Doctoral Thesis

Structure and function in tendon experimental studies on the ultrastructural determinants of tendon biomechanical function

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Structure and function in tendon: experimental studies on the ultrastructural determinants of tendon biomechanical function

A dissertation submitted to the ETH Zürich for the degree of Doctor of Sciences presented by Samuela Rigozzi Dipl. Ing. Mat. Sc. EPFL born 13th June, 1979 citizen of Blenio TI accepted on the recommendation of Prof. Dr. Jess G. Snedeker, examiner Prof. Dr. Ralph Müller, co-examiner

2011
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April 2011
Samuela Rigozzi
Summary

Tendon is a connective tissue that transmits mechanical loads and forces from muscle to bone and in doing so enables locomotion and enhances joint stability. It is a living tissue and responds to mechanical forces by changing its metabolism as well as its structural and mechanical properties. Injuries to tendon, including overuse (tendinopathy), affect millions of people in occupational and athletic settings. After injury, tendon can heal itself; however the healed tendon typically does not reach the biochemical and mechanical properties of the tendon prior to injury. The actual healing dynamics of tendon has been reported to be more complicated than other soft tissues and does not recover to the same degree compared to before the injury.

An injured tendon usually shows scartissue in the repaired area which results in higher stiffness and tensile strength then the surrounding healthy tissue. In some cases, even people who had a tendon rupture and were treated with controlled immobilization (i.e. a plaster), had a very high incidence of re-rupture compared to people who were surgically treated. A comprehensive understanding of the pathological and biomechanical processes that underlie tendon function and pathology is essential to improved clinical diagnosis and treatment of tendon pathology. For this reason a deeper investigation of the tendon components is needed to elucidate the structure-function relationships in tendons.

Tendon structure, composed of collagen fibrils embedded within a proteoglycan (PG) rich extracellular matrix (ECM), is the key to its mechanical behavior. Collagen fibers are the major constituent of tendon and are believed to bear most of the tensile load. The PGs are thought to mediate the organization of collagen ultrastructure and facilitate force transmission along the discontinuous collagen fibrils. A considerable number of scientific investigations have already been performed to define the influence of ultrastructure on the mechanical properties of tendon. However, the large body of published experimental evidence yields conflicting conclusions with regard to the fundamental ultrastructural determinants of tendon behavior. Thus, the aim of this thesis is to elucidate these contrasting results by taking a “bottom up” approach, by which the basic intermolecular forces of binding within the ECM between the collagen fibrils and PGs are considered, in conjunction with the tensile
mechanical properties of individual fibrils. This may help reconcile the conflicting and counterintuitive results that have thus far been observed in laboratory experiments, and will ultimately help inform clinical diagnosis and treatment of connective tissue disorders. Specifically, a new protocol to mechanically test Achilles tendon was developed and validated in order to quantify the mechanical contribution of the PG concentration in tensile load. Tendons were chemically digested of their PG secondary chains — the glycosaminoglycans (GAGs) — and compared with the native ones. Results showed that along the longitudinal axis the mechanical behavior was heterogeneous in tensile load, especially at the muscle and the bone insertion, highlighting the importance of performing local strain analysis with regard to tensile tendon mechanics. The contribution of PGs to tensile tendon mechanics at the macroscale was not straightforward and points to a heterogeneous and complex structure-function relationship in tendon.

Additionally, two inbred strains of mice showing similar macroscopic mechanical behavior but different elastic modulus were further investigated in their collagen fibril morphology to understand their mechanical contribution. New image-analysis tools were designed to parameterize the collagen fibril morphology. The group with higher elastic modulus, structurally exhibited a larger mean collagen fibril radius, smaller specific fibril surface (i.e. the perimeter of the fibril in contact with the non-collagenous ECM), and a lower concentration of GAGs. As in previous studies, larger collagen fibril radius appeared to be associated with a stiffer tendon, but this functional difference could also be attributed to reduced potential surface area exchange between fibrils and the surrounding PG rich matrix, in which the hydrophilic GAG side chains may promote inter-fibril sliding.

Finally, the interactions between the PGs and the collagen fibrils were studied at the ultrascale in their physiological matrix. A new experimental approach combining macroscopic mechanical loading of tendon with a morphometric ultrascale assessment of longitudinal and cross-sectional collagen fibril deformations was developed and validated. Tendons with different PG concentration were submitted to different target strains at the macroscale and chemically fixed. Collagen fibrils were characterized with atomic force microscopy (AFM). The mechanical contribution of PG at the ultrascale was quantified by comparing the difference in the collagen fibril elongation and diameter between native and GAG-depleted tendon. Tendons with a lower concentration of GAG showed greater elongation in the collagen fibril.

In conclusion, in conjunction with all the specific studies performed in this thesis, GAG concentration did not significantly influence the overall tendon mechanical response. However, at the ultrascale GAGs were responsible for changes in collagen
Summary

fibril elongation, which increased at lower GAG concentration. Thus, GAGs seem not to link the collagen fibrils in order to mechanically transfer forces between them in tension, but rather hydrate the ECM, and promote collagen fibril sliding under tension, most likely to prevent damage.
Riassunto

Il tendine è un tessuto connettivo che trasmette i carichi meccanici e le forze dai muscoli alle ossa e, in tal modo, consente la locomozione e migliora la stabilità articolare. Si tratta di un tessuto vivente che risponde alle sollecitazioni meccaniche, modificando il proprio metabolismo e le sue proprietà strutturali e meccaniche. La lesione del tendine, compreso un uso eccessivo (tendinopatia), colpisce milioni di persone in un contesto professionale ed atletico. Dopo una lesione, il tendine può ristabilirsi spontaneamente, ma generalmente la sua guarigione non raggiunge le proprietà biochimiche e meccaniche precedenti la lesione. La dinamica effettiva della guarigione del tendine è nota per essere più complicata di quella di altri tessuti molli e per non recuperare la rigidezza sufficiente, come prima della lesione.

Un tendine infortunato mostra, di solito, tessuti cicatrizzati nella zona riparata che si traducono in una maggiore rigidezza e resistenza alla trazione. In alcuni casi si è notato che le persone che hanno subito una rottura del tendine e sono state trattate con immobilizzazione controllata (gesso) hanno avuto un’incidenza molto elevata di rottura, rispetto alle persone che sono state trattate chirurgicamente. Una comprensione completa dei processi patologici e biomeccanici che stanno alla base del funzionamento e la patologia del tendine sono essenziali per una migliore diagnosi clinica e un trattamento della patologia tendinea. Per questo motivo, uno studio più approfondito delle componenti del tendine è necessario per chiarire i rapporti tra struttura e funzione dei tendini.

La struttura del tendine, composta di collagene racchiuso all’interno di una matrice extracellulare (MEC) ricca di proteoglicani (PG), è la chiave per la sua efficienza meccanica. Le fibre di collagene sono le costituenti principali del tendine costrette a sopportare la maggior parte del carico di trazione. Si pensa che i PG siano i mediatori dell’organizzazione dell’ultrastruttura collagenosa e facilitino la trasmissione della forza lungo le fibrille discontinue di collagene. Una notevole quantità di ricerche scientifiche sono già state eseguite per definire l’influenza dell’ultrastruttura sulle proprietà meccaniche del tendine. Tuttavia, la grande quantità di risultati sperimentali pubblicati finora propongono conclusioni contrastanti per quanto riguarda i dati fondamentali del comportamento ultrastrutturale del tendine. L’obiettivo di
Riassunto

Questa tesi è quello di spiegare questi risultati contrastanti partendo da un approccio “di base”, per cui vengono prese in considerazione le forze intermolecolari di base dei legami all’interno della MEC, tra le fibrille di collagene e i PG, oltre alle proprietà di resistenza meccanica individuali delle fibrille. Questo può aiutare a riconciliare i risultati contrastanti che sono stati finora osservati negli esperimenti di laboratorio, e in ultima istanza, contribuire a confermare la diagnosi clinica per i trattamenti dei disturbi ai tessuti connettivi.

In particolare, è stato sviluppato e validato un nuovo protocollo per testare meccanicamente i tendini d’Achille, al fine di quantificare il contributo meccanico della concentrazione di PG sottoposti a un carico di trazione. I tendini sono stati trattati chimicamente per eliminare parzialmente le catene secondarie dei PG — ovvero i glicosamminoglicanici (GAG) — e confrontati con quelli naturali. I risultati hanno mostrato un comportamento meccanico eterogeneo, in tensione lungo l’asse longitudinal, soprattutto all’inserimento del muscolo e dell’osso, evidenziando l’importanza di eseguire un’analisi locale della deformazione rispetto alla resistenza meccanica del tendine. Macroscopicamente, il contributo dei PG alla deformazione del tendine in trazione meccanica ha indicato un rapporto eterogeneo e complesso tra la struttura e la funzione dei tendini.

