Acta Biomaterialia 50 (2017) 293–301

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Evidence of structurally continuous collagen fibrils in tendons

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ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form 5 December 2016
Accepted 3 January 2017
Available online 5 January 2017

Keywords:
Collagen fibril length
FIB-SEM
Focused ion beam
Serial block face-scanning electron microscopy
tendo m. stapedius

ABSTRACT

Tendons transmit muscle-generated force through an extracellular matrix of aligned collagen fibrils. The force applied by the muscle at one end of a microscopic fibril has to be transmitted through the macroscopic length of the tendon by mechanisms that are poorly understood. A key element in this structure-function relationship is the collagen fibril length. During embryogenesis short fibrils are produced but they grow rapidly with maturation. There is some controversy regarding fibril length in adult tendon, with mechanical data generally supporting discontinuity while structural investigations favor continuity. This study initially set out to trace the full length of individual fibrils in adult human tendons, using serial block face-scanning electron microscopy. But even with this advanced technique the required length could not be covered. Instead a statistical approach was used on a large volume of fibrils in shorter image stacks. Only a single end was observed after tracking 67.5 mm of combined fibril lengths, in support of fibril continuity. To shed more light on this observation, the full length of a short tendon (mouse stapedius, 125 μm) was investigated and continuity of individual fibrils was confirmed. In light of these results, possible mechanisms that could reconcile the opposing findings on fibril continuity are discussed.

Statement of Significance

Connective tissues hold all parts of the body together and are mostly constructed from thin threads of the protein collagen (called fibrils). Connective tissues provide mechanical strength and one of the most demanding tissues in this regard are tendons, which transmit the forces generated by muscles. The length of the collagen fibrils is essential to the mechanical strength and to the type of damage the tissue may experience (slippage of short fibrils or breakage of longer ones). This in turn is important for understanding the repair processes after such damage occurs. Currently the issue of fibril length is contentious, but this study provides evidence that the fibrils are extremely long and likely continuous.

1. Introduction

Connective tissues play a crucial role in maintaining the structure of our bodies. The feat of literally keeping us in shape is made no less impressive by the fact that it is performed to a large extent by a single group of proteins called collagens. While there are 28 different types of collagen in vertebrates, it is a subgroup of fibrillar collagens (type I, II, III, V, XI), which is mainly responsible for providing mechanical strength on larger scales [1,2]. The different fibrillar collagens have similarities in their structure and general behavior, and although important differences exist, they all form elongated triple-helical molecules that aggregate in a highly ordered stagger to form fine threads called fibrils [3,4]. These fibrils are the main building blocks of large connective tissues such as skin, bone, cartilage, and tendon. Tendons are possibly some of the simplest connective tissues in terms of structure and composition, consisting almost entirely of parallel type I collagen fibrils [2], with a low rate of turnover [5,6]. In spite of this apparent simplicity, a fundamental question remains unanswered; how long are the fibrils? The diameters of tendon collagen fibrils are routinely measured by transmission electron microscopy (40–400 nm) [7]; however, determining the length has proven more difficult primarily

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http://dx.doi.org/10.1016/j.actbio.2017.01.006
1742-7061/

because individual fibrils are too thin to image by light microscopy methods, they cannot be extracted intact from tissues [8], and they are too long to trace by conventional serial section transmission electron microscopy. Had the length of mature fibrils been on the order of tens or hundreds of microns, it would undoubtedly have been determined many years ago as is the case in embryonic tissue where the lengths are indeed in this range [9].

Tendon is known to have poor healing capabilities, regenerating slowly and often with incomplete recovery [10,11]. Several factors likely play a role in this poor healing ability, including low cell density and vascularization [12] but the tendon structure itself may also play a role due to difficulty in aligning, tensioning, and interweaving new fibrils across a rupture site. For this reason, the length of collagen fibrils is of importance for tendon regeneration, and it is also important in determining how load is distributed through lateral and longitudinal transfer mechanisms.

The question of fibril length in mature tissue has been addressed in the past by several different approaches. Direct structural investigations have attempted to trace the length of fibrils with scanning electron microscopy of fracture surfaces [13] as well as serial sectioning with transmission electron microscopy [14]. Fibril length has also been estimated indirectly based on calculations from mechanical [15] or structural [14] properties. Other studies did not numerically estimate the length but concluded that they are discontinuous by structural and mechanical relations [16,17]. The findings generally agree that mature collagen fibrils are long (>1 mm); however, the direct structural studies find values high enough to indicate that fibrils may be structurally continuous whereas those based on more indirect methods tend to support discontinuity (length < 10 mm).

The aim of the present study was to determine if mature human tendon collagen fibrils are structurally continuous, by tracing individual collagen fibrils over distances in the centimeter range using automated electron microscopy serial imaging techniques. Tracing individual fibrils over such distances turned out not to be possible, instead continuity was investigated in a much shorter (125 μm) mouse tendon (*tendo m. stapedius*) in combination with statistical analyses of mature human tendon fibrils traced over 25 μm segments.

