A Mouse Model of Geriatric Fracture Healing: Toward the Elucidation of Aged Fracture Healing Deficiencies

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Introduction

Fragility fractures represent a significant obstacle in medical care leading to significant morbidity, mortality, and cost both to the individual and society. As the population ages, the incidence of these injuries is expected to increase. It is known that fracture healing capacity decreases with age; an 80 year-old may take months longer to heal the same fracture as an 18 year-old. Improved understanding of the aging process in the skeletal system and advancing technology now allow detailed investigation into the differences in bone healing observed across a lifespan. Currently, no well-established animal model exists to study mechanistic changes responsible for aged fracture healing. The objective of this study was to characterize the phenotypic differences in healing due to chronologic aging in our murine model—and to provide a validated foundation for the rational investigation of the cellular and molecular mechanisms that affect aged fracture healing. Such a model will allow for the identification of pathways that can be manipulated—and therapeutically targeted—to accelerate and improve aged fracture healing characteristics.

Methods

Model Design: C57BL/6 mice at 5-months of age are reproductively and skeletally mature—"young" adults. 25-month old (m/o) C57BL/6 mice represent the age of 25% survival and therefore a "geriatric" mouse (life expectancy represents 50% survival). All mice were obtained from the National Institute of Aging (NIA Aged Rodent Colonies, Bethesda MD). Surgical Model and Sample Preparation: 84 5-m/o and 85 25-m/o C57BL/6 mice underwent bilateral closed, transverse tibial diaphysis fractures with intramedullary pin fixation (IACUC approved), as previously described1. Tibiae were harvest, at 5, 10, 15, 20, 25, 30, and 40 days post fracture (DPF).

Genetic Analysis: The fracture callus was isolated from the tibial diaphysis at 0, 10, and 20 DPF. RNA was purified with RNeasy system including a DNase digestion. First strand cDNA was then synthesized. Analysis by real-time quantitative PCR (qPCR) was conducted utilizing Fast SYBR Green and the 7500 Fast Instrument (Applied Biosystems). Probes were utilized against target genes including Osteocalcin, Collagen 2a, SOX9, and Osterix. RNA was also sent for microarray analysis.

Histology: Formalin fixed tibiae were decalcified in 15% Formic Acid. Specimens were embedded in paraffin and cut into 5-μm longitudinal sections. Sections were stained with Fast Green FCF/Safranin-O to identify cartilage matrix and Masson's Trichrome to identify bone and osteoid tissue. 2x images were captured with an Olympus BX51 inverted microscope and SPOT Advanced imaging software. Using ImageJ software (NIH), callus size, cartilage area and composition, and bone area and composition were quantified. Two sections per sample were analyzed.

Micro Computed Tomography (μCT Analysis): Using a SancoMedical vivaCT 40 (San Antonio, TX) tibiae were imaged at a voxel size of 21 μm. Callus was spatially segmented from cortical bone. 3D images of the mineralized callus were rendered and callus and connectivity measurements were measured.

Statistical analysis: paired comparisons were performed using two-tailed student t-test with significance set as $p<0.05$(*).

Results

Histology: 5-m/o mice produce a larger callus ($p=0.03$) with more cartilage area ($p=0.03$) and show a trend towards a larger cartilage per callus area ($p=0.11$) than do 25-m/o mice at 10 dpf. At 20 dpf, 5-m/o mice still have a larger callus ($p=0.02$), but no longer contain more cartilage with the trend of cartilage per callus area reversed toward the 25-m/o mice having a larger percentage ($p=0.17$). No differences in the type of cartilage (hypertrophic, mature, or neo) were observed. Analysis of bone and osteoid tissue did not demonstrate significant differences in the proportion of bony callus between 5-m/o and 25-m/o mice at either 10 or 20 DPF. μCT: 5-m/o mice produce a significantly greater total callus volume (TV) (15, 20, 25, and 30 DPF)