Inoltre, due gruppi di topi “inbred strain”, che mostrano simile comportamento macroscopico ma hanno un diverso modulo elastico, sono stati ulteriormente esaminati nella loro struttura, per investigare l’influenza delle fibrille di collagene. Nuovi strumenti di analisi d’immagine per parametrizzare la morfologia delle fibrille di collagene sono stati progettati. Il gruppo con il più alto modulo elastico ha esibito strutturalmente un raggio medio delle fibrille di collagene più grande, una superficie fibrillare specifica (ovvero il perimetro delle fibrille in contatto con la matrice non collagenosa) più piccola e una minore concentrazione di GAG. Come in precedenti studi, un maggiore raggio di fibrille di collagene sembra essere associato ad un tendine rigido, ma questa differenza funzionale potrebbe anche essere attribuita alla riduzione del potenziale di superficie di scambio tra fibrille e la matrice circostante ricca di PG, le cui catene idrofile secondarie possono promuovere lo scivolamento inter-fibrillare aumentando l’idratazione.

Infine, le interazioni tra i PG e le fibrille di collagene sono state studiate a livello microscopico, nel loro ambiente fisiologico. Un nuovo approccio sperimentale, che unisce il carico meccanico del tendine a livello microscopico, è stato sviluppato e validato, con una valutazione morfometrica delle deformazioni longitudinali e trasversali delle fibrille di collagene a livello nanoscopico.

Le fibrille di collagene sono state caratterizzate con il microscopio a forza ato-
Riassunto

mica, per tendini con differenti concentrazioni di PG che sono stati sottoposti a diversi valori di deformazione macroscopica. Il contributo meccanico dei PG a livello nanoscopico è stato quantificato confrontando la differenza di lunghezza nella caratteristica periodicità strutturale — il cosiddetto d-periodo — e nel diametro delle fibrille tra tendini naturali e tendini impoveriti di GAG. I tendini con una minore concentrazione di GAG hanno mostrato un maggiore allungamento nelle fibrille di collagene.

In conclusione, riassumendo tutti gli studi specifici effettuati in questa tesi, la concentrazione di GAG non influenza significativamente la risposta globale meccanica del tendine. Tuttavia, a livello microscopico, i GAG sono responsabili dei cambiamenti nell’allungamento delle fibrille di collagene, che risulta maggiore per una concentrazione minore di GAG. Questi ultimi sembrano quindi non legare le fibrille di collagene al fine di trasferire meccanicamente forze tra loro in tensione, come si credeva in precedenza, ma piuttosto idratare la MEC, e migliorare lo scorrimento tra le fibrille di collagene per evitare lesioni probabilmente dovute all’eccessiva frizione.
Chapter 1

Introduction

Thesis motivation

A considerable number of scientific investigations have already been performed to define the influence of ultrastructure on the mechanical properties of tendon. However, the large body of published experimental evidence yields conflicting conclusions with regard to the fundamental ultrastructural determinants of tendon behavior. For example, numerous studies suggest that increased fibril diameter is primarily responsible for increased tendon stiffness and strength [1, 2, 3, 4], while others indicate that the total fibril surface area and the network of proteoglycans (PGs) are responsible for it [5, 3]. The use of transgenic “knockout” mouse models has permitted the focused investigation of specific ultrastructural components, such as the proteoglycan decorin, which has historically been hypothesized to be a critical factor in fibril organization and load sharing between adjacent fibrils. However, the findings from these knockout studies have often been counterintuitive, implying that fundamental assumptions regarding the manner in which fibrils are interconnected in the extracellular matrix (ECM) must be re-evaluated.

The present study takes a “bottom up” approach, by which the functional properties of tendons are considered in conjunction with the basic intermolecular binding forces between the collagen fibrils and PGs in the ECM. The accurate investigation of multiscale structural properties may help reconcile the conflicting and counterintuitive results that have thus far been observed in laboratory experiments, and will ultimately help inform clinical diagnosis and treatment of connective tissue disorders.

This dissertation relies heavily on the mouse as a model for human tendon structure and function. The use of the mouse as a model for human musculoskeletal diseases has increased in popularity since the mouse genome was well characterized [6, 7, 8]. The advantages of using the mouse model are that various strains have been observed to exhibit disease state characteristics similar to those found
in humans, and that the mouse is easily accessible to manipulation of the genetic makeup by either gene knockout, gene overexpression (transgenes), or genetic breeding strategies [9]. With the exception of identical twins, the genetic background in humans varies significantly from one individual to another, making studies of genetic involvement in a given tendon phenotype difficult. Well characterized animal lines, such as murine inbred strains are therefore used as an approach to study more heterogeneous populations [10].

The project

The concept for this study was born from a collaboration of the Institute for Biomechanics within the European Union Integrated Project “Adult mesenchymal stem cell engineering for connective tissue disorders; from the bench to the bed side”. This project involved a consortium of 26 European laboratories, that was focused on the use of mesenchymal stem cells (MSCs) to repair injured or pathological bone, tendon and cartilage. As part of this project, the Institute for Biomechanics, performed quantitative biomechanical assessment of tendon function, and was responsible for determining whether MSC therapies are in fact accelerating or improving the healing process. Initial research within the EU project made it clear that more information regarding the ultrastructural mechanics of tendon was required in order to better direct clinical intervention strategies. While it is possible to determine whether a particular therapy restores tendon function, the process yields little information as to why a treatment works or not. Specifically, it was unknown whether restoration of normal distributions of collagen fibrils should be a primary clinical goal, or whether an increased/decreased concentration of GAGs in the ECM would more effectively improve the biomechanical performance of healing tendon.

Specific aims

In an effort to improve novel tendon treatments, this doctoral thesis aims to investigate how the individual molecular tendon components contribute to the functional properties of tendon tissues. Tendon component influences on the mechanical behavior are individually investigated through various combinations of selective chemical digestion, microscopic observations, and tensile testing. The focus on each single component can in fact be related with specific diseases that could occur in the human body. For instance, the behavior of GAG-depleted tendon could be associated with the tendon of older people, in which the amount of PG naturally decreases with age [11], or it is also found to be lower for tendon with pathologies that lead to
early injuries [12]. In the work, murine tendons are observed from the macro down to the micro- and the nano-scale to quantify structural differences at these levels of structural hierarchy and relate them to the mechanical response.

The foci of the project can be summarized by the following three specific aims:

**Specific aim 1** Investigate the functional role of proteoglycan sidechains in tensile mechanics at the macroscale and quantify their contribution.

**Specific aim 2** Define the collagen fibril morphological parameters that influence the tendon tensile mechanical response.

**Specific aim 3** Relate the interaction between the PGs and collagen fibrils in their native network at the nanoscale with the overall mechanical response.

This work involved the development and validation of suitable experimental set-ups, protocols, and image analysis tools to achieve these aims. The following sections explain in more detail the outline of the thesis from the basic physiology of tendon, the strategies for investigating the mechanical role of the tendon components, and summarize our current understanding of the tendon structure-function relationships.

**Outline of the thesis**

The thesis is structured into the following five chapters: an introduction, a chapter of general background information, targeted studies to answer open questions, and a concluding synthesis of the work performed and outcomes achieved.

**Chapter 1** is an introduction to the motivations behind the thesis. It includes the basic information that inspires the project outline, and a listing of the specific aims.

**Chapter 2** provides the necessary background on tendon structure related to the mechanical properties and the state of the art that frames the research performed in the thesis.

In the following chapters the influence of the tendon components on the mechanical properties are investigated with different techniques starting from the tissue-organ level to the material level. Specifically, the following studies were carried out:

**Chapter 3** is an analysis at the tissue-organ level that mainly focuses on the mechanical response as a correlation to different component parameters. In **Chapter 3.1** the investigation at the macroscopic level, of the mechanical influence of proteoglycans on tendon behavior is presented. Specifically, this influence is correlated with the PG content in the tissue. Tensile tests are performed on both natural
and GAG-depleted tendon. The tendon is locally analyzed to give quantitative information about the structural heterogeneity of the tissue (i.e., regional GAG content), and whether any difference is reflected in the functional properties.

**Chapter 4** is an analysis at the material level, of the mechanical response of the collagen fibrils to elucidate the force transfer mechanism between them. **Chapter 4.1** focuses on how fiber morphology influences the mechanical response to tensile load. Two genomic inbred mouse strains, showing different fiber morphology, are compared with respect to their mechanical properties. Transmission electron microscopy (TEM) pictures of the tendon cross-section are quantitatively analyzed and parameterized to extract information about collagen fibril diameter, fibril density, and interface between collagen and matrix. The two groups are tested in tension to correlate the morphology with the corresponding mechanical properties without any chemical tissue alteration.