2. Materials and methods

2.1. Human tendons

As part of a previously published study [18], tendon tissue from males 18 to 32 years old were collected during routine anterior cruciate ligament reconstruction, and for the present study a patellar tendon and a hamstring tendon were investigated. The experiment was approved by the ethical committee and informed consent was obtained from the patients as previously described [18]. The patellar tendon was stored frozen at −20 °C in PBS until use while the hamstring tendon was prepared fresh. Frozen storage damages cellular structures but had no observable effect on the collagen. For the patellar tendon, a single fascicle was fixated in glutaraldehyde fixative (2% v/v in 50 mM sodium phosphate buffer, pH 7.2) under low tension (0.2 N) to improve long range alignment. For technical reasons described in the discussion we gave up on using stacks in the mm range obviating the need for long range alignment. Consequently the hamstring tendon was fixated directly in glutaraldehyde without tension.

2.2. Mouse stapedius tendon

Two mature 12-week old mice were pre-anaesthetized with inhalation of Halothane 3% (Halocarbon Laboratories, River Edge, NJ, USA). Anesthesia was induced by intraperitoneal injection with Pentothal Sodium (Abbott Scandinavia AB, Sweden), 55 mg/kg body weight. The mice were fixed by vascular perfusion through the left ventricle of the heart with glutaraldehyde fixative for 5 min. Following fixation, the stapes and the stapedius muscle with the interposed tendon were isolated by microdissection from the middle ear (Fig. 1A). The samples were subsequently transferred to a hypotonic fixative (1% glutaraldehyde in 25 mM cacodylate buffer, pH 7.2) for 1 h to help swell the tendon. Finally, the samples were post-fixed, stained, dehydrated, and embedded as described below.

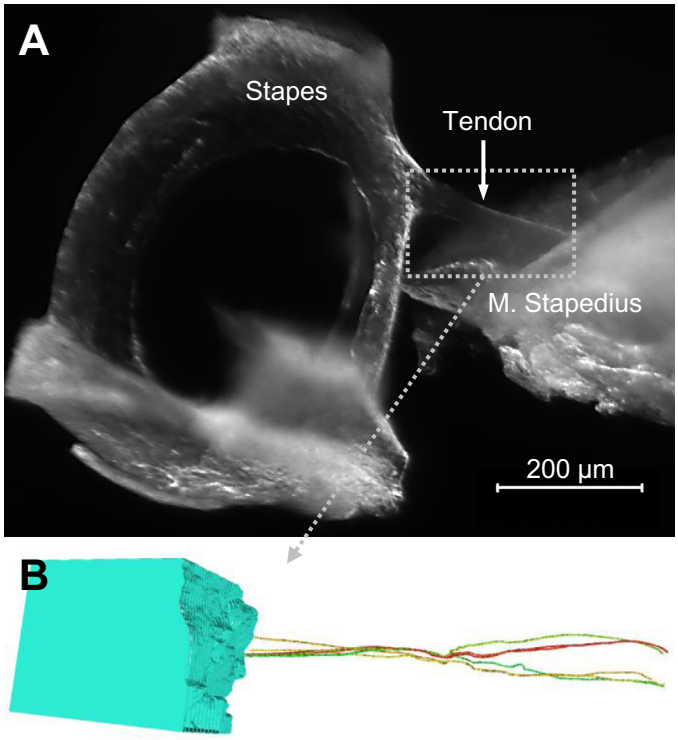

Fig. 1. Mouse stapedius tendon. (A) Stereo microscope image of an isolated stapedius tendon attached to bone (Stapes) and muscle (M. Stapedius). (B) Electron microscope 3D rendering of the 5 collagen fibrils, which were traced through the length of the mouse stapedius tendon. The region to the left is the mineralized fibrocartilage at the bone end. See also Supplementary video 1.

2.3. Sample preparation

Staining and embedding samples for FIB-SEM followed a protocol similar to that of Starborg et al. [19]. Following primary fixation, samples were washed (3 × 20 min) in 0.15 M phosphate buffer (pH 7.2), fixed in 1% OsO_4 with 0.05 M $K_3Fe(CN)_6$ in 0.12 M cacodylate buffer (pH 7.2) (1 h), and subsequently washed in distilled water (3 × 15 min). To enhance the stain, samples were treated with 1% tannic acid in 0.1 M cacodylate buffer (pH 7.2) at 4 °C (1 h), washed in distilled water (3 × 15 min) and stained in 1% OsO_4 in distilled water (1 h) followed by another wash in distilled water (3 × 15 min). Finally, the samples were *en bloc* stained with 1% uranyl acetate in distilled water at 4 °C over night and subsequently washed in distilled water (3 × 15 min). The stained samples were dehydrated in a gradient of ethanol (70% 2 × 15 min, 96% 2 × 15 min, 100% 3 × 15 min), transferred to propylene oxide (2 × 15 min) and subsequently infiltrated with a gradient of epoxy resin (TAAB 812, TAAB Laboratories Equipment Ltd, England) in propylene oxide (25% 40 min, 50% 40 min, 75% 40 min, 100% over night).