Journal of Structural Biology 176 (2011) 9–15

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Mechanical response of individual collagen fibrils in loaded tendon as measured by atomic force microscopy

S. Rigozzi [a], A. Stemmer [b], R. Müller [a], J.G. Snedeker [a,c,*]

[a] Institute for Biomechanics, ETH Zurich, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland
[b] Nanotechnology Group, ETH Zurich, Tannenstrasse 3, 8092 Zurich, Switzerland
[c] Orthopedic Research Laboratory, University of Zurich, Balgrist, Forchstrasse 340, 8008 Zurich, Switzerland

ARTICLE INFO

Article history:
Received 14 February 2011
Received in revised form 4 July 2011
Accepted 5 July 2011
Available online 13 July 2011

Keywords:
Collagen fibril
Atomic force microscope
Collagen network
Tendon

ABSTRACT

A precise analysis of the mechanical response of collagen fibrils in tendon tissue is critical to understanding the ultrastructural mechanisms that underlie collagen fibril interactions (load transfer), and ultimately tendon structure–function. This study reports a novel experimental approach combining macroscopic mechanical loading of tendon with a morphometric ultrascale assessment of longitudinal and cross-sectional collagen fibril deformations. An atomic force microscope was used to characterize diameters and periodic banding (D-period) of individual type-I collagen fibrils within murine Achilles tendons that were loaded to 0%, 5%, or 10% macroscopic nominal strain, respectively. D-period banding of the collagen fibrils increased with increasing tendon strain (2.1% increase at 10% applied tendon strain, $p < 0.05$), while fibril diameter decreased (8% reduction, $p < 0.05$). No statistically significant differences between 0% and 5% applied strain were observed, indicating that the onset of fibril (D-period) straining lagged macroscopically applied tendon strains by at least 5%. This confirms previous reports of delayed onset of collagen fibril stretching and the role of collagen fibril kinematics in supporting physiological tendon loads. Fibril strains within the tissue were relatively tightly distributed in unloaded and highly strained tendons, but were more broadly distributed at 5% applied strain, indicating progressive recruitment of collagen fibrils. Using these techniques we also confirmed that collagen fibrils thin appreciably at higher levels of macroscopic tendon strain. Finally, in contrast to prevalent tendon structure–function concepts data revealed that loading of the collagen network is fairly homogenous, with no apparent predisposition for loading of collagen fibrils according to their diameter.

1. Introduction

The mechanical properties of tendon must be optimized to avoid injury and efficiently transfer load from muscle to bone (Lichtwark and Barclay, 2010). Tendon is hierarchically structured (Fig. 1), being mainly composed of collagen fibrils that are organized into functional units (fibers, or fascicles) which are further organized according to the specific functional demands of the tendon itself (Kastelic et al., 1978). As the primary load-bearing protein in tendon, the nature of collagen load distribution has been widely investigated at multiple size scales (Gupta et al., 2010; Ottani et al., 2002; Sasaki and Odajima, 1996; Screen et al., 2004). Particular attention has been directed toward understanding the behavior of the collagen fibrils themselves (Puxkandl et al., 2002) as well as how the bundled collagen fibrils collectively respond to applied macroscopic loads (Fratzl et al., 1998).

At the macroscopic scale, the most common structure–function analyses of tendon have been performed using tensile test to failure of single tendon fibers (fascicles) extracted from rat tail tendon (Derwin and Soslowsky, 1999; Fessel and Snedeker, 2009). More complex anatomical structures composed of intertwined fibers have also been characterized (Rigozzi et al., 2009; Soslowsky et al., 1999) and modeled (Kahn et al., 2010; Wren and Carter, 1998) in efforts to understand the interaction between these structures under load. It has been reported that only 40% of macroscopic (fiber level) tendon elongation can be attributed to straining of individual collagen fibrils, and the majority of tendon strain would thus appear to be due to inter-fibril movements, such as the progressive alignment of the collagen fibrils in the direction of applied load (Fratzl et al., 1998; Gupta et al., 2010; Puxkandl et al., 2002). Although mechanical properties of single fibrils have been investigated by one-dimensional tensile testing using optical tweezers (Wang et al., 2005) and atomic force microscopy (AFM) (Graham et al., 2004; Yang et al., 2007), the nature by which collagen fibrils interact within their network to accommodate applied tendon strains remains largely unknown (Gupta et al., 2010). Part of this uncertainty lay in the

* Corresponding author at: University of Zurich, Balgrist, Forchstrasse 340, CH-8008 Zurich, Switzerland. Fax: +41 44 386 1109.
E-mail address: jsnedeker@research.balgrist.ch (J.G. Snedeker).