In **Chapter 4.2** a novel imaging method needed to monitor collagen fibril deformation is validated and presented. This method combines the mechanical tests performed at the macroscale and presented in the previous chapter with an atomic force microscopy (AFM) image analysis that allows quantification of the collagen fibril deformation while in their physiological environment. In **Chapter 4.3** the novel imaging method is implemented to investigate collagen fibril elongation occurring within the tendon fascicles dependent on the PG concentration. Specifically, changes in the collagen fibril elongation in relation to the PG concentration are analyzed with AFM. Two groups of tendons are chemically fixed in their relaxed position and at increasing target strains. Images are taken for both natural and GAG-depleted tendon. The measured fibril elongation for equal applied-strain is correlated to GAG content and compared.

In **Chapter 5** the thesis concludes with a synthesis of the key findings, a discussion that combines all the results and compares them with previous studies, and possible future work.

**References**


Chapter 2

Background
2.1 An analogue for tendon

2.1.1 Structure function

In everyday life objects or materials that are used are chosen for their function, which is defined by their structure. The same construction materials can be used to build different structures like bridges or skyscrapers that have a different function. Stepping further into the structure, from a general perspective, all materials are made of atoms. Depending on its atomic structure, the same atom can form materials with totally different functions as in the case of diamond and graphite, shown in Figure 2.1.

![Diagram showing cubic and hexagonal structures with carbon atoms forming diamond and graphite, respectively.](image)

Figure 2.1: Function related to the structure of the same ground material. The carbon atom with a different atomic structure is the ground material of two totally different materials like diamond and graphite.

This simple example shows how the structure and function of a material are related. Even if all the components of a material are known, the structure is critical to understand and predict its function. This study will focus on the structure at the microscale of a tendon, whose structure and function can be compared with a rope, as explained in the following section.
2.1.2 The rope

Ropes are used to bind together many things, connect two objects to their ends with a knot or as a security for sports, like mountaineering or climbing. Some of their functions include attaching, carrying, fastening or lifting. In all of these tasks, ropes are subjected to tensile load. The key to the performance of ropes in tension is in their unique structure: a core of fibers, which are twisted or braided together at different levels to provide elasticity; the core is further covered with an additional layer of intertwined fibers to protect them from local damage due to cuts or friction (see Figure 2.2).

![Figure 2.2: Schematic section of a rope.](image)

Similar to ropes, tendons attach bones to muscles in the human body to “carry” the force produced by the muscle to “lift” the bone. Their performance is also a feature of their structure composed of fibers grouped together at different levels to give elasticity and strength, embedded into a sheath that allows the tendon to glide and protect the core from friction.

Tendon and rope structures differ in two main aspects: tendons cannot be tied at their ends but consist of a single structure that binds two different tissues (the muscle and the bone); and tendon fibers — for example, those in the Achilles tendon — are parallel to each other rather than twisted or braided. These apparently minor differences in the structure are mechanically challenging. The challenge is in providing a continuous structural transition between a soft elastic muscle to a hard stiff bone without losing energy during locomotion. For this reason a lot of interest is being directed across the research world into the tendon structure-function relationship that is discussed in the following section.
2.2 Tendon function

2.2.1 Anatomical role

The tendon’s main role is to transmit muscle generated force to a bone and enable locomotion [1]. In the human body there are two kinds of tendon: positional tendons and energy storing tendons. Positional tendons principally transmit force to bone to move joints and they are relatively inextensible under physiological loads. The energy-storing tendons also act like a spring to store elastic energy and increase the efficiency of locomotion [2] and are required to stretch and recoil under physiological loads to ensure return of stored energy [3]. The difference between these two tendon groups is also reflected in the mechanical properties. The energy-storing tendons show a significantly lower elastic modulus (E-modulus) than the positional tendons, in order to support optimization for their specific physiological roles [4].

The Achilles tendons is an example of an energy-storage tendon [5], and it will be the main subject of this study. A detailed description of its mechanical behavior under tensile load will be described in the following section.

2.2.2 Tensile behavior

Tendon under tensile load presents a typical stress-strain curve that can be explained by three different mechanical behaviors summarized in three main regions displayed in Figure 2.3: the toe region, the heel region and the elastic region. In the toe region, the macroscopic “crimp” formed by the fascicle disappears, while there are no further structural changes visible with the light microscope at larger strains. This crimp pattern of the tendon fascicle is believed to play a major role in the mechanical behavior of tendon, specifically for small loads that correspond to the toe region [6, 7]. It has been suggested that the crimp provides a shock dissipation mechanism that is evident in the compliant toe region of the load-deformation curve [7, 8]. Local variations in tissue stress have been associated with region-specific differences in crimp angle [9, 10]. It has also been shown that patients clinically presenting a rupture of the Achilles tendon possess significant differences in collagen fibril diameter distribution and crimp when compared to non-ruptured controls [11].

In the heel region an increase in the E-modulus is observed. Some studies have found that as in the fascicles, at the fibril level there are also micro crimps, so-called kinks [12, 13]. A change in the E-modulus in the heel region is therefore believed to be a change in the intermolecular spacing or in the degree of order in the fibril gap regions. X-ray studies reported no net reduction in the intermolecular spacing
Background

within the fibrils but a dramatic reduction in the entropic disorder under tension [13, 14, 6]. In other words the kinks present in the fibril’s gap straighten with applied load before any significant elongation is observed in the fibrils themselves [12, 13, 15]. This mechanism is basically a form of rubber elasticity, due to a further loss of freedom of motion of the gap zone segments between the collagen molecules. In the elastic region the whole tendon structure is already under tension and exhibits a linear mechanical response in relation to the applied load.

Figure 2.3: Typical stress-strain curve and corresponding images at the macroscale (visualized using polarized light) and microscale (structural changes occurring at the fibrillar level) of a rat tail fascicle. In the toe region the macroscopic crimps disappear, in the heel region the molecular kinks in the gaps regions progressively straighten, while in the elastic region the intermolecular spaces elongate and the fibril order increases. Figure adapted from Fratzl [6].
2.3 Tendon structure

Tendons are made of groups of fascicles that are connected to the muscles. Each muscle ends in a different fascicle that can have a different orientation in the structure depending on the bone to which it is attached. Specifically, tendon has a multi-level hierarchical structure composed of tendon units, fascicles, fiber bundles, fibrils and collagen molecules that run parallel to the tendon’s long axis (Figure 2.4).

![Figure 2.4: Organization of tendon as identified from nanoscale imaging techniques (x-ray diffraction, electron microscopy) to microscale (scanning electron microscopy, visible light microscopy). This classic figure is adapted from Kastelic [16].](image)

The fascicles are wrapped by a thin walled sheath: the endotendon. The low friction coefficient of the endotendon allows the independent relative movement of the fascicles within the tendon [17]. Each fascicle presents the same structural characteristics showing a “crimp” pattern along the longitudinal axis. Crimping can be observed as a periodic banding of dark lines using polar light microscopy (Figure 2.3), and have been quantified in several studies [18, 19, 14] as a period ranging from 10 to 20 µm, with an amplitude of 3 to 4 µm, and an angle ranging between 30 to 40 degrees. Their function is to give elasticity from the unloaded state to the elongated state without losing tensile strength [6]. Within the fascicle, the collagen fibrils embedded in a proteoglycan-rich matrix form the extracellular matrix (ECM).
2.3.1 Collagen

Collagen is the most abundant protein in mammals. About one quarter of the protein in the body is collagen, which is also the main component of all connective tissues [20]. Collagen provides structure to our bodies, protecting and supporting the softer tissues and connecting them with the skeleton. There are several types of collagen present in the human body as shown in Table 2.1.

<table>
<thead>
<tr>
<th>Type</th>
<th>Polymerized form</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril forming</td>
<td>fibril</td>
<td>bone, skin, tendons, ligaments, cornea,</td>
</tr>
<tr>
<td></td>
<td>I fibril</td>
<td>internal organs</td>
</tr>
<tr>
<td></td>
<td>II fibril</td>
<td>cartilage, intervertebral disc, notochord,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vitreous humor of the eye</td>
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<tr>
<td></td>
<td>III fibril</td>
<td>skin, blood vessels, internal organs</td>
</tr>
<tr>
<td></td>
<td>V fibril</td>
<td>associated with type I</td>
</tr>
<tr>
<td></td>
<td>XI fibril</td>
<td>associated with type II</td>
</tr>
<tr>
<td>Fibril associated</td>
<td>IX lateral association with type II</td>
<td>cartilage</td>
</tr>
<tr>
<td></td>
<td>XII lateral association with some type I</td>
<td>tendon, ligaments, some other tissues</td>
</tr>
</tbody>
</table>

Type I collagen constitutes around 60% of the dry mass of tendon and 95% of the total collagen [21]. Fibrils are easily recognizable by their characteristic, so-called, d-

Figure 2.5: Schema and micrographs of the d-period. The schema on the left shows the d-period regular stripe visible on the collagen fibrils surface along the longitudinal axis. On the right side, experimental view of the d-period with different imaging techniques: atomic force microscope (AFM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM).
period, i.e. a regular stripe on the fibrils surface along the longitudinal axis as showed in Figure 2.5. This d-period is formed by the peculiar axial packing of the collagen molecules, as illustrated in Figure 2.6. First the cell secretes a procollagen that is converted to collagen by removal of the propeptide ends with enzymes. Lateral forces induce the collagen molecules to spontaneously self-assemble into fibrils. Eventually, the fibrils are stabilized by covalent cross-linking initiated by enzymes (lysil-oxidase) [22]. The structural characteristic of the fibrils is explained by the Hodge-Petruska model as a result of the molecule staggering [23].

