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Characterizing Local Collagen Fiber Re-Alignment and Crimp Behavior Throughout Mechanical Testing in a Mature Mouse Supraspinatus Tendon Model

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Abstract

Background—Collagen fiber re-alignment and uncrimping are two postulated mechanisms of tendon structural response to load. Recent studies have examined structural changes in response to mechanical testing in a postnatal development mouse supraspinatus tendon model (SST), however, those changes in the mature mouse have not been characterized. The objective of this study was to characterize collagen fiber realignment and crimp behavior throughout mechanical testing in a mature mouse SST.

Method of Approach—A tensile mechanical testing set-up integrated with a polarized light system was utilized for alignment and mechanical analysis. Local collagen fiber crimp frequency was quantified immediately following the designated loading protocol using a traditional tensile set up and a flash-freezing method. The effect of number of preconditioning cycles on collagen fiber re-alignment, crimp frequency and mechanical properties in midsubstance and insertion site locations were examined.

Results—Decreases in collagen fiber crimp frequency were identified at the toe-region of the mechanical test at both locations. The insertion site re-aligned throughout the entire test, while the midsubstance re-aligned during preconditioning and the test's linear-region. The insertion site demonstrated a more disorganized collagen fiber distribution, lower mechanical properties and a higher cross-sectional area compared to the midsubstance location.

Conclusions—Local collagen fiber re-alignment, crimp behavior and mechanical properties were characterized in a mature mouse SST model. The insertion site and midsubstance respond differently to mechanical load and have different mechanisms of structural response. Additionally, results support that collagen fiber crimp is a physiologic phenomenon that may explain the mechanical test toe-region.

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Disclosure Statement

The authors have no conflicts of interest and nothing to disclose.

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Keywords

Collagen fiber crimp; fiber alignment; supraspinatus tendon; inhomogeneous; preconditioning

1 Introduction

Collagen fiber re-alignment and uncrimping are two postulated mechanisms of tendon structural response to load. Crimp morphology is thought to be related to tendon mechanical behavior (Rigby, 1964; Rigby et al., 1959; Viidik, 1972; Woo et al., 2000). In previous work, a technique to quantify fiber crimp frequency at specific, quantifiable points throughout a mechanical test was presented and a decrease in crimp frequency was identified during the toe-region of the mechanical test in a developmental mouse supraspinatus tendon model at 4, 10, and 28 days old (SST) (Miller, 2012a). Similarly, a study examining collagen fiber re-alignment throughout postnatal development suggested that where fiber re-alignment occurs during mechanical testing may depend on tendon age and matrix maturity (Miller et al., 2012). Local differences in crimp behavior and collagen fiber re-alignment as well as collagen organization and mechanical properties have been identified throughout postnatal development in the mouse SST (Miller, 2012a; Miller et al., 2012). However, collagen fiber crimp, collagen fiber re-alignment and mechanical properties have not been characterized in a mature mouse SST model. While the rat SST is a well-established model for examining clinical problems in the shoulder, the mouse model has advantages including a wide range of genetically modified strains and an established developmental model to further elucidate structure-function relationships.

Recently, a correlation between collagen fiber re-alignment and preconditioning has been identified (Miller, 2012b; Quinn and Winkelstein). Additionally, at late development, comparisons in crimp frequency between the preload and after preconditioning changed in response to increasing the number of preconditioning cycles, indicating that the number of preconditioning cycles applied may affect tendon structural response to load (Miller, 2012a). However, the effect of increasing the number of preconditioning cycles has not yet been examined in the mature SST. Further, it is not known how the residual stress of the tendon from the in vivo state to the excised test configuration affects local crimp frequency. Therefore, the objective of this study was to determine 1) where collagen fiber re-alignment and uncrimping occur throughout the mechanical test; 2) if collagen fiber re-alignment and crimp behavior are affected by increasing number of preconditioning cycles; and 3) if crimp behavior, re-alignment or mechanical properties vary by location in a mature mouse SST model. We hypothesize that 1) the largest shift in re-alignment will occur during preconditioning, but realignment will also occur in toe- and linear-regions with uncrimping occurring primarily in toe-region; 2) crimp frequency will decrease with increasing number of preconditioning cycles; and 3) the insertion site will demonstrate a more disorganized collagen distribution, lower mechanical properties and higher crimp frequency compared to midsubstance location.