1047-8477/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2011.07.002

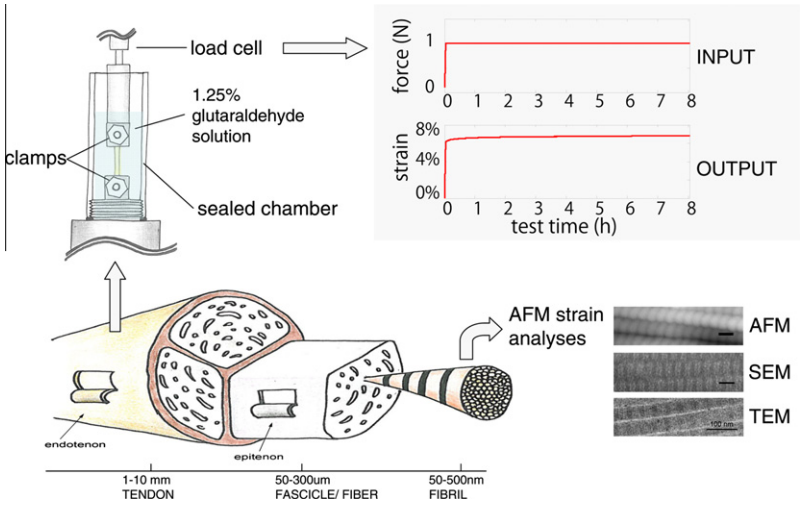

Fig.1. Experimental setup for fixing the strained tendon at a given load. The tendon is clamped to a universal tensile test machine in a chamber that is filled with 1.25% glutaraldehyde solution, while the tendon is under constant load for 8 h. The applied constant load (input) and the tendon strain response (output) are recorded. The fixed tendon is sliced and the fibril strain is measured with an AFM. The images show the D-period observed with AFM, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

limited ability of current methods to discern in situ strains in individual collagen fibrils.

Macroscopic tendon extension is enabled by straining and sliding mechanisms that simultaneously occur at different length scales. At the tissue level this is enabled by straining of and sliding between collagen fibers (Fig. 1). Within the fibers, ultra-scale straining and sliding of collagen fibrils also occurs, and this is partly regulated by the proteoglycan rich, non-collagenous matrix (Fessel and Snedeker, 2010; Rigozzi et al., 2009; Screen et al., 2005). The relative straining associated with collagen molecule stretch can be monitored by assessing D-period length changes (Gupta et al., 2010; Puxkandl et al., 2002).

Attempts to directly relate tendon ultrascale structure to tissue-scale function have employed various experimental approaches including optical and scanning electron microscopy of sectioned tendon tissue (Franchi et al., 2010; Kukreti and Belkoff, 2000; Okuda et al., 2009), AFM on isolated fibrils (Graham et al., 2004; Habelitz et al., 2002), or scattering X-ray spectroscopy on tendon fibers (Fratzl et al., 1998; Gupta et al., 2010; Misof et al., 1997; Puxkandl et al., 2002). Scanning electron microscopy studies of tendon sections allow excellent visualization of the collagen structures, but quantitative measures of collagen fibril dimensions (and eventually mechanical collagen strains) is limited by perspective distortion. Transmission electron microscopy (TEM) studies are better suited for morphometric analysis, but due to difficulties in aligning the sectional plane with the longitudinal axis of collagen fibrils, have been used almost exclusively for morphometric analysis of collagen fibril cross-section (Morgan et al., 2006; Rigozzi et al., 2010). X-ray spectroscopy has been successfully used to infer collagen fibril strain in loaded tendon fibers (Fratzl et al., 1998; Gupta et al., 2010; Puxkandl et al., 2002), but visualization of individual fibrils is not possible, precluding direct investigation of collagen network deformation. Finally, while AFM can provide very precise morphometric quantification of single fibril collagen topology and mechanics (Graham et al., 2004; Habelitz et al., 2002), AFM has not yet been applied in studying collagen fibril strains in loaded tendon. Thus no study has yet implemented a method to accurately quantify the ultrascale mechanical response of individual collagen fibrils embedded within a loaded tendon.

The motivation for this study was therefore to introduce a metrically accurate AFM visualization approach to characterize mechanical strain response of individual collagen fibrils within the fibril network comprised by a tendon fiber. We specifically hypothesized that such an approach might elucidate the reported lag between collagen fibril strain and applied tendon load by providing a more direct visualization of fibril load distribution within the network. To investigate this hypothesis, we measured collagen fibril strains in populations of individual fibrils within a loaded tendon, relying on D-period banding distance as a proxy measure of collagen fibril strain.

2. Materials and methods

2.1. Sample preparation

All animal experiments were reviewed and approved by local and state authorities (Kantonales Veterinäramt Zürich, Zürich, Switzerland). Achilles tendons were harvested from 19-week-old female C57BL/6 inbred (wild type) mice. These inbred mice were selected as an experimental model for their strong genomic homology to humans (Copeland et al., 1993; Erickson, 1989; Nadeau and Taylor, 1984), for their low genetic variability, and for their potential use as a genetic background in future structure–function investigations using knock-out or transgenic mice. Each tendon was laid onto a piece of paper and mounted in a saline filled testing chamber designed to prevent sample dehydration. Mechanical tests were performed with a universal testing machine (Zwick 1456, Ulm, Germany). Using previously described methods (Rigozzi et al., 2010), five ($n = 5$) tendons were preconditioned with 10 cycles of 0–10% nominal strain (defined as incremental tendon elongation under load, normalized as a percentage of the original unloaded tendon length) before ramp loading to failure. From these curves the "linear" region of the tendon material response (Fig. 1) corresponding to reversible (non-damaging) deformation at the fibril level (Fratzl et al., 1998) was defined for subsequent investigations. Later tendon test groups ($n = 5$ each) were stretched to target strain levels in 5% increments covering this range (i.e. 0%, 5%, and 10% nominal strain). All tendons were fully hydrated in phosphate buffered saline (PBS) solution prior to chemical fixation with 1.25% glutaraldehyde solution (G5882, Sigma, St. Louis, MO) added to the test chamber just prior to application of mechanical loading to the target strain. Tendons were then maintained under constant load for 8 h until the structure was fixed (Liao and Vesely,