![Diagram of collagen fibril formation](image)

**Figure 2.6: Formation and self-assembly of collagen fibrils.** Procollagen is secreted from the cell and cleaved at the propeptide ends to form a collagen molecule. The fibril is formed by the spontaneous parallel self-assembly of the collagen molecules. As a last step the fibrils are stabilized by covalent cross-linking, which is initiated by lysil oxidase.

The length of a collagen molecule (~ 280 nm) was recognized to be 4.4 times greater than this characteristic d-period of 67 nm. This discrepancy between the collagen molecule’s length and arrangement suggests the concept of a quarter-stagger, consisting of overlap and gaps between the molecules as shown in Figure 2.7. The
Background

collagen molecules spontaneously assemble laterally with the same configuration forming overlapping and low density stripes in the fibril that exhibits a regular periodicity.

Figure 2.7: Schematic representation of a tendon fibril. The spontaneous lateral arrangement of the collagen molecules results in an overlap of the molecules (labeled with O). The gap region (labeled with G) appears in one molecule out of five and results in the d-period. Figure is adapted from Hodge-Petruska [23].

2.3.2 Proteoglycans

As mentioned previously, collagen fibrils are embedded in the ECM, which contains another important constituent: the proteoglycans (PGs). PGs have a core protein to which one or more glycosaminoglycan (GAG) side chains are covalently bonded. In Figure 2.8 a schematic of a PG aggregate, to which several GAGs such as dermatan sulfate (DS), chondroitin sulfate (CS) and heparan sulfate (HS) are covalently bonded. Various types of PG are present in the tendon and they constitute 1% to 5% of the tendon dry mass. One of the so-called small leucine rich PGs, decorin, is widely distributed in the tendon and it is known to modulate the formation and the final sizes of the fibrils in the ECM [24, 25, 26, 27, 28, 29]. In the tensile region of the tendon, decorin is the predominant PG, while in the region where tendon inserts or wraps around bone both small leucine rich PGs —decorin and biglycan— and larger PGs like aggrecan are present [25, 30, 31]. Decorin has only one GAG chain of either chondroitin or dermatan sulfate. DS constitutes 60% of the GAG mass in the tensile area and CS constitutes 65% of the GAG mass in the bone insertion zone [30].

GAGs are unbranched polysaccharide chains, which are strongly hydrophilic. Their high density of negative charge attracts sodium cations (Na\(^+\)) present in the
Figure 2.8: Schema of a proteoglycan aggrecan and decorin. The aggrecan is a large protein, to which several glycosaminoglycans are covalently connected to the core protein, while the decorin is the smallest PG and has only one GAG chain. Adapted from Alberts et al [32].

tissue, that osmotically attract a large amount of water in the matrix. The osmosis creates a swelling pressure that enables the matrix to bear compressive forces as in the case of the cartilage where the collagen network traps the PG chains and holds them “in place” in the ECM [33]. Thus, PGs keep the matrix hydrated [34, 35, 36]. The composition and organization of the PG-rich matrix is believed to influence the sliding of the collagen fibrils [37, 38] and be directly implicated in the viscoelastic behavior of the tendon [39].

In the ECM Scott, [40] suggested that there are interactions between PGs and collagen fibrils. Later on Ruggeri [41] proposed that PGs are like side-chains that connect the collagen fibrils enabling transfer forces between them. Decorin and its DS and CS chains, being the main PGs found in tendons, have been described by several authors as being directly implicated in the mechanical integrity of tendon [42, 43, 44, 13, 45], and have since became the main subject of study for models trying to explain the tendon mechanical response.

2.4 Structure-function models

2.4.1 Composite material theory

The relationship between the distribution of collagen fibrils and functional behavior of tendon has been investigated in numerous experimental studies. Several of these suggest that increased fibril diameter is a primary determinant of improved tendon stiffness and strength [46, 18, 47, 48]. More recent studies have shown however, that despite a generally small fibril diameter, healing tendon gains strength with an increasing degree of fibril area fraction (Figure 2.9d), and that factors apart from fibril diameter may be critical to improved tendon strength [47]. Consistent with this concept, a different study indicated that Achilles tendons of Growth/Differentiation
Factor 5 (GDF5) deficient mice showed drastically reduced functional performance while being composed of nearly normal fibril diameter distributions and fibril area fractions [49]. Generally these studies indicate that biochemical deficiencies in the non-collagenous ECM may be a primary causative factor in certain tendon pathologies.

If the tendon is considered to be a composite material made of stiff fibers embedded in softer matrix, the E-modulus of the material can be calculated with the following equation:

\[
E = E_f \frac{S_f}{S} + E_m \frac{S_m}{S}
\]  

(2.1)

\[
and \ S = S_f + S_m
\]  

(2.2)

where \( E_f \) is the E-modulus of the fibers, \( E_m \) is the E-modulus of the matrix, \( S_f \) is the surface of the fiber, \( S_m \) is the surface of the matrix and \( S \) the total area of the material. Isolated PGs are known to form a gel-like substance with a shear stiffness of about 0.05 kPa. This is eight orders of magnitude lower than the tensile modulus of collagen (2.9 ± 0.1 GPa) [50], therefore \( E_f \gg E_m \). If this equation
can be applied to calculate the E-modulus of a tendon, the collagen density should be the main source that influences its stiffness. If this is true the collagen fibril diameter without information on the fibril fraction area is not a sufficient parameter to determine tendon strength.

In fact, the mechanical properties of tendon are based not only on the orientation, density, and length of the collagen fibrils, but also on intra- and inter-molecular cross-links between other components of the ECM and the collagen fibrils [51, 50, 52, 53, 54]. The biochemical makeup of the ECM influences the functional mechanical properties including the viscoelastic characteristics of the tissue and its response to impulsive loading [9, 55]. Molecular cross-linking in connective tissue involves a complex enzymatic interplay between the ECM components during growth and maturation, and is regulated, at least in part, by physical activity [56, 57, 58, 59, 60].

Based on experimental analysis, studies support the idea that PGs connect the fibrils to transfer load [24, 41, 43, 42, 27]. After load application, a relative movement between fibrils and a corresponding change in the fibril-PG configuration was observed. Specifically, Liao showed a skewness angle of interfibrillar PGs that increased with applied load [43].

### 2.4.2 Shear-lag theory

The shear-lag theory assumes that there are perfect connections between the fibers and the matrix, and that both fibers and matrix show an elastic behavior. Thus, the shear forces between adjacent fibers, distributed uniformly along the entire length of the fiber, transfer the load into the surrounding matrix [61, 62, 63]. Connective tissue modeling [64] has introduced this concept in an effort to illuminate the role of interstitial GAGs. Thus, consistent with this theory, the cumulative effect of these weak forces could highly influence the mechanical strength of the tissue. The single fibril tensile contribution in the network would be then determined by the efficiency of the shear forces transmission between adjacent fibrils through PGs, which is related to their concentration in the structure (see figure 2.10).

Theoretical models have also been introduced based on this theory. These models quantified the binding force of decorin to the collagen fibril [45], or the force transferred between fibrils [44] in the range of some micronewtons. If we considered tendons as composite materials composed of stiff fibers surrounded by a softer matrix, the cumulative effect of these weak forces could highly influence the mechanical strength of the tissue.
2.4.3 Open question

While the actual contribution of PGs to tensile tendon mechanics remains largely undefined, a simplified estimate of the PG linkage density in a single tendon fascicle gives an understanding of the potentially important role played by PG interconnections. Since tendon fibrils have an average fibril diameter of approximately 50 nm and a d-period of 67 nm, one d-period of a single fibril can be held in a cubic volume of 50 nm $\times$ 50 nm $\times$ 67 nm. In a cylindrical fascicle with a diameter of 0.5 mm and a length of 10 mm, and assuming that half of the volume is occupied by fibrils and that each d-period corresponds to a single PG, there would be approximately 6 trillion PG linkages within a single tendon fascicle. Thus even with extremely small intermolecular forces, the capacity for a substantial cumulative effect exists.

Concerning the Achilles tendon, other studies have shown that there is a difference in GAG concentration between the tensile and the insertion regions of the tendon. Specifically, the tensile region is reflected with a larger abundance of the small PGs such as decorin, and the compressed region have a more fibrocartilaginous phenotype with large PGs of aggrecans [4]. This results in a concentration of GAGs in the insertion region that can be up to 25 times higher than in the midsubstance [30, 65, 66, 67]. This implies that the mechanical properties along the tendon might be mediated by GAG content, or may simply reflect compressive loading at the
Recent conflicting studies, comparing native and GAG-depleted tendon groups, challenge this theory. Indeed no evidence of the PG contribution to the tensile properties of the tissue were proved [68, 69, 70]. Both studies found no influence related to the concentration of PG to the mechanical response of soft tissue at the macroscale. It thus remains unclear whether or not the increase in the skewness angle of interfibrillar PGs with applied load is a conformational change in PG arrangement under stress.