2 Methods

2.1 Sample Preparation

This study was approved by the University of Pennsylvania IACUC. Postnatal mice in a C57/BL/6 background (Jackson Laboratory) were bred in house. All litters were reduced to 6 pups within 1 day of birth to reduce variance from litter size (Festing, 2006). Pups were weaned 21 days after birth and separated by sex. A sample was defined as a collective litter of 6 female pups from the same breeder pair. Ten tendons were used to examine structural changes throughout testing within a sample including 3 tendons for cross polarizer testing

where three different mechanical testing preconditioning protocols (5, 10, 20 cycles) were randomly assigned and 7 tendons for crimp analysis ($N=9$ for each of the 10 tests). SSTs were removed under a stereomicroscope from postnatal mice at 90 days old. Excess tissue was removed with the tendon still attached to the humeral head, which was trimmed to a small bone chip. Tendon cross-sectional area was measured using a custom built laser-based device (Favata, 2006). Verhoeff's stain was used to mark the tendon insertion site and midsubstance locations with a 2.5 mm gauge length. Both ends of the tendon were secured with cyanoacrylate adhesive between two pieces of sandpaper. Tendons were secured in the grips and loaded into a tensile testing system (Instron, Norwood, MA).

2.2 Reference Configuration Dissection Protocol

One tendon from each breeder pair was prepared to serve as a reference configuration for the mechanical testing points. These samples were flash-frozen while still attached to the muscle and humeral head to provide an "in situ" reference configuration for comparative analysis. After the joint was exposed, the shoulder was externally rotated and secured with tape to maintain position throughout the dissection. Trapezius and deltoid muscle were removed to expose the joint. The acromioclavicular ligament was cut and the clavicle was peeled back to expose the SST and subscapularis tendons. The infraspinatus and subscapularis tendons were sharply detached at their insertion sites. The entire remaining shoulder joint was thoroughly sprayed with flash-freezing spray (Decon Laboratories, King of Prussia, PA). With the joint frozen, the humerus-tendon-muscle complex was sharply detached from the body. The joint was quickly positioned in a freezing tray, embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and flash frozen with liquid nitrogen. Samples were cut into 8 μm sections and stained with Hematoxylin and Picrosirius Red. In graphical representation, this point is referred to as the "in situ" or IS point.

2.3 Mechanical Testing

A 10 N load cell was used for all tests and the following protocol was used: preloaded to a nominal load of 0.02N, followed by either 5, 10 or 20 cycles of preconditioning from 0.02–0.05N at a rate of 0.1%/second, followed by a 60 second hold. Finally, a ramp-to-failure was applied at a rate of 0.1%/second. As described previously, a structural fiber recruitment model was used to determine strains to represent the toe- and linear-region for alignment and crimp analysis (Miller, 2012a; Miller et al., 2012; Peltz et al.). The transition region was modeled at 50% fiber recruitment and a point in the linear-region was modeled at 75% fiber recruitment.

2.3.1 Crimp Testing—To analyze crimp behavior, samples were sprayed with phosphate buffered saline throughout the testing process to maintain constant hydration. Collagen fiber crimp was assessed at 6 different points throughout the mechanical test: preload, after 5, 10, and 20 cycles of preconditioning and at the toe- and linear-regions. Tendons were snap-frozen while mounted in the testing device immediately following the designated tensile loading protocol to obtain a "snapshot" of crimp at the desired testing point (Miller, 2012a; Thornton et al., 2002). Samples were sharply detached at the insertion site and top grip and immediately placed in tissue freezing medium. Samples were cut into 8 μm sections and stained with Hematoxylin and Picrosirius Red.

