rats, the bone marrow of two rats and in both the tissue and bone marrow of one rat. All rats inoculated with *Acinetobacter Baumannii* tested negative for the inoculated bacteria, however (2/14) bone marrow samples and (6/14) muscle tissue cultures demonstrated growth of other bacteria.

Our results suggest that the addition of MRSA bioburden significantly accelerates the timing and augments the level of HO development. Modest increases in HO formation were observed when wounds were infected with *Acinetobacter baumannii*. Follow-on studies for the next year will be focused on characterizing the timing of HO development at the histological, cell and molecular cell signal levels in tissue from animals subjected to traumatic injury plus bacterial challenge with *Acinetobacter baumannii*, MRSA and combination of both strains. These experimental will be critical in order to define the optimal timing for prophylaxis intervention.

Task 3b. Test drug effectiveness, regimens and systemic versus local delivery (months 8-30).

Current objective: In order to optimize the timing of drug-based prophylactic intervention following blast-related combat injury, it is important to define the (1) early histological changes in soft tissue architecture, vascularity, collagen deposition and cartilage development and their correlation with later ectopic bone development following blast-related traumatic injury;(2) the blast-related gene expression patterns in traumatized tissues subsequent to calcium deposition, tissue mineralization and ectopic bone formation and (3) and the expression profile of early chondrogenic and osteogenic gene transcripts in traumatized tissue compare to early known proteomic indicators of ectopic bone development.

Results, Progress and Accomplishments with Discussion:

In a series of studies using our rat HO model, consisting of blast exposure (120kPa \pm 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 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) was 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.

All rats developed HO evident by the mCT, and the ectopic bone volume increased until D-28. Histological analysis at day-3 showed edema, degenerative necrosis, and marked cellular infiltration consistent with an acute inflammatory response. By day 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. 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 PPAR γ 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.

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. Based on these findings, we propose definitive treatment targeted at inhibiting vasculogenesis of the new synthesized cartilage should commence within 7 days of injury, preferably between 5-7 days to avoid any adverse effects on physiologic early wound healing processes such as tissue revascularization and granulation tissue development. The ability to correlate molecular events, with histologic and morphologic changes will help researchers and clinicians in understanding the heterotopic ossification process, ascertain how applicable the findings are to the human wound-healing scenario, and will be critically important in formulating therapeutic interventions that target early chondrogenic, angiogenic and osteogenic signaling components of ectopic bone development.

In another set of pilot experiments, we evaluated the effect of palovarotene (2 mg/kg orally via gavage every second day for 14 days starting 5-days post injury) on inhibition of HO formation in our combat-related traumatic injury model of HO. Our preliminary findings from pilot studies completed last quarter suggest reduce radiographic evidence (microCT) of HO formation during the first 30 days post injury. This observation was confirmed by histological analysis. The number of animals treated in the pilot was too few to draw any concrete conclusions at this time plus animals need to be followed on for at least 3-4 months to make sure treatment inhibited rather than just delayed HO development.

Task 3c. Analyze wound healing and muscle repair (18-36).

We have not specifically conducted any in-depth experimentation to evaluate the short-term and/or long-term and effects of therapeutic intervention with palovarotene on wound healing and muscle repair following blast-related traumatic injury.

KEY RESEARCH ACCOMPLISHMENTS

- Defining the early cellular and molecular signaling development phases of HO development in the blast-related HO model.
- Based on collective research findings, we have defined the therapeutic window for targeting early chondrogenesis, vasculogenesis and osteogenesis to inhibit the synthesis of cartilage and early ectopic bone formation without adverse effects on physiologic early wound healing processes such as tissue revascularization and granulation tissue development.
- Characterized the effects of bioburden, specifically *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) infection, on the rate development and severity of ectopic bone formation (HO).
- Demonstrated in a small pilot study that the administration of palovarotene appears to attenuates ectopic bone formation when administered orally every other day for 2-weeks starting 5-days post injury.

CONCLUSION

The initial year of this research project has dedicated the majority of its effort to establishing and histologically characterizing the blast-related HO model at the cellular and molecular cell signaling levels as well as assessing the effects of *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) infection on the development and augmentation of ectopic bone formation. The ability to correlate molecular events, with histologic and morphologic changes has helped us researchers and clinicians in understanding the heterotopic ossification process, ascertain how applicable the findings are to the human wound-healing scenario, and are critically important in formulating therapeutic interventions that target early chondrogenic, angiogenic and osteogenic signaling components of ectopic bone development. As a result, the initial critical objectives and milestones for each specific task of Aim 3 of the partnership grant have been established and validated.

- The team is now poised for publishing these keys findings in several publications within the next 3-6 months.
- Larger follow-on experiments to validate our preliminary results using palovarotene are now underway.
- In year 2, we will complete comprehensive experiments aimed at the histological assessment of early HO formation rat blast trauma/amputation model in the presence of bioburden. Analysis and review of the findings at various time points (days 0, 1, 3, 5, 7, 10, 14, 21 and 28 post-injury) in regard to histological evidence of early cartilage, blood vessel and ectopic bone development.
- In year 2, we will assess the effects of palovarotene treatment (start day-1 and day-5 post injury) on the early developmental phases of HO development in our blast-related trauma injury model (\pm bioburden) in relationship to concurrent wound reparative/healing processes such as neoangiogenesis, granulation tissue formation and wound dehiscence.
- In year 2, increase the number of abstract submissions for poster and oral presentation at national and international meetings from the investigative team.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Lay Press

Nothing to report.

Peer-Reviewed Scientific Journals

Nothing to report.

Invited Articles

Nothing to report.

Abstracts

1. Abstract entitled "Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model" submitted for presentation consideration at the Orthopaedic Research Society meeting to be held March 28-31, 2015 in Las Vegas, NV. (25-08-2014)
2. Abstract entitled "Early Histological and Molecular Characterization of the Local Tissue Microenvironment Following Blast-Related Post-Traumatic Injury in a Rat Model of Heterotopic Ossification" submitted for presentation consideration at the Orthopaedic Research Society meeting to be held March 28-31, 2015 in Las Vegas, NV. (25-08-2014)

Presentations

Nothing to report.

INVENTIONS, PATENTS AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Nothing to report.

OTHER ACHIEVEMENTS

Nothing to report.

REFERENCES

No references cited

APPENDICES

1. n/a