These results confuse the question of how force transmission between components occurs. Some support the idea that PGs transfer force between fibrils [40, 44] while others that they rather influence the sliding behavior of the fibril [37, 71]. Another open question is whether the major source of the tendon stiffness is regulated by the collagen fibrils [46, 72], or the PG network [51, 49]. Although the mechanical properties and structure of tendon are well characterized, it remains unclear how the structure and organization of the tendon leads to its properties. In the following chapters, the tendon structure and its related functions will be analyzed from the tendon macroscopic level (1 - 10 mm), progressing to the fibril level (50 - 400 nm) in order to elucidate these questions.

References


Background


Background


Background


Background

Chapter 3

Tissue-organ level
3.1 Local strain measurement reveals a varied regional dependence of tensile tendon mechanics on glycosaminoglycan content

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Abstract:
Proteoglycans (PG) and their associated glycosaminoglycan (GAG) side-chains are known to play a key role in the bearing of compressive loads in cartilage and other skeletal connective tissues. In tendons and connective tissues that are primarily loaded in tension, the influence of proteoglycans on mechanical behavior is debated due to conflicting experimental evidence that alternately supports or controverts a functional role of proteoglycans in bearing tensile load. In this study we sought to better reconcile these conflicting data by investigating the possibility that GAG content is differentially related to tensile tendon mechanics depending upon the anatomical subregion one considers. To test this hypothesis, we quantified the mechanical consequences of proteoglycan disruption within specific tendon anatomical subregions using an optical-mechanical measurement approach.

Achilles tendons from adult mice were treated with chondroitinase ABC to obtain two groups consisting of native tendons and GAG depleted tendons. All the tendons were mechanically tested and imaged with high-resolution digital video in order to optically quantify tendon strains. Tendon surface strains were locally analyzed in three main subregions: the central midsubstance, and the proximal and distal midsubstance near the muscle and bone insertions respectively. Upon GAG digestion, the tendon midsubstance softened appreciably near the bone insertion, while elastic modulus in the central and proximal thirds was unchanged. Thus the contribution of PGs to tensile tendon mechanics is not straightforward and points
Introduction

Tendon is composed mainly of type I collagen embedded in a proteoglycan (PG)-rich extracellular matrix (ECM). Type I collagen constitutes around 60% of the dry mass of tendon and 95% of the total collagen. The fibrils are discontinuous segments that do not run the length of the tendon [1] implying that collagen fibrils must transfer forces to neighboring fibrils to be mechanically viable [2]. In stretched tendon, the overall tissue strain is larger than the strain in the individual fibrils, indicating that relative fibril movements within the matrix must take place [3, 4].

Aside from collagen, another important constituent of tendon ECM are PGs and their associated glycosaminoglycan (GAG) side-chains such as dermatan sulfate (DS), chondroitin sulfate (CS), or keratan sulfate (KS). PGs constitute 1% to 5% of tendon dry mass. One of these, decorin, is widely distributed in the tendon and is known to modulate the formation and final sizes of the fibrils [1, 5, 6, 7, 8]. Decorin is the predominant PG in the tensile region of tendon, while in the region where tendon inserts or wraps around bone other PGs like biglycan and aggrecan are also present [9, 10, 7]. Decorin has only one GAG, either CS or DS. DS constitutes 60% of the GAG mass in the tensile area and CS constitutes 65% of the GAG mass in the bone insertion zone [9].

Decorin and its sulfated GAG chain have been directly implicated in the mechanical integrity of tendon. In the tendon ultrastructural model first proposed by Ruggeri and Benazzo [11], decorin bonds are believed to interconnect neighboring collagen fibrils in order to transfer forces between them. There has been further evidence to support this model [2, 12, 13]. Conversely, some recent studies have indicated that this model may not hold true [14, 15].

In the current study we attempt to examine the functional role, if any, of GAGs in the tensile mechanics of the murine Achilles tendon. Our hypothesis was that DS/CS content would correspond to tendon elastic modulus due to GAG cross-linking of the collagen fibrils. We further hypothesized that regional variations
in tendon elastic modulus are partly due to relative differences in the anatomical distribution of GAGs. To investigate these hypotheses a method to measure local tendon strains was developed and validated, and then applied to investigate the mechanical repercussions of targeted enzymatic digestion of DS/CS.

3.1 Local strain measurement

Materials and Methods

Tendon dissection and preparation

Achilles tendons were harvested from female 19-week-old C57BL/6 inbred (wild type) mice reviewed and approved for use by the local authorities for all levels of investigation. This mouse model was selected because inbred mice present genetic homogeneity and consequently low skeletal variability [16]. Forty tendons from 20 mice were dissected with the calcaneous and the gastrocnemius/soleus muscles intact. The tendon sheaths were also maintained in order to preserve the natural anatomical structure and relative orientation of the individual tendon bundles (critical for reliable mechanical testing, unpublished data). Muscle fibers were then carefully removed to expose the intramuscular tendon fibers.

Tendons from both legs were randomly allocated to the experimental groups. All specimens were treated as independent samples since previous work in our laboratory has shown variance between contralateral sides to be similar to variance between animals (unpublished data). A control group of 20 tendons was incubated for 12 h at 37°C in 6ml of Tris Hydrochloride 0.1M Sodium Acetate 0.1M buffer (pH 8). A treated group of 20 tendons was incubated for 12 h in 6ml Tris buffer + 0.15U/ml chondroitinase ABC (C2905, Sigma-Aldrich, Switzerland), pH 8, in order to partially digest the GAG [17].

All tendons were then rinsed in phosphate buffered saline solution (PBS) and briefly soaked in a PBS / indigo ink mixture. By this method small regions of the tendon surface were “permanently” stained (in an apparently random fashion), thus providing markers that facilitated optical strain analysis. Specimens were prepared for tensile testing by bonding the intramuscular fibers to paper with cyanoacrylate adhesive, leaving approximately 5 mm of exposed tendon midsurface between the calcaneous and the bound intramuscular fibers [18]. After mechanical testing, the tendon midsurface was dissected proximally at the clamp interface and distally near the bone insertion, wrapped in saline soaked gauze and immediately frozen at -18°C for eventual analysis of GAG content.

The frozen tendon was later thawed, carefully cut into thirds, briefly soaked in PBS (approximately 5 seconds), blotted lightly with a paper towel, and immediately
weighed. All tendon wet weight was determined using the same protocol. Additionally, to define the relationship between wet weight and dry weight, three tendons were first wet-weighed as above and then weighed after lyophilization. Dry weight corresponded to 40 ± 4% of the wet weight. This is consistent with the literature [15, 19] and indicated good reproducibility of the wet weighing protocol.

After weighing, the tendon subregions were digested for 16h with 500U/ml papain solution in buffer (0.1M disodium hydrogen phosphate, 0.01M EDTA disodium salt, 14.4mM L-cysteine) at 60°C. GAG content was determined spectrophotometrically (Cary 50, Varian, Zug, Switzerland) at 525 nm following binding to dimethylmethylen blue dye (DMMB) [20] using CS as standard. DMMB binds to negatively-charged sulfated groups, such as CS/DS or KS.

**Biomechanical testing**

Mechanical tests were performed with a universal testing machine (Zwick 1456, Ulm, Germany). Specimens were clamped for testing using a previously described technique that minimizes failure at the clamping interfaces [18], with the calcaneous mounted at 30° dorsiflexion to approximate a neutral anatomical position. Tendons were tested in saline at 37°C using controlled displacement (10 mm/min). Tendons were preconditioned with 10 cycles of 0-10% nominal strain before ramp loading to failure. Non-contact measurements of tendon cross sectional area were obtained by frontal and lateral imaging of each tendon at a preload of 0.1 N with telecentric lenses (Zeiss Visionmes 16.1, Zeiss, Jena Germany, pixel resolution ∼ 0.015 mm). Cross-sectional area was determined from these measurements at the smaller measured diameter by assuming an elliptical cross-section. Force was then normalized by the cross-sectional area to yield nominal stress values.

Tendon strains were measured using both relative changes in clamp-to-clamp distance (i.e. “machine-based strain”) as well as optically measured strains in the tendon midsubstance as described in the following section. Figure 3.1 shows a parameterized material curve obtained from a typical tensile test (shown with machine-based strains). These were analyzed for ultimate stress, failure strain (strain at ultimate stress), and elastic modulus (slope of the best fit line to the “linear region” of the material curve, as defined below).