2.3.2 Cross Polarizer Testing—For alignment and mechanical analysis, samples were placed in a room temperature phosphate buffered saline bath and loaded into a tensile testing system integrated with a polarized light setup, consisting of a linear backlight (Dolan-Jenner, Boxborough, MA), 90°-offset rotating polarizer sheets (Edmund Optics, Barrington, NJ) on either side of the sample, and a digital camera (Basler, Exton, PA) (Lake et al., 2009). As described previously, sets of 13 images were acquired every 20 seconds as the

polarizers rotated through a 125° range for measurement of fiber alignment during loading (Lake et al., 2009; Miller, 2012b; Miller et al., 2012). Images were acquired every 5 seconds for optical strain analysis as described previously (Derwin et al., 1994).

2.4 Data Analysis

To determine crimp frequency, 2 slides were imaged with a polarized light microscope for each sample and analyzed at the insertion site and midsubstance locations using custom software (Matlab, Natick, MA) (Miller, 2012a). For each location analyzed, the results from the 2 slides were averaged and crimp frequency is reported. Fiber alignment was calculated from image sets as previously described (Lake et al., 2009; Miller et al., 2012). Circular variance (VAR) is a measure of the spread of the distribution of collagen fiber alignment, where a higher value of VAR indicates a more disorganized collagen fiber distribution (Lake et al., 2009; Miller, 2012b; Miller et al., 2012; Zar, 1999). VAR was calculated for fiber distributions before and after preconditioning (5, 10, 20 cycles), at the transition region, and in the linear-region. Fiber re-alignment during preconditioning was evaluated by comparing VAR values before preconditioning to after preconditioning for each preconditioning protocol. Similar methods were used to determine fiber re-alignment during the toe- and linear-regions of the stress-strain curve.

2.5 Statistical Analysis

Imputation by predictive mean matching was performed to compare changes in crimp frequency throughout the mechanical testing protocol for each litter (Landerman et al., 1997; Little, 1988; Yuan, 2011). A 2-way repeated-measure ANOVA was used (region of test and location). Post-hoc t-tests were used to evaluate changes in crimp frequency, mechanical properties and cross-sectional area. Bonferroni corrections for multiple statistical comparisons were made for each hypothesis and data is presented as mean $\pm$ standard deviation. Shapiro-Wilk tests indicated that VAR values were non-normally distributed so nonparametric tests were used. VAR data was analyzed as paired comparisons and presented as median $\pm$ interquartile range. Changes in VAR were compared for tendon location and throughout the mechanical test. Bonferroni corrections were again applied for multiple comparisons.

3 Results

3.1 Crimp

The 2-way ANOVA identified that the effect of time (throughout the mechanical test) was significant, while location (insertions versus midsubstance) was not found to be significant. No significant interactions were identified. Significant decreases in crimp frequency were identified at the toe-region with all preconditioning protocols at both locations (Fig. 1). No significant changes in crimp frequency were identified at additional points throughout the mechanical test or with increasing number of preconditioning cycles. However, histology and average values of crimp frequency throughout the test demonstrate a mild decrease in crimp frequency with increasing number of preconditioning cycles at the midsubstance location (Fig. 2).

3.2 Alignment and Mechanics

No differences in collagen fiber re-alignment or mechanics were found between protocols with varying number of cycles. Therefore, to increase power, data was pooled across the protocols for the alignment and mechanical analysis.

At the insertion site, collagen fiber re-alignment occurred throughout the entire mechanical test (during preconditioning, toe- and linear-regions) (Fig. 3). At the midsubstance location,

collagen fiber re-alignment occurred during preconditioning and the linear-region of the mechanical test (Fig. 3). Locally, the insertion site was more disorganized than the tendon midsubstance throughout the entire mechanical test (Fig. 4). The insertion site cross-sectional area (0.12 ± 0.04) was larger than the midsubstance location (0.08 ± 0.03). Both toe- and linear-region moduli were larger at the midsubstance location compared to the insertion site (Fig. 5).

