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A mechanistic study for strain rate sensitivity of rabbit patellar tendon

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ABSTRACT

The ultrastructural mechanism for strain rate sensitivity of collagenous tissue has not been well studied at the collagen fibril level. Our objective is to reveal the mechanistic contribution of tendon's key structural component to strain rate sensitivity. We have investigated the structure of the collagen fibril undergoing tension at different strain rates. Tendon fascicles were pulled and fixed within the linear region (12% local tissue strain) at multiple strain rates. Although samples were pulled to the same percent elongation, the fibrils were noticed to elongate differently, increasing with strain rate. For the 0.1, 10, and 70%/s strain rates, there were $1.84 \pm 3.6\%$, $5.5 \pm 1.9\%$, and $7.03 \pm 2.2\%$ elongations (mean $\pm$ S.D.), respectively. We concluded that the collagen fibrils underwent significantly greater recruitment (fibril strain relative to global tissue strain) at higher strain rates. A better understanding of tendon mechanisms at lower hierarchical levels would help establish a basis for future development of constitutive models and assist in tissue replacement design.

1. Introduction

Tendons are soft connective tissue exhibiting nonlinear viscoelastic properties, with a hierarchical structure ranging from the entire tendon to fascicle to collagen fiber to collagen fibril to the collagen molecule (Fig. 1)(Butler et al., 1978; Wang, 2006; Kastelic et al., 1978; Screen et al., 2004). This intricate structural hierarchy gives tendon the viscoelastic characteristics of most soft tissues, i.e., strain rate sensitivity, stress relaxation, and creep. Many mechanistic studies have been carried out to understand the underlying mechanisms of these viscoelastic tissue behaviors (Butler et al., 1978; Wang, 2006; Cohen et al., 1976; Woo, 1982; Yamamoto et al., 1992; Johnson et al., 1994; Duenwald et al., 2009).

In tendon mechanics, the central role is played by Collagen Type I, which is the major component of tendon and makes up approximately 70–80% of the tissue dry mass(Kannus, 2000; Yamamoto, 1999). Collagen fibril diameter distribution changes with age and affects the stiffness of tendon (Derwin and Soslowsky, 1999). As the key structural protein component, the collagen molecule consists of a triple helix formed by three polypeptide α-chains with a length of ~300 nm and a diameter of ~1.5 nm(Petruska and Hodge, 1964). Quarter staggering of the collagen molecules gives rise to the collagen fibril with a banding appearance also known as the D-period(Petruska and Hodge, 1964).

There have been previous studies on kinematics of collagen fibrils under uniaxial loading (Sasaki and Odajima, 1996a,b; Graham et al., 2004; Shen et al., 2008; van der Rijt et al., 2006; Yang et al., 2008, 2007 Hansen et al., 2009). Fibril mechanisms have been studied mainly using X-ray diffraction techniques and TEM. Increasing load has been shown to result in elongated collagen fibrils, demonstrated by an increased D-period (Sasaki and Odajima, 1996a,b; Folkard et al., 1987; Fratzl et al., 1998; Liao et al., 2005). When collagen fibrils undergo load, the D-period elongates due to intrinsic fibril mechanisms of molecular elongation and slippage (Sasaki and Odajima, 1996a). Recently, combining multi-scale measurements (inter-fiber sliding and intra-fiber elongation along with fibril elongation), Gupta et al. (2009) created a novel multiscale model to describe how tendon distributes load between its fiber and fibril hierarchies during stress-relaxation. Recently, fascicle viscoelastic models were proposed by Lucas et al. (2009); Elliott et al. (2003).

