Achilles Tendon Impingement Elicits Spatially Heterogeneous Patterns of Aggrecan Metabolism

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INTRODUCTION: Tendon impingement generates a unique mechanical strain environment with markedly elevated transverse compressive strain[1] that drives anabolism of large, glycosaminoglycan (GAG)-rich macromolecules such as aggrecan, yielding a localized fibrocartilage phenotype with compressive load bearing capacity[2]. While this fibrocartilaginous tissue is present in impinged regions of healthy tendon, aberrant proteoglycan metabolism associated with excessive fibrocartilage formation is a hallmark feature of tendinopathy, which frequently colocalizes to regions of tendon impingement[3]. Accordingly, tendon impingement is clinically recognized as an extrinsic factor in the initiation and progression of tendinopathy. Nevertheless, mechanobiology underlying tendon impingement remains understudied, which obfuscates our understanding of tendinopathy pathogenesis and impedes the development of superior preventative and therapeutic modalities. Interestingly, prior studies have demonstrated that simple uniaxial compression applied to excised tendon explants not only stimulates aggrecan biosynthesis, but also regulates proteolytic turnover[2,4]. This is noteworthy considering altered proteoglycan catabolism has been described in tendon disease[5]. Despite these observations, aggrecan metabolism within complex and spatially heterogeneous patterns of mechanical strain generated by tendon impingement has yet to be characterized. In this regard, our lab has developed a novel murine hind limb explant model for studying mechanobiology secondary to impingement of the calcaneus upon the Achilles tendon insertion through passively applied ankle dorsiflexion while maintaining viability within the tendon over at least 7 days. By preserving anatomical structures of the impinged region *in situ*, this model allows for controlled prescription of mechanical impingement to reproduce multiaxial strain patterns and can be interfaced with ultrasound or multiphoton imaging to visualize and quantify tissue strain across length scales[1]. Previously, we have established significant change in GAG staining and altered collagen network organization indicative of fibrocartilage formation secondary to impingement within this model. Here, we seek to investigate the molecular basis of enhanced GAG staining elicited by impingement within this model, which we hypothesize is attributed to change in aggrecan metabolism. Moreover, we hypothesize that impingement drives deposition of aggrecan into the pericellular space.

METHODS: All animal studies were approved by the University Committee for Animal Resources. After euthanasia, all mice (C57BL/6, <1 year) were immediately dissected to isolate hind limb explants, removing the skin and plantaris tendon. **Explant Culture:** Hind limbs were pretreated in culture media (Dulbecco's Modified Eagle Media + 1% penicillin-streptomycin + 2 μM ascorbic acid) with 100 nM of dexamethasone[6] at 37°C for 48 hours. After 48 hours, dexamethasone was no longer supplemented in media. From each mouse, one hind limb explant was cultured for an additional 7 days in the absence of externally applied load, while the contralateral limb was positioned into our experimental platform to place the Achilles tendon insertion under static impingement (ankle dorsiflexion). **Immunofluorescence:** Limbs were embedded in paraffin and serial tissue sections were obtained at 10 μm thickness in sagittal orientation, tracking depth through the tendon. Level-matched sections from contralateral pairs of limbs (unloaded vs. static impingement, n = 5 pairs) were incubated with rabbit polyclonal anti-Aggrecan antibodies targeting either the G2 domain (Bioss, BS-11655R, 1:100) or the chondroitin sulfate domains (Chemicon®, AB1031, 1:100) followed by secondary antibodies conjugated with AlexaFluor 555, with DAPI counterstaining. Given constitutive aggrecanase activity and rapid aggrecan turnover within tendon[7], we expect the antibodies targeting the G2 domain to label many GAG-rich aggrecan fragments that are prevalent in fresh and culture tendon tissue. The other antibodies target an amino acid sequence spanning a high-affinity site of aggrecanase proteolysis early within the chondroitin sulfate 2 domain, and we expect these to label newly synthesized, non-degraded aggrecan within the pericellular space. Fluorescence microscopy images were analyzed to quantify average fluorescence intensity within spatial quadrants defining the compressive tendon fibrocartilage (CTF) at the tendon insertion (**Fig. 1**). Together, these quadrants separate the CTF from the adjacent attachment zone fibrocartilage (AZF) and extend 800 μm proximally along the deep tendon border. **Statistics:** Data within spatial quadrants were compared using repeated measures two-way ANOVA with Šídák's multiple comparison test. $p \leq 0.05$.

RESULTS: Immunofluorescent labeling of the aggrecan G2 domain indicated statistically significant differences in average fluorescence intensity data within quadrant 1 after 7 days of static impingement compared to contralateral unloaded tendons (**Fig. 1**). Immunofluorescent labeling of the chondroitin sulfate region prone to aggrecanase cleavage indicated statistically significant differences in average fluorescence intensity data within quadrants 2-4 after 7 days of static impingement compared to contralateral unloaded tendons (**Fig. 2**).