4 Discussion

This study quantified local fiber re-alignment and crimp behavior in a mature mouse SST model. Uncrimping of collagen fibers was confined to the toe-region of the mechanical test at both the insertion site and midsubstance locations supporting previous work in the developmental mouse SST (Fig. 1) (Miller, 2012a). Additionally, this result provides further support for the theory that the uncrimping of collagen fibers may explain the toe-region and contribute to the nonlinear behavior of tendons (Houssen et al.; Miller, 2012a; Rigby, 1964; Rigby et al., 1959; Screen et al., 2004; Viidik, 1972; Woo et al., 2000).

As expected, the insertion site re-aligned throughout the entire mechanical test (Fig. 3). Surprisingly, the midsubstance location did not re-align during the toe-region (Fig. 3). The insertion site is thought to experience more complex, multi-axial loads in vivo, resulting in a more disorganized collagen fiber distribution (Lake et al., 2009; Miller, 2012b; Miller et al., 2012; Thomopoulos et al., 2003), which may require additional collagen fiber re-alignment at the insertion site. Therefore, the midsubstance location may “pause” recruiting fibers following preconditioning to allow collagen fibers to uncrimp during the toe-region, thus giving the insertion site additional time to recruit fibers before transitioning into the linear-region. Alternatively, fibers initially recruited in the midsubstance during preconditioning may already be failing by the end of the toe-region as crimp histology identified regions of fiber damage at the transition point in some samples. Assuming these fibers were initially aligned in the direction of load or required a minimal amount of re-orientation, the fibers would be tensioned almost immediately and may fail earlier in the ramp-to-failure. Their early failure would require the recruitment of additional fibers in the linear-region at the midsubstance location. Further, at both locations, collagen fiber re-alignment occurred during preconditioning, supporting previous studies which identified a correlation between preconditioning and collagen fiber alignment (Miller, 2012b; Miller et al., 2012; Quinn and Winkelstein).

In addition, this study found no significant differences in crimp frequencies at the preload compared to points after preconditioning (5, 10 or 20 cycles). This suggests that the uncrimping of collagen fibers occurs primarily in the toe-region, regardless of the number of preconditioning cycles. However, histology and the reported average values of crimp frequency across all litters indicate that, while not significant, decreases in crimp frequency with increasing number of preconditioning cycles may be present at the midsubstance location in mature tendon (Fig. 2). This is supported by previous observations noting similar behavior and merits further study (Houssen et al.; Miller, 2012a).

As expected, a more disorganized collagen fiber distribution and lower moduli values were identified at the insertion site compared to the midsubstance location (Figs. 4 & 5). The local differences in organization and modulus support results in the human and mature rat SST (Lake et al., 2009; Miller, 2012b). Interestingly, unlike the mature rat SST (Miller, 2012b), the midsubstance and insertion site in the mouse SST demonstrated different re-alignment behaviors (Fig. 3). This may suggest that the locations experience different loading conditions in vivo, which may initiate local remodeling, resulting in different fiber network configurations including alterations in collagen fiber alignment as well as cross-links, fiber-

fiber and fiber-matrix interactions. These potential changes in collagen microstructure between locations may affect their ability to respond to load simultaneously or in the same manner.

While significant changes in alignment and mechanics were identified, no changes in crimp frequency were found with location, supporting previous results at 4, 10 and 28 days old (Miller, 2012a). This suggests that crimp may not be affected by multi-axial loads seen at the insertion site and crimp may be a mechanism of tensile loading instead of compressive or multi-axial loading. The magnitude of crimp response was decreased at insertion compared to midsubstance, supporting previous results at 28 days old (Miller, 2012a). Previous speculation that fiber re-alignment may be a more dominant or earlier mechanism at the insertion site than crimp is supported here (Miller, 2012a; Sellaro et al., 2007). It is possible that fibers at the midsubstance have finished their initial collagen fiber re-alignment and begin to uncrimp with increasing cycles of preconditioning. In this study, the entire tendon was flash frozen at 5 and 8% strain to represent points in the toe- and linear-regions (determined by the structural model (Peltz et al.)). While these are appropriate strains to represent each of the corresponding regions, it is possible that the insertion site and midsubstance locations are at different stages of the toe-region, which may provide explanation for these differences in structural behavior.