Noncollagenous components in tendon are proteoglycans, elastin, water, and fibroblasts. Tendon's proteoglycan (PG) network is composed of highly negatively charged glycosaminoglycans (GAGs), which are matrix components that regulate fluid flow and attribute to viscoelasticity within the tendon (Elliott et al., 2003; Puxkandl et al., 2002; Screen et al., 2005; Robinson et al., 2004; Yin and Elliott 2004; Weiss et al., 2002). There has been theoretical modeling and experimental observations supporting the interfibrillar mechanical role of PG bridges (Liao and Vesely 2007; Redaelli et al., 2003; Scott, 2003). Some question the noncollagenous matrix's mechanical role, stating collagen fibrils span the whole tissue and load is not

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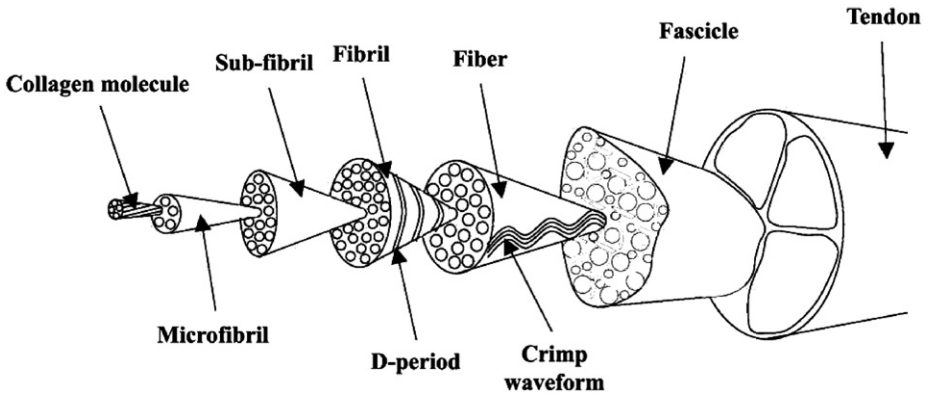

Fig. 1. Tendon's hierarchical structure adopted in current study. (Adapted from Kastelic et al. (1978) and Screen et al. (2004).

transferred to PGs (Provenzano and Vanderby, 2006). Note that mechanical role of PGs is still debatable and in need of research.

In the present study, our objective is to reveal the mechanistic contribution of tendon structural component to tissue strain rate sensitivity, specifically to examine the collagen fibril elongation in tendon tissues that were loaded at various strain rates. The interruption mechanical testing (IMT) and scanning electron microscopy (SEM) were applied to assess the collagen fibrils in the chemically fixed tendon fascicles that have been loaded to the same strain level at various ramping rates. A better understanding of tendon mechanisms at lower hierarchical levels can help us better to understand tissue structure–function relation and shed light on biomimic design of tissue replacements that are optimized in strength and robustness (Ackbarow and Buehler, 2009).

2. Methods

2.1. Sample preparation

Three mature Japanese white rabbits were used in this study. The procedures of sacrifice and experiment were approved by the MSU Institutional Animal Care and Use Committee (IACUC). Animals were humanely euthanatized by administration of Beuthanasia solution (100 mg/ml perntabarbitol, 1 ml/10 lbs) intravenously in the ear vein or by intracardiac injection. Prior to euthanasia, each rabbit was sedated with an intramuscular injection of ketamine (10 mg/kg) and medetomidine (0.5 mg/kg). Immediately after euthanasia, both hind limbs were harvested using sharp dissection. Each limb was wrapped in gauze soaked in a phosphate saline solution and sealed in an airtight plastic bag. These were then stored at -30 °C until testing procedures were executed. Prior to testing, the legs were thawed at room temperature for 2 h fully hydrated with PBS. Three tendons, all from different animals, were used in the study for interrupted mechanical testing and SEM analysis (D-period measurement). The remaining patellar tendon samples were used for mechanical testing only (Fig. 2).

Both the proximal and distal insertion portions of the patellar tendon and the fascia surrounding the tendon were removed. The middle portion of the tendon was carefully extracted and then trimmed into four fascicles with the patella end attached to a holder and the other end grasped tightly with forceps (cut with an approximate width of 1 mm (Yamamoto, 1999)). All fascicles used in the study were cut from the inner portions (the central 4 mm) of the tendon to prevent location variability within the tendon (Williams et al., 2008). One tendon from each animal yielded 4 fascicles (extracted from the inner portion of tendon), among which 3 fascicles were used for tensile testing and 1 was used as a control sample. The specimens were kept moistened with PBS during the sample extraction procedure and testing.