DISCUSSION: Spatial changes in immunofluorescent labeling of G2-containing aggrecan reflect changes in GAG staining previously quantified using Toluidine blue histology following 7 days of static impingement within this model, with significant differences in quadrant 1 where we have previously measured maximum transverse compressive strains, adjacent to the calcaneus. This supports our hypothesis that GAG enrichment generated by impingement within our model is provided by increased aggrecan deposition, particularly in regions of tendon that experience high magnitudes of compressive strain. Immunofluorescent staining of a region spanning the chondroitin sulfate 1 and 2 domains (CS1/2) that contains a high-affinity site of aggrecanase proteolysis appears to label newly synthesized, non-degraded aggrecan confined to the pericellular space, with significantly increased deposition within quadrants 2-4 secondary to 7 days of static impingement. This data may support the hypothesis that cells in these regions are not adapted to elevated magnitudes of compressive strains produced by exaggerated and sustained impingement within this model and respond by depositing aggrecan into their pericellular matrix, which could have implications for strain transfer to the cell. In the future, we plan to relate changes in aggrecan metabolism to changes in nanomechanical compressive properties within the pericellular and extracellular matrix with atomic force microscopy.

SIGNIFICANCE: The data presented here characterize spatial change in aggrecan metabolism secondary to Achilles tendon impingement, which will inform future efforts to identify potential targets for intervention in the treatment of impingement-associated tendinopathies.

REFERENCES: [1]Mora 2022 (J Biomech). [2]Robbins 1997 (Arch Biochem & Biophys). [3]Cook 2012 (Br J Sports Med). [4]Vogel 1996 (Conn Tissue Res). [5]Corps 2012 (Ann Rheum Dis). [6]Connizzo 2020 (J Ortho Res). [7]Samiric 2004 (Matrix Bio)

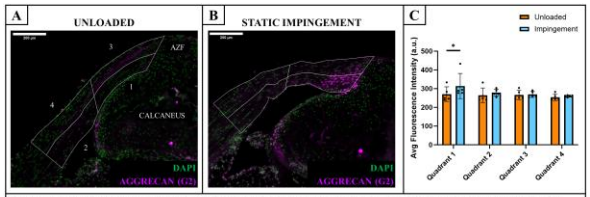

Figure 1: Aggrecan (G2 domain) immunofluorescence. Immunolabeled aggrecan appears magenta, DAPI labeled nuclei appear green. **(A)** Representative image of immunofluorescent labeling of aggrecan and aggrecan fragments retaining the G2 domain following 7 days of unloaded culture. For analysis, the compressive tendon fibrocartilage (CTF) at the impinged Achilles tendon insertion onto the calcaneus (labeled) is captured by 4 quadrants outlined in white (labeled 1-4), which delineate the CTF from the distal and adjacent attachment zone fibrocartilage (AZF). The region encompassed by these quadrants extends 800 μm proximally along the deep tendon border from its junction with the calcaneus. **(B)** Representative immunofluorescent staining of aggrecan and aggrecan fragments retaining the G2 domain following 7 days of static impingement. Enhanced staining of G2-containing aggrecan is appreciated most notably in quadrant 1. **(C)** Quantification of average fluorescence intensity within each quadrant. Impingement generated significant change in G2-containing aggrecan within quadrant 1 where we have previously quantified significantly elevated GAG staining and maximum transverse compressive strains. **Static impingement vs. unloaded: n = 5 pairs of explants. * $p \leq 0.05$.**

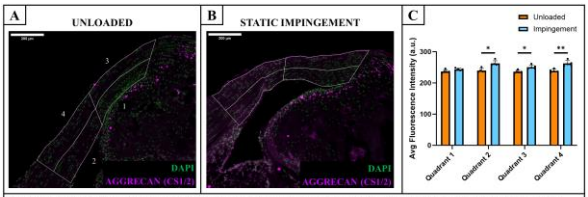

Figure 2: Aggrecan (chondroitin sulfate 1/2 domains) immunofluorescence. Immunolabeled aggrecan appears magenta, DAPI labeled nuclei appear green. **(A)** Representative image of immunofluorescent labeling of aggrecan that has not been cleaved at a high affinity site for aggrecanase proteolysis at the beginning of the chondroitin sulfate 2 domain following 7 days of unloaded culture. Quadrants utilized for analysis are outlined in white and labeled 1-4 (see **Fig. 1**). **(B)** Representative immunofluorescent staining of non-degraded aggrecan following 7 days of static impingement. Immunolabeled non-degraded aggrecan is primarily confined to the pericellular space and appears enhanced in proximal quadrants 2 and 4 most notably. **(C)** Quantification of average fluorescence intensity within each quadrant. Impingement generated significant change in non-degraded aggrecan within quadrants 2-4. **Static impingement vs. unloaded: n = 5 pairs of explants. * $p \leq 0.05$. ** $p \leq 0.01$.**