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In situ fibril stretch and sliding is location-dependent in mouse supraspinatus tendons

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ABSTRACT

Tendons are able to transmit high loads efficiently due to their finely optimized hierarchical collagen structure. Two mechanisms by which tendons respond to load are collagen fibril sliding and deformation (stretch). Although many studies have demonstrated that regional variations in tendon structure, composition, and organization contribute to the full tendon's mechanical response, the location-dependent response to loading at the fibril level has not been investigated. In addition, the instantaneous response of fibrils to loading, which is clinically relevant for repetitive stretch or fatigue injuries, has also not been studied. Therefore, the purpose of this study was to quantify the instantaneous response of collagen fibrils throughout a mechanical loading protocol, both in the insertion site and in the midsubstance of the mouse supraspinatus tendon. Utilizing a novel atomic force microscopy-based imaging technique, tendons at various strain levels were directly visualized and analyzed for changes in fibril d-period with increasing tendon strain. At the insertion site, d-period significantly increased from 0% to 1% tendon strain, increased again from 3% to 5% strain, and decreased after 5% strain. At the midsubstance, d-period increased from 0% to 1% strain and then decreased after 7% strain. In addition, fibril d-period heterogeneity (fibril sliding) was present, primarily at 3% strain with a large majority occurring in the tendon midsubstance. This study builds upon previous work by adding information on the instantaneous and regional-dependent fibrillar response to mechanical loading and presents data proposing that collagen fibril sliding and stretch are directly related to tissue organization and function.

1. Introduction

Tendon's primary function is to transmit mechanical load and displacement from muscle to bone (Lichtwark and Barclay, 2010). It is able to perform this function due to its finely tuned hierarchical structure, composed of collagen fibrils organized into fibers or fascicles and further bundled to form tendon proper (Kastelic et al., 1978). Macroscopic structure-function studies of tendon have shown that mechanical changes occurring at lower scale levels are likely responsible for the complex non-linear and viscoelastic response of full tendon (Derwin and Soslowsky, 1999; Fessel and Snedeker, 2009; Lake et al., 2009; Lake et al., 2010; Rigozzi et al., 2009; Robinson et al., 2004). Recent evidence suggests that tendons are able to withstand high forces by employing a number of unique mechanisms occurring at many of the fibril and fiber length scales, including uncrimping, re-alignment, sliding, and deformation or stretch (Connizzo et al., 2013b; Gupta et al., 2010; Miller et al., 2012b; Screen et al., 2013). Although collagen fiber uncrimping and re-alignment have been studied extensively in recent literature (Connizzo et al., 2013a; Connizzo et al., 2013b; Miller et al., 2012a; Miller et al., 2012b; Miller et al., 2012c; Miller et al., 2012d), the quantification of collagen fiber and fibril sliding and stretch has been studied less due to the experimental difficulties, particularly the inability to visualize individual collagen fibrils in vivo during mechanical loading.

Mechanical properties of single collagen fibrils have recently been investigated using several different technologies (Eppell et al., 2006; Graham et al., 2004; Tang et al., 2010; Yang et al., 2007). Although these studies substantially improved our understanding of mechanics of individual collagen fibrils, they do not replicate the in vivo environment of collagen, where collagen fibrils are interacting with other collagen fibrils and with the surrounding extracellular matrix proteins. Furthermore, it has been reported that fiber-level elongation cannot be solely attributed to the deformation of the individual collagen fibrils, suggesting fibril-fibril and fibril-matrix interactions are likely responsible for this discrepancy (Fratzl et al., 1998; Puxkandl et al., 2002). Recent investigations utilizing atomic force microscopy have successfully measured d-period length changes as a quantitative

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measure of collagen fibril stretch in situ (Li et al., 2013; Rigozzi et al., 2011). This work introduces a significant advancement in the literature, allowing for the ability to study fibril stretch under various types of mechanical loading as well as with cases of altered structure, such as disease, aging, or injury (Li et al., 2013). However, these studies have primarily investigated fiber sliding during or following stress relaxation or creep events (Gupta et al., 2010; Li et al., 2013; Rigozzi et al., 2011; Screen et al., 2013). The strain rate dependence of tendon identifies that the timing and rate of loading, in addition to the magnitude, is extremely important to tendon's response. Furthermore, since tendons have been known to rupture clinically due to a single traumatic event or impact (Moller et al., 1996), the instantaneous response to load, as well as the ability to repetitively undergo that impact stress, is critical to the overall function and has not been investigated.

