Understanding the Biological Basis of Contracture in a Mouse Single Hindlimb Immobilization Model

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INTRODUCTION:

Arthrofibrosis is characterized by excessive collagen production and adhesions that result in restricted joint motion (stiffness) and debilitating pain. Stiffness and contracture formation are exceedingly common after joint injury, surgery, and immobilization. Current treatments are limited to early mobilization after injury, physical therapy, dynamic splinting, manipulation under anesthesia and surgical contracture release, but at present there are no pharmacologic interventions that can reliably reduce or prevent joint stiffness. This is at least in part due to our limited understanding of the biological mechanisms that lead to the development of fibrosis and stiffness.

We have previously developed a non-surgical mouse model of contracture formation through hindlimb immobilization in 3D-printed clamshell casts. This model produces contractures of the knee and ankle, which vary in reversibility depending on animal age and duration of immobilization. Using this model, we now seek to understand the biological mechanisms that lead to immobilization-driven contracture formation through motion testing, histopathology, and gene expression analyses.

METHODS:

Wild-type 14-19 week-old C57BL/6J female and male mice were subjected to the single hindlimb immobilization model using custom, 3D-printed clamshell cast for 2 or 3 weeks prior to cast removal. All experiments were performed according to the guidelines of the American Veterinary Association and were approved by the Institutional Animal Care and Use Committee. Casts were changed and knee motion was assessed weekly using a custom, 3D-printed motion measurement system, designed to measure joint motion using a constant force. Mice were euthanized on the day of cast removal or at 4 days after cast removal, and tissues were harvested for histology (n=4 each time point) or gene expression analyses (n=9, 3-week casting, harvest 4 days after removal). Contralateral limbs were used as controls. Tissues were processed and sectioned using established protocols and stained with Hematoxylin and Eosin (H&E), Masson's Trichrome, Picrosirius Red or Safranin O/Fast Green. For gene expression analyses tissues from the anterior compartment (including fat pad, capsule and adjacent synovial tissues) were retrieved immediately after euthanasia, while bathed in RNAlater. Total RNA was isolated using Trizol, treated with DNase (Ambion), and purified using the Qiagen MinElute Kit. RNA was used for NanoString analyses using the mouse nCounter Fibrosis panel and the accompanying nSolver 4.0 software. Changes in selected genes were further validated using RTqPCR. Quantitative histomorphometry was done using quPath, and statistical analyses were completed in GraphPad Prism.

RESULTS:

Single hindlimb immobilization with the 3D-printed clamshell cast for 3 weeks produced sustained contracture formation in both male and female mice, with an average knee motion loss of 31.9 ± 6.5° for male mice and 37.3 ± 15.6° for female mice, consistent with previously reported results. Hindlimb immobilization resulted in deposition of excess collagen-rich extracellular matrix (ECM) in the anterior fat pad and posterior knee, confirmed and quantified using by Masson's Trichrome and Picrosirius Red Stains. Histopathological assessment of the immobilized joints showed thickening of the posterior capsule and replacement of the anterior fat pad with reactive fibrous stroma. These changes were present in both male and female mice and were more pronounced after 3 weeks of immobilization, compared to limbs immobilized for 2 weeks.

Multiplexed NanoString gene expression analyses identified more than 230 differentially expressed genes in limbs immobilized for 3 weeks relative to contralateral control limbs. We observed a pronounced upregulation in genes associated with collagen biosynthesis and modification, ECM synthesis and degradation, transforming growth factor beta (TGFβ) signaling, and epithelial mesenchymal transition (EMT) signaling pathways. This was accompanied by downregulation of fatty acid metabolism and peroxisome proliferator activator receptor gamma (PPAR-γ) pathways, which is consistent with the replacement of adipose tissue with fibrotic stroma. The differential expression of selected genes identified by NanoString was confirmed using qPCR.

DISCUSSION AND CONCLUSION:

Here, we have provided an in-depth characterization of the histologic and gene expression changes that occur in a non-surgical mouse single hindlimb immobilization model of joint fibrosis and contracture. Our results clearly demonstrate that mouse single hindlimb immobilization is associated with the development of knee contracture and peri-articular fibrosis. Consistent with larger animal studies of contracture formation, we see thickening of the posterior capsule with deposition of collagen. Similar also to the human phenotype, we see fibrosis of anterior knee structures including the infrapatellar fat pad. Importantly, we see histopathological and gene expression changes that are consistent with studies evaluating joint fibrosis in human tissues. These changes include fibroblast activation, upregulation of collagen synthesis and

modification, ECM synthesis and degradation and TGF-β signaling, as well as downregulation of fatty acid metabolism and PPAR-γ pathways. This animal model represents a reproducible and cost-effective preclinical platform that can be readily employed to further define the mechanisms underlying the developing of fibrosis, stiffness and contracture formation, and to test therapeutic interventions.