**Local strain analysis**

High-speed digital video (VT Cam, AOS Technologies AG, Switzerland) was recorded to quantify the engineering strain in the anatomical subregions illustrated in Figure 3.2. Two frames were selected corresponding to the beginning and end of
3.1 Local strain measurement

Figure 3.1: Typical stress-strain curve for an Achilles tendon during uniaxial tensile testing (left). Optical strains were extracted from video frames corresponding to points A and B indicated in the linear region of the test curve. A is the end of the toe region, defined at 0.8 N; B corresponds to 80% of the ultimate load. Displacement markers (highlighted here for emphasis) were automatically detected according to regions of high image contrast.

Figure 3.1: Typical stress-strain curve for an Achilles tendon during uniaxial tensile testing (left). Optical strains were extracted from video frames corresponding to points A and B indicated in the linear region of the test curve. A is the end of the toe region, defined at 0.8 N; B corresponds to 80% of the ultimate load. Displacement markers (highlighted here for emphasis) were automatically detected according to regions of high image contrast.

the linear region of the test curve (Figure 3.1). The first frame (A) corresponded to tendon loading at 0.8 N (approximately the end of the “toe” region), while the last frame (B) corresponded to 80% of the ultimate load — a measure that reliably marked the end of the linear tendon response.

A semi-automatic script was written to quantify surface deformation and corresponding surface strains. A minimum of 13 displacement markers were chosen on the surface, with all markers visible in both frames. After mapping these points of interest, the program connected all displacement markers with a line segment. Following loading, the resulting elongation of each marker segment was decomposed to determine the engineering strain component along the functional axis of the loaded tendon. In order to minimize pixel rounding errors, only marker pairs with a minimum separation of 30 pixels were considered (1 pixel ∼ 0.015 mm). Further, only vector angles that did not deviate more than 30° from the tendon functional axis were considered.

The accuracy and precision of the semi-automated local strain analysis was investigated using an isotropic rubber test phantom submitted to the tendon test protocol. Optically derived local strains were highly consistent with structural values obtained from the testing machine, and gave accurate and precise local strain values for all considered marker pairs with less than 5% measurement error. The
local strain algorithm was also submitted to a repeatability study, and indicated an absolute error lower than 2% for independently analyzed trials (same user) of individual force-video datasets.

**Statistical analysis**

A Lilliefors test was used to determine whether the data distribution was normal. Normally distributed data were compared using a pair-wise t-test with Holm correction for multiple comparisons. A pair-wise Wilcoxon rank sum test was used to compare groups with non-parametric distribution. Relationships between the two groups were computed with single and multiple linear regression analyses. A \( p \)-value < 0.05 was considered to be significant.
3.1 Local strain measurement

Results

GAG Digestion

The GAG assay showed that in the control group there was a statistically significant difference in GAG concentration between the subregions (Figure 3.3). Chondroitinase ABC treatment most affected the middle subregion, which lost 54% of GAG content. The proximal and distal subregions were digested of 47% and 44% of the total amount respectively. After digestion, the remaining GAG concentration was found to be similar in all tendon subregions.

![Figure 3.3: Chondroitin sulfate weight per wet tendon weight (µg/mg) for the three midsubstance regions as determined by GAG assay. The relative subregion contribution to total midsubstance GAG content is also shown as a percentage. All columns connected by a star (⋆) are statistically different at p < 0.05.](image)

Biomechanical testing

Two samples from each per group were excluded for cases in which a calcaneous avulsion or intramuscular tendon fiber tear were observed. The others failed in the midsubstance. Video analysis revealed no slippage at the clamp. In general, the natural tendons demonstrated a sudden failure of a twisted bundle that was
visible through the sheath. For the digested group, the rupture was less dramatic, and did not obviously involve fiber bundle torsion. A mean cross-sectional area of $0.58 \pm 0.14 \text{ mm}^2$ was measured for the control group, while for the digested group it was $0.48 \pm 0.10 \text{ mm}^2 \ (p < 0.05)$.

Table 3.1: Summary of the measured parameters.

<table>
<thead>
<tr>
<th>Groups</th>
<th>$L_0$ (mm)</th>
<th>Cross-sectional area (mm$^2$)</th>
<th>Ultimate force (N)</th>
<th>Ultimate stress (MPa)</th>
<th>Strain at failure (%)</th>
<th>Stiffness (N/mm)</th>
<th>Elastic modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.5$\pm$0.7</td>
<td>0.58$\pm$0.14*</td>
<td>6.4$\pm$2.1*</td>
<td>11.1$\pm$4.9</td>
<td>36$\pm$17</td>
<td>33.8$\pm$20.8</td>
<td>61.8$\pm$38.8</td>
</tr>
<tr>
<td>Digested</td>
<td>5.2$\pm$0.9</td>
<td>0.48$\pm$0.10*</td>
<td>5.0$\pm$1.8*</td>
<td>11.5$\pm$5.6</td>
<td>36$\pm$19</td>
<td>25.4$\pm$19.8</td>
<td>57.9$\pm$45.9</td>
</tr>
</tbody>
</table>

Values are expressed as mean $\pm$ standard deviation. * Statistical significant for $p < 0.05$.

Effects of GAG digestion on tensile tendon mechanics

Measurements of tendon modulus and failure behaviors satisfied tests of fit to a normal distribution and were analyzed using Students $t$-test to compare group means. Optical measurements of midsubstance tendon strains were not normally distributed according to Lilliefors test, and were thus analyzed using appropriate parametric statistical tests. As summarized in Table 3.1, a 50% digestion of GAGs over the entire tendon was associated with a significant decrease in ultimate load, but with no statistical change in ultimate tensile stress, failure strain, stiffness, or elastic modulus when strains were normalized to machine gage length (clamp to clamp distance). Conversely, normalization of data using optical strain measurements of the tendon midsubstance indicated a significant decrease of 46% in elastic modulus after digestion ($p < 0.05$, Table 3.2). In both control and digested tendons, the optically-based

Table 3.2: Elastic modulus values for the tendon midsubstance and its three component subregions, based on local strain measurements.

<table>
<thead>
<tr>
<th>Modulus MPa</th>
<th>Midsubstance</th>
<th>Proximal third</th>
<th>Central third</th>
<th>Distal third</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>117 (50;225)*</td>
<td>120 (57;221)</td>
<td>92 (45;195)</td>
<td>131 (66;242)*</td>
</tr>
<tr>
<td>Digested</td>
<td>67 (30;117)*</td>
<td>78 (34;214)</td>
<td>114 (39;245)</td>
<td>69 (21;117)*</td>
</tr>
</tbody>
</table>

$p$-value $<0.05$ 1.000 1.000 $<0.001$

Values are expressed as median (first percentile; third percentile). * Denotes a significant $p$-value at the indicated level.
3.1 Local strain measurement

Figure 3.4: (A) Median stress vs strain curves for control and GAG digested murine Achilles tendon. After digestion, the GAG-depleted group (dashed gray) shows a lower modulus than the control group (black). (B) The regression line indicates the relationship between the machine-based elastic modulus and the optically-based elastic modulus for both the control and the digested groups.

Elastic modulus was nearly twice that of the machine-based modulus. The median stress vs. strain curves for both control and digested tendon as well as the correlation between optically-based and machine-based moduli are summarized in Figure 3.4.

Figure 3.5: Box plot of (A) the strain and (B) the elastic modulus in loaded tendon, as measured in the proximal, central and distal region of the tendon midsubstance. The distal subregion of GAG-depleted tendon was statistically different from corresponding controls with $p$-value < 0.05.
A more localized analysis of the midsubstance subregions revealed non-homogeneous distributions of strains in the tendon (Figure 3.5A). Enzyme digestion differently affected the more proximal subregions of the midsubstance when compared to the most distal third. Specifically, GAG digestion did not affect the strain distributions within the proximal and central midsubstance but significantly increased the tendon strains closer to the bone insertion. Thus the observed 46% decrease in tendon modulus associated with GAG removal was attributable to decreased modulus towards the bone insertion rather than over the entire tendon (Figure 3.5B).

**Discussion**

In this paper we have investigated the mechanical role of GAGs in tendon under tensile load. Local mechanical properties were correlated to local GAG content in distinct subregions of the tendon. It was shown that partial removal of CS/DS does not alter distribution of strains in the proximal and middle portions of the midsubstance, but does have a significant effect at the distal region closer to the bone insertion.

While localized analysis of tendon optical strains were correlated to clamp-to-clamp measurements of tendon strain (control tendons: $R^2=0.63$, $p < 0.001$) the strains in the midsubstance were typically half that of machine based tendon strains. Some of the discrepancy between optical and machine strain measures may be attributed to slippage at the tendon/clamp interface — a well known technical issue that can plague the mechanical testing of soft tissues [21, 22]. However analysis of video data indicated a lack of clamp slippage with our employed clamping technique [18]. Therefore we rather attribute the optical/machine strain measurement discrepancy in the current study to relative movements between internal tendon substructures.