No significant differences were identified between crimp frequency at the reference configuration, or “in situ” condition, and after the preload. The lack of significant difference identified here indicates that the crimp frequency behavior demonstrated throughout the mechanical test may be applicable to the crimp behavior in vivo.

This study suggests that the uncrimping of collagen fibers may be an in vivo structural response to mechanical load and not solely an artifact of ex vivo mechanical testing and changing the residual stress experienced by the tendon. This study is not without limitations. First, the structural fiber recruitment model was utilized to determine average strain values for the entire tendon samples. While the average values are within the parameters to represent the toe- and linear-regions of both the insertion site and midsubstance locations, it is possible that the locations were at different “points” in the toe-region. However, the chosen method improves consistency within this experiment and allows for comparisons with other experiments, in addition to permitting conclusions to be drawn across collagen fiber alignment and crimp measurements. This study examined collagen fiber re-alignment and crimp behavior throughout only one mechanical testing protocol. Future studies are necessary to determine the effects of collagen fiber re-alignment and crimp behavior in response to different loading protocols and at additional strains in the toe- and linear-regions at both locations. Additionally, while sample were immediately flash-frozen following loading, we cannot guarantee that no stress relaxation occurs whatsoever while the sample was freezing. However, consistent, significant changes in crimp frequency were still identified across samples. Further, histology from this project was stained in multiple batches, though the analysis methods performed were consistent and repeatable. As previously described, the quantitative software normalizes each image by the average pixel intensity and establishes exclusion criteria based on the standard deviation of pixel fluctuations to exclude artifact and only include fluctuations representing fiber crimp (Miller, 2012a). This method minimizes the potential effect of changes in stain intensity on the histological analyses. Finally, while the reference configuration samples were flash-frozen while still attached to the humerus and supraspinatus muscle, adjacent structures, such as the deltoid, the infraspinatus and subscapularis, were removed. The removal of these structures may have changed the supraspinatus tendon loading and subsequently, the in vivo crimp frequency. Additionally, the tone of the supraspinatus muscle may have changed post-sacrifice and altered the crimp frequency. Future studies are needed to further examine

crimp frequency and fiber distribution of the supraspinatus tendon at its native state in the body.

In conclusion, this study quantified local mechanical properties as well as collagen fiber realignment and crimp behavior throughout mechanical testing in a mature mouse SST model. Additionally, this study identified that collagen fiber realignment and crimp behavior were different at each location suggesting that the insertion site and midsubstance locations may respond to load differently and may have different microstructural and biochemical compositions. Future studies are needed to further characterize local compositional and microstructural properties to examine these differences in behavior and elucidate their potential effects on clinical treatments and diagnosis.

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Fig. 1. Crimp throughout the mechanical test at insertion site and midsubstance locations demonstrates that collagen fiber crimp decreased at the toe-region with all preconditioning protocols. (*= $p < 0.016$, **= $p < 0.001$, ***= $p < 0.0001$).

Fig. 2. Histology demonstrates that while collagen fiber uncrimping occurs primarily in the toe-region, a mild decrease in crimp frequency is also identified with increasing number of preconditioning cycles.

90 Day Re-Alignment

Fig. 3. Circular variance (VAR) for a representative sample at the insertion site and midsubstance locations shows significant collagen fiber re-alignment throughout the entire test at the insertion site and during preconditioning and the linear-region at the midsubstance. A larger value of circular variance indicates a more disorganized collagen fiber distribution.

Fig. 4.

Circular variance values throughout the test demonstrate that the insertion site location is more disorganized throughout the entire mechanical test compared to the midsubstance location. (BP: Before preconditioning; AP: After preconditioning; Trans: Point at the transition strain; Linear: Point in the linear-region). (***= $p < 0.0001$).

Fig. 5. A lower linear modulus was present at the insertion site compared to the midsubstance location. (***)= $p < 0.0001$.