2.2. Interruption tensile mechanical testing

Tendon fascicles were mounted onto the Biomomentum Mach-1 mechanical testing system (Biomomentum Inc., Canada). Special grips were designed to secure the specimen and minimize slippage (Fig. 3). The fascicles were then measured for their cross-sectional areas in the midsubstance using NIH Image J digital imaging program. All tested fascicles had grips initially at 7 mm apart giving a ratio of the samples' dimensions to be around 7:1 (length vs. width), with each sample slightly varying in diameter. Fascicles were more elliptical in shape than assuming a completely round cross-sectional area. Area was defined as $A=(\pi/4)\times a\times b$, where a and b are the width and depth of the fascicle.

The fascicles were preconditioned in order to provide the specimens with similar loading histories and reduce variations in response to loading. Each sample was preloaded at 0.01 N at 0.01 mm/s; and zero strain (gauge length) was defined at this load. Each specimen was then preconditioned to a strain of 2%, for 10 cycles at 1 Hz (Yamamoto, 1999). The mechanical data were recorded in engineering stress and engineering strain. Control samples were subjected to the same preconditioning protocol and then fixed at a zero load in the fixative bath for the same amount of time (4 h). For the 4 fascicles yielded from each tendon, one was used as load free control and three were loaded to 20% clamp-to-clamp strain at 0.1, 10, and 70%/s strain rates. This corresponded to a 12% local strain in the midsubstance. Immediately after the test, the tendon was fixed in 1.25% glutaraldehyde for 4 h. The tangent modulus was taken from the linear portion of the curve at approximately 5% strain. Because of the inhomogeneous nature of tissue strain field from clamp-to-clamp, we monitored the local strain of midsubstance by placing two markers using permanent ink vertically on the fascicle surface and imaging the marker movement before and after the elongation. The local strain was calculated by measuring the distances between the centroids of two markers.

Each rabbit used in the study yielded one leg for interruption tensile testing and D-period analysis and one leg devoted just for mechanical testing for modulus calculations. Mechanical data from the interruption testing fascicles were also used in calculating the modulus ($n=6$).

2.3. Scanning electron microscopy

Samples for SEM were fixed in 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), rinsed with 0.1 M phosphate buffer, and post-fixed with 2% osmium tetraoxide in 0.1 M phosphate buffer. Samples were dehydrated in a graded ethanol series and then were critical point dried in a Polaron E 3000 CPD. They were then mounted on aluminum stubs and sputter coated with gold–palladium. Specimens were imaged using a JEOL JSM-6500 FE scanning electron microscope.

2.4. D-period analysis

After imaging, the samples were analyzed using Image J software (Image J 1.41, National Institutes of Health, USA). SEM photographs were taken from $\times$ 20,000 to $\times$ 30,000 magnification. The average number of D-periods for each fibril (40 ± 9) was averaged by Image J using the image's scale bar and a scale factor calculated by SF=(distance in pixels)/(known distance (nm)). The elongation of collagen fibrils were compared among different strain rates to show the differences in fibril recruitment. Note that, in this study, fibril recruitment was defined as the amount of fibril strain relative to the global tissue strain.

For each interruption tensile test, five images randomly located in the specimen's midsubstance were used to measure the fibril periodicity (Fig. 4). However, in order to avoid stereological bias, the imaging areas needed to have collagen fibrils predominately aligned along the image plane. Obviously, collagen fibrils with a large oblique orientation (out of plane) could potentially underestimate the D-period.

Six fibrils from each image were selected for D-period measurement. Again, collagen fibrils within plane orientation were picked for an accurate measurement. The sample size was 90 fibrils for each strain rate (Fig. 5), while the total number of fibrils analyzed for the control group was 50. This was due to the crimping nature of load free tendon and the difficulty of locating in-plane undistorted fibrils (Fig. 6-a).