From the recorded video we were able to see that murine Achilles tendons were formed from intertwined primary fiber bundles that twisted in a coordinated fashion under load. These bundles had unique functional axes, and were surrounded and interconnected by fascias. Our measurements were not sufficiently sensitive to detect fiber uncrimping [23, 24, 25] or to determine whether inter-fascicle slippage occurred [26, 27, 28, 29]. These factors, as well as collagen fibril slippage at the ultrascale [2, 3, 30], have been described as providing a potentially important functional contribution to macroscopic tendon behavior.

Although optical measurement of soft tissue strains is becoming more standard and can improve analytical precision in examining tendon substructures, these mea-
measurements are still limited by the assumption that surface strains are indicative of the strains inside the various tendon subregions. As was alluded to above, hierarchical collagen structures yield complex tissue stress and strain patterns due to progressive recruitment and eventual failure of constituent fiber bundles and do not generally represent the response of a continuum material. Despite this important limitation we believe that useful insight into local tendon mechanics can still be extracted.

Some biomechanical studies on the murine Achilles tendon [31, 15, 16] have reported higher strains at failure, lower failure loads and a lower stiffness than was measured in the current study. This discrepancy might be a consequence of the experimental set-up, since our tests were performed in PBS at 37°C, while the cited studies reporting stiffer/stronger tendons tested at room temperature and moistened with lightly sprayed PBS. Since we focus here on relative differences between natural and GAG-depleted tendon under equivalent (and approximately physiological) loading conditions, concerns regarding discrepancies in the mechanical testing approach are of secondary importance.

We observed a statistically lower concentration of GAGs in the proximal subregion compared to the two subregions closer to the bone. While this is consistent with the doctrine that the concentration of GAGs depends on the level of compressive forces acting in that region, the differences we observed were considerably smaller than previously reported values of GAG content at the bone insertion [32, 33, 9]. This discrepancy may be attributed to the fact that the tendon (midsubstance) considered in the present study was dissected proximally from the actual bone-tendon interface where a more fibrocartilage-like ECM is present [10].

The tensile mechanical effects of GAG digestion were indicated as a decrease in ultimate load and elastic modulus. However, a focused analysis of strains within three subregions of the midsubstance indicated that the change in modulus was not uniformly distributed over the entire tendon. More precisely, the optical strains increased toward the bone insertion upon GAG removal, while the central and proximal subregions were relatively unaffected. This inhomogeneous mechanical response to partial GAG removal may reflect the complexity of the transition from tendon to bone [34]. This histological and mechanical transition complicates the interpretation of our data with respect to a mechanical (e.g. cross-linking) role of GAG in tendon. Interpretation of the mechanical effects of enzyme digestion in the central portion of the midsubstance is more straightforward. In contrast to the bone region the central subregion is constituted of a largely homogenous cell phenotype, and these fibroblast-like cells produce a parallel-fibered ECM that is optimized to effectively
transmit tensile load.

Our finding that the tensile properties of the central portion of the tendon mid-substance are insensitive to DS/CS content supports some recent mechanical studies on individual tail tendon fascicles [15] and intact human ligaments [14], in which GAGs have been enzymatically removed. Importantly, these findings collectively contradict hypotheses that have emerged from modeling and ultrascale experimental studies investigating a potential mechanical role of decorin as a cross-linker that is fundamental to tensile tendon mechanics [2, 12, 13, 11]. It is well established that decorin structurally interconnects type-I collagen fibrils at discrete binding periods, but our results indicate that these interconnections (or lack of them) do not quantitatively contribute to tensile tendon mechanics. We conclude rather that decorin content is probably associated with other well-described functional roles such as tissue hydration [32] and structural organization [5]. By regulating tissue water content, cell mediation of decorin content may partially dictate tendon mechanics by promoting/inhibiting inter-fibril gliding or through dissipative (viscoelastic) mechanisms that were not directly investigated here.

In conclusion, our results indicate that GAG content may affect elastic tensile tendon mechanics in the distal third of the midsubstance, but apparently not in more proximal midsubstance subregions. This study presents evidence against an essential role of PGs as collagen fibril cross-linkers in resisting tensile load. It must be noted that more complex mechanical behaviors (such as stress relaxation, creep, fatigue resistance, shock dissipation, etc.) may in fact be regulated by CS/DS content, and there is some evidence indicating that GAG composition reflects these functional demands [6]. Finally, the demonstrated variability in structure and function of the various tendon subregions highlights the importance of a local strain measurement at regions of interest and indicates that structural measurement is by itself inappropriate for assessing tendon midsubstance behavior.

**Conflict of interest statement**

All authors have no conflict of interest and nothing to disclose.

**Acknowledgement**

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Tissue-organ level


Chapter 4

Material level
4.1 Collagen fibril morphology and mechanical properties of the Achilles tendon in two inbred mouse strains

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Abstract:
The relationship between collagen fibril morphology and the functional behavior of tendon tissue has been investigated in numerous experimental studies. Several suggest that larger fibril radius is a primary determinant of higher tendon stiffness and strength; others have shown that factors apart from fibril radius (such as fibril-fibril interactions) may be critical to improved tendon strength. In the present study, we investigate these factors in two inbred mouse strains that are widely used in skeletal structure-function research — C57BL/6J (B6) and C3H/HeJ (C3H). The aim was to establish a quantitative baseline that will allow one to assess how regulation of tendon extracellular matrix architecture affects tensile mechanical properties. We specifically focused on collagen fibril structure and glycosaminoglycan (GAG) content — the two primary constituents of tendon by dry weight — and their potential functional interactions. For this purpose, Achilles tendons from both groups were tested to failure in tension. Tendon collagen morphology was analyzed from transmission electron microscopy (TEM) images of tendon sections perpendicular to the longitudinal axis. Our results showed that the two inbred strains are macroscopically similar, but with higher elastic modulus in C3H mice (p < 0.05). Structurally, C3H mice showed a larger collagen fibril radius compared to the B6 (96 ± 7 nm and 80 ± 10 nm respectively). Tendons from C3H mice also showed smaller specific fibril surface (0.015 ± 0.001 nm/nm² vs. 0.017 ± 0.003 nm/nm² in the
B6 tendons, $p < 0.05$), and accordingly lower concentration of GAGs ($0.60 \pm 0.07 \mu g/mg$ vs. $0.83 \pm 0.11 \mu g/mg$, $p < 0.05$). As in other studies of tendon structure and function, larger collagen fibril radius appears to be associated with stiffer tendon, but this functional difference could also be attributed to reduced potential surface area exchange between fibrils and the surrounding proteoglycan rich matrix, in which the hydrophilic GAG side chains may promote inter-fibril sliding. This study provides an architectural and functional baseline for a comparative murine model that can be used to investigate the genetic regulation of tendon biomechanics.

Keywords:
Achilles tendon, biomechanics, collagen fibrils morphology, glycosaminoglycans, inbred strain

Introduction

The relationship between the distribution of collagen fibrils and functional behavior of tendon has been investigated in numerous experimental studies. Several of these suggest that increased fibril radius is a primary determinant of improved tendon stiffness and strength [1, 2, 3, 4]. More recent studies have shown however, that despite a generally smaller fibril radius in healing tendon, it gains strength with an increase in the fibril area fraction, and that factors apart from fibril radius may be critical to improved tendon strength [3]. Consistent with this concept, an earlier study indicated that Achilles tendons of growth differentiation factor 5 (GDF-5) deficient mice showed drastically reduced functional performance while being composed of nearly normal fibril radius distributions and fibril area fractions [5]. Generally these studies indicate that biochemical deficiencies in the non-collagenous extracellular matrix (ECM) may be a primary causative factor in certain tendon pathologies.

Two main classes of extracellular macromolecules make up the tendon matrix: proteoglycans (PGs), which play a complex role in force transmission and maintenance of tendon tissue structure [6, 7], and collagen fibrils. Studies that have investigated the relationship between structural and mechanical properties have generally focused on one major component; either collagen or PGs, with studies focusing on collagen fibril morphology being more common. Some of the earliest studies have related collagen fibril morphology and mechanical properties [1, 8, 9], and these suggest that tendon ability to bear high stress is positively related to the percentage of large fibrils in the tissue, while creep-inhibition properties are related to the percentage of small fibrils. These studies suggest that tendon is thus able to fulfill
its specific functional demands by regulating an appropriate collagen architecture. On the other hand, various studies report that a high concentration of negatively charged PG side chains — the glycosaminoglycans (GAGs) — is related to an increased osmotic pressure, and enhanced tissue hydration [10]. This tissue hydration could separate individual fibril bundles and minimize shear stress as fibrils move relative to one another [11]. In other words, PGs may lubricate the structure at the molecular level, facilitate inter-fibril slippage, and thus make the tissue more compliant.