In addition, fibril sliding and deformation have primarily been studied during the linear region of the mechanical test. Due to the prevalence of collagen uncrimping and re-alignment during the initial toe region (Miller et al., 2012b; Miller et al., 2012c), it is likely that these other fibrillar responses are also occurring. Finally, while many studies have demonstrated that the specific transition in composition, structure and collagen organization from the midsubstance to the insertion site contributes significantly to the full tendon's mechanical response (Lake et al., 2009; Lake et al., 2010; Shaw and Benjamin, 2007), the location-dependent response to mechanical load at the fibril level has not been studied. Therefore, the purpose of this study was to quantify the instantaneous response of collagen fibrils throughout a mechanical loading protocol both in the insertion site and midsubstance of the mouse supraspinatus tendon. We hypothesized that more fibril stretch will occur at the insertion site than the midsubstance (higher strains) and that more fibril sliding will occur at the midsubstance than at the insertion site.

2. Methods

2.1. Sample preparation

Fifteen C57BL/6 mice at 150 days of age were used in this study (IACUC approved). Supraspinatus tendons from both shoulders of each mouse were used for this study, but no two tendons from the same animal were used in the same testing group to ensure independence of samples. All soft tissues were removed from around the tendon, leaving the supraspinatus tendon attached to the humerus. Tendon cross-sectional area was then measured using a custom laser-based device (Peltz et al., 2009). The humerus was then embedded in an acrylic tube with PMMA. A second coating of PMMA was applied to prevent failure at the growth plate. The proximal end of the tendon was glued between two pieces of sandpaper with an initial gauge length of 2.5 mm and both the tendon and the acrylic pot were placed in custom grips for tensile testing (Fig. 1A,B), as described previously (Connizzo et al., 2013a).

All samples were kept hydrated using phosphate buffered saline (PBS) and were then loaded in a tensile testing system (Instron, Norwood, MA) for mechanical testing. A 10 N load cell was used for all tests with a resolution of 0.01 N. All tendons underwent a preload to 0.02 N and ten cycles of preconditioning from 0.02 N to 0.04 N followed by a 60 second hold before the ramp to failure. Tendons were then divided into six groups and stretched to a randomly assigned grip-to-grip strain value (0, 1, 3, 5, 7, or 10%) at a rate of 0.1% strain per second. Tendons were then frozen using freezing spray (McMaster-Carr Electrical Cleaning and Maintenance Aerosol, Product #7437K43), removed from the mechanical testing setup, and placed in a specimen dish with tissue freezing medium. The sample was kept frozen during this process using freezing spray and the dish was then submerged in liquid nitrogen to complete the flash-freezing phase. Tendons were then stored at −20 °C until they were sectioned in a cryostat microtome in the coronal anatomical plane at 20 μm and sections were then again kept frozen at −20 °C until further processing. Frozen sections were then immersed in cold 10% neutral buffered formalin for four minutes for fixation and allowed to dry prior to imaging.

2.2. Atomic force microscopy (AFM)

Tendons were imaged in air using Peak Force Quantitative Nanomechanical Mapping mode using a Dimension Icon AFM (BrukerNano, Santa Barbara, CA). Imaging of 2 μm × 2 μm regions was performed using Bruker ScanAsyst Fluid+ probes (nominal spring constant $k \approx 0.70$ N/m, radius $R \approx 2$ nm). Tendons were scanned at 2-3 regions across the width of the insertion site and midsubstance of the tendon. The insertion and midsubstance locations were determined consistently by taking images within the bottom quarter (about 0.5 mm) of the specimen for the insertion site and the top quarter of the specimen for the midsubstance region (a single sample region shown in Fig. 1C). Scans were also taken from 4–6 sections throughout the depth of the tendon.