Poster No: 0645
2013 Annual Meeting
Orthopaedic Research Society
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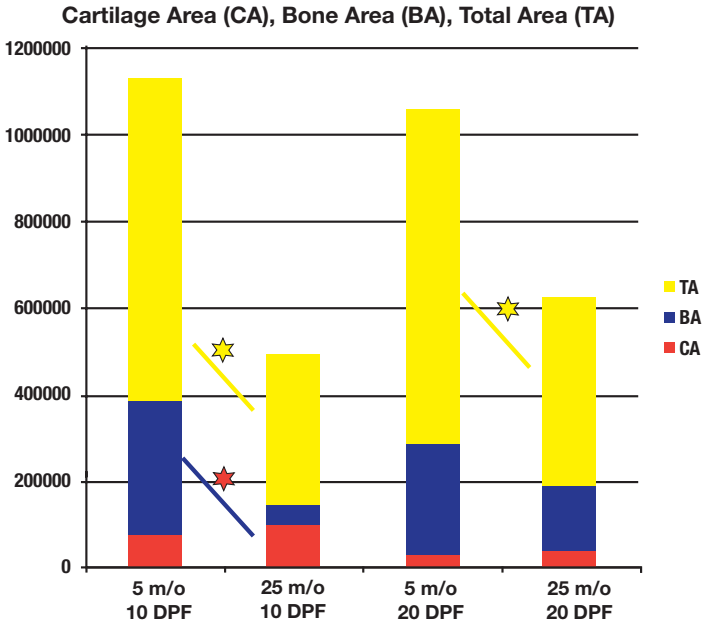

Figure 1. Histologic analysis of total area, cartilage area, and bone area of callus in 5 and 25-m/o mice at 10 and 20 DPF.

and bone volume (BV) (10, 15, 20, 25, and 40 DPF) than 25-m/o mice. TV could not be reliably measured at 10 DPF. Calluses formed by 5-m/o mice have a lower degree of anisotropy and a significantly greater polar moment of inertia (PMI) (10, 15, 20 and 25 DPF) than that of 25-m/o mice. Bone mineral content is significantly higher in young mice at 15, 20, 25, 30, and 40 DPF.

Gene Expression: Chondrogenic markers Sox9 and Col2α show upregulation at both 10 and 20 DPF when compared to unfractured 5-month old bone with the greatest upregulation

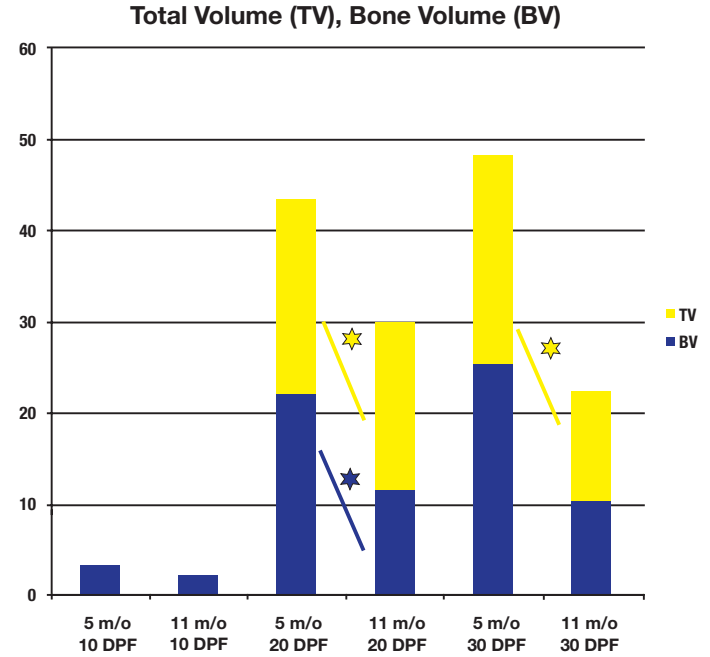

Figure 2. μCT analysis of TV and BV of callus in 5 and 25-m/o mice at 10, 20, and 30 DPF.