2.3. Data analysis

Several parameter maps were produced from imaging, including Height, Peak Force, Peak Force Error, Modulus, LogModulus, Dissipation, and Adhesion. Analysis on approximately two tendons (~20 images) showed no difference between the different parameter maps for measurement of d-period. Due to the strength of contrast from the LogModulus and Adhesion maps, these two maps were used to analyze all specimens. Custom MATLAB software (MathWorks, Natick, MA) was written to allow for the measurement of d-period length for many fibrils in a single image and for the ability to enhance the processing technique in the future. Images were first contrast enhanced by equalizing the histogram of intensities locally using a default MATLAB function (adapthisteq) with the default parameters. A line

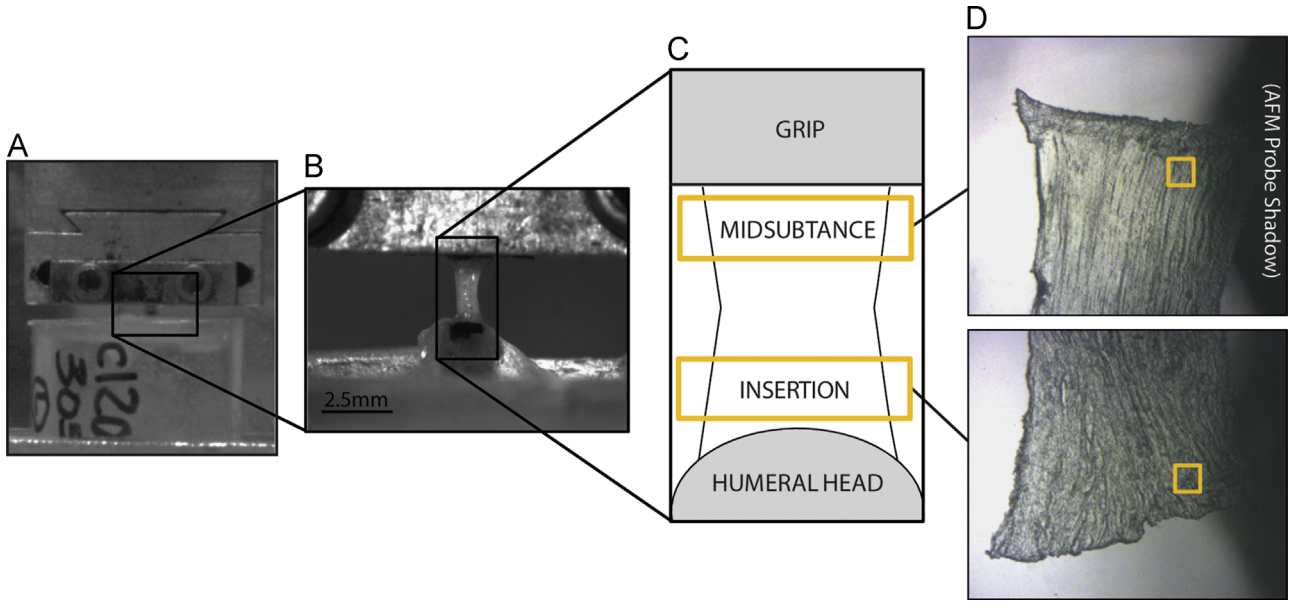

Fig. 1. (A) Testing image of supraspinatus tendon prepared for mechanical testing. (B) Zoomed-in view of supraspinatus tendon. (C) Diagram depicting insertion site and midsubstance regions for d-period analysis. (D) Microscope images depicting typical scan regions for the midsubstance (top image) and insertion site (bottom images).