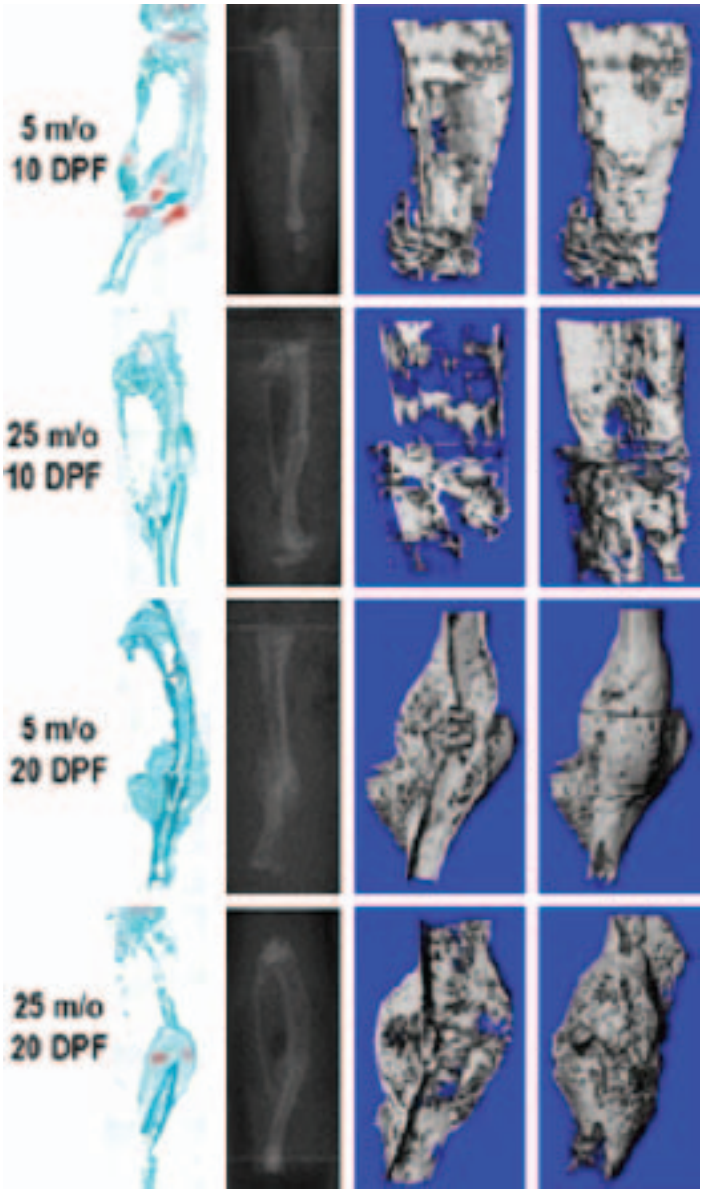

Figure 3. Representative images. From left to right: Fast Green/Safranin-O stained, μCT scout, μCT mid-callus and callus 3D reconstruction.

at 10 DPF in both age groups. Osteogenic makers Ocn and Osx also demonstrate upregulation in both age groups at 10 and 20 DPF when compared to 5-m/o unfractured bone. Early microarray analysis indicates there are measurable differences in gene expression patterns (Affymetrix) between young and aged fracture healing.

Discussion

Our histology demonstrates more robust callus formation in young mice at all time points examined. The observation that cartilage is increased in the callus of young mice at 10 DPF but decreased at 20 DPF relative to aged mice, suggests that 5-m/o mice undergo more robust and rapid remodeling. The μCT data shows significantly greater TV and BV in young mice at most time points. Considering the decreased anisotropy

and increased PMI demonstrated by the young mice suggests that, not only do the young mice produce a more robust healing response, but that this response results in bone of enhanced structural integrity compared with that of aged mice. Preliminary analysis of phenotypic chondrogenic and osteogenic gene expression reveals a profile consistent with formation and resorption of cartilage and formation of bone matrix consistent with endochondral bone formation in both young and old mice. Our characterization reveals a generally intact fracture healing machinery in the geriatric mice but one that remodels into bone more slowly and never to the same quality or extent as seen in the young.This model provides a validated system for further study of the specific age-based differences in fracture—and to test alternations that enhance healing in the elderly. Indeed, initial expression array analysis suggests that there are discernable and significant differences in the gene regulation in these populations, providing targets for further study and potential therapeutic manipulation.

Significance

Understanding the biological differences in fracture healing in geriatric population will provide a rational basis for potential therapeutic intervention.

Acknowledgements

These investigations were supported by NIA R03AG040670, Penn Center for Musculoskeletal Disorders (NIAMS P30AR050950), the Jahnigen Scholar in Aging award and the McCabe Foundation.

References

1. Taylor DK, Meganck JA, Terkhorn S, et al. Thrombospondin-2 influences the proportion of cartilage and bone during fracture healing. *J Bone Miner Res* 2009;24:1043-54.