In the present study, we evaluated the ultrastructural and mechanical properties of the Achilles tendon in the two inbred mouse strains C57BL/6J (B6) and C3H/HeJ (C3H). Adult B6 and C3H mice have a similar body size and weight and their skeletons are similarly sized, but they show morphologically distinct skeletal traits [12]. Despite differences in adult peak bone density and whole bone cross-sectional area, they have similar bone mechanical properties at the structural level [13]. We hypothesized that, as with bone, these two genotypes would show differences in tendon architecture while exhibiting similar macroscopic mechanical behavior — and thus provide a useful comparative (genetic) model for investigating tendon structure-function. In quantifying tendon structure, we focused on defining the collagen fibril morphology in terms of both fibril radius and the potential for fibril-to-fibril interactions. This allows one to extract information about the relative functional contribution of not only the collagen, but also other macromolecules like the PG decorin, which attaches to the surface of the collagen fibrils at regular intervals [14]. The aim of this study was to thus define an architectural and functional baseline for comparison between two inbred mouse strains as a model for a genetic basis of tendon structure and function.

Materials and Methods

Animal model

This study was reviewed and approved by the local and state authorities for all levels of animal investigation. We used two inbred mouse strains with markedly different skeletal phenotypes, where B6 represents the low bone mass and C3H represents the high bone mass phenotype [12, 15, 16, 17, 18]. Ten B6 and ten C3H female mice were purchased from the Harlan breeding facility (Horst, The Netherlands). Mice were sacrificed by CO$_2$ inhalation at 19 weeks of age. The animals were then stored at -20°C and thawed at room temperature just before dissection of the Achilles tendon. Twenty Achilles tendons were harvested from each of the two strains.
Collagen fibril structure analysis

The tendon connected to the soleus muscle was dissected and fixed in a solution of 2% glutaraldehyde for 30 minutes and rinsed three times with 0.1M cacodylate buffer (pH 7.4). After fixation, tendons were postfixed with 2% osmium oxide for 30 minutes and then rinsed three times in distilled water. The samples were stained with ethanolic uranyl acetate (2% uranyl acetate/50% ethanol) overnight and then twice rinsed with distilled water. Dehydration was continued through a cold (4°C), graded ethanol series. The tendons were infiltrated and embedded through propylene oxide in a fresh mixture of Epon and ethanol; polymerized for 2 days in the oven at 60°C.

Figure 4.1: Representative transmission electron micrograph for the two inbred strain; B6 and C3H, and its binarized reconstruction on the right. The binarized reconstruction is processed to calculate the fibril radius, the collagen area fraction, the interfibrillar distance, the specific fibril surface and the fibril contact area. The color bar indicates the pixel size of the fibril radius that is converted to nm. The black bar indicates 400 nm.
4.1 Collagen fibril morphology

Sections were cut with 50 to 70 nm thickness perpendicular to the longitudinal axis of the tendon with a diamond knife mounted to an ultramicrotome (Reichert Ultracut E, Wetzlar, Germany). Sections taken approximately 1 mm proximal from the calcaneous attachment were mounted to Butvar-coated 1 × 2 mm slot grids, contrasted with 2% uranyl acetate and lead citrate according to the method of Reynolds [19]. The region of interest (ROI) was identified at ×5’000 magnification. Using a transmission electron microscope (TEM), seven micrographs per tendon were then taken at a final magnification of ×40’000 as shown in Figure 4.1. The fourteen animals thus yielded a total of 98 micrographs at 100 kV.

A script was written in Matlab (The MathWorks, version 7.4) to automatically quantify morphological parameters, as schematized in Figure 4.2. The original image was automatically calibrated, treated with a Gauss filter (width, σ = 1.2; support, s = 3 voxels), and thresholded according to the algorithm of Trussell [20] to give a binary image that was stored and used to measure the geometric parameters of the fibrils. The measured parameters described below can be divided in two groups: classic parameters widely described for collagen morphology characterization (collagen fibril radius, collagen area fraction), and newly introduced morphometric parameters describing the potential for lateral interactions between the collagen fibrils (interfibrillar distance, specific fibril surface, fibril contact area). The collagen fibril radius of each collagen fibril was represented by the minor collagen radius, to avoid any error due to oblique fibril angle in relation to the image plane. Additionally, the fibril radii of the 7 images from each animal were cumulatively summed to give an overall indication of the distribution. The cumulative curves were shown to give a supplementary overall indication of the distribution within the two strains. Collagen area fraction was defined as the total fibril area in the image divided by the total image area (yielding a dimensionless parameter). The interfibrillar...
**Material level**

**distance** was defined as the mean spacing between fibrils expressed in nm. This value was calculated by applying a distance transform on the gap between the fibrils [21], resulting in the average distance between a given fibril and all neighboring fibrils that can be connected by an uninterrupted line segment (Figure 4.2C). The **specific fibril surface**, expressed in nm/nm², is a normalized value consisting of the total perimeter of all fibrils within a given image divided by the total image area (Figure 4.2D). The **fibril contact area** is defined as the percentage of the total fibril perimeter that is in contact with neighboring fibrils (Figure 4.2E). Two fibrils are considered in contact if the total perimeter of each cannot be distinguished as an isolated circle on a binary image. The exchange area between the collagen fibrils and the outer ECM is calculated by applying a Canny filter on the filtered image [22], the Canny filter detects the edges of the fibrils by applying two thresholds to the gradient: a high threshold for low edge sensitivity and a low threshold for high edge sensitivity. The filter starts from the low sensitivity and then grows it to include connected edge pixels from the high sensitivity result to fill in gaps in the detected edges.

Table 4.1: Summary of measured morphological parameters measured for B6 and C3H mice

<table>
<thead>
<tr>
<th></th>
<th>Mean radius (nm)</th>
<th>Collagen area fraction (%)</th>
<th>Interfibrillar distance (nm)</th>
<th>Specific fibril surface (nm/nm²)</th>
<th>Fibril contact area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>79.7±9.7*</td>
<td>72.5±3.8*</td>
<td>10.8±1.6</td>
<td>0.017±0.003*</td>
<td>33.0±4.2</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>95.7±7.0*</td>
<td>73.1±3.0*</td>
<td>10.9±2.1</td>
<td>0.015±0.001*</td>
<td>34.5±2.5</td>
</tr>
</tbody>
</table>

* Statistical significant for p < 0.05

**GAG analysis**

To relate the morphological measures of potential for fibril-to-fibril interactions to PG content, we assessed GAG content. Tendons were weighed and digested for 16 h with 500 U/ml papain solution in buffer (0.1M disodium hydrogen phosphate, 0.01M EDTA disodium salt, 14.4mM L-cysteine) at 60°C. GAG content was determined spectrophotometrically (Cary 50, Varian, Zug, Switzerland) at 525 nm following binding to dimethylmethylene blue dye (DMMB) [23] using chondroitin sulfate as the standard.

Cupromeronic blue dye (CB) was used to visualize GAG architecture with TEM according to reported studies [24, 25]. Briefly, freshly dissected Achilles tendons connected to the soleus muscle were stained overnight with 1% CB in 0.2M acetate...
buffer (pH 5.6). After rinsing in the same solution without CB, samples were immersed in 0.5% Na₂WO₄ in buffer for 1 h and then overnight in 0.5% Na₂WO₄ in 30% ethanol. To stain the collagen fibrils, samples were stained with 1% uranyl acetate and fixed with the Reynolds technique as mentioned above [19].

**Biomechanical testing**

Mechanical tests were performed with a universal testing machine (Zwick 1456, Ulm, Germany). Tendons were preconditioned with 10 cycles of 0-10% nominal strain before ramp loading to failure at 10 mm/min, as described earlier [7]. Force displacement data from the ramp-to-failure test were analyzed for ultimate stress, failure strain, and elastic (E) modulus.

**Statistical analysis**

A Lilliefors test was used to determine whether data were adequately fitted to a normal distribution. For normally distributed data, an unpaired t-test was used to test the significance of the two strains on measured morphological and mechanical parameters. Otherwise a Wilcoxon rank sum test was used to test significance between the strains. A p-value < 0.05 was considered to be significant.

Two-sample Kolmogorov-Smirnov testing was used to compare the distribution of the collagen fibril mean radii between the cumulative curves of the two inbred mouse strain. The distributions were considered significantly different for p-values < 0.05.

**Results**

**Collagen fibril structure analysis**

Images from the two inbred strains were compared, and the results are summarized in Table 4.1. Results show that C3H mice have a significantly larger mean fibril radius than B6, while the collagen area fraction of the ECM and the lateral spacing between fibrils can be considered the same for both groups. Specific fibril surface was significantly higher in the B6 strain, but effective fibril contact area was statistically similar. Figure 4.3 shows cumulative distribution curves for fibril radii for each mouse. The curves for the B6 strain have a steeper slope, indicating that they contain more small-radius fibrils than the C3H. There was a statistically significant inter-strain difference for the curves.
Figure 4.3: Cumulative distribution curves for B6 and C3H mice. B6 generally contain a majority of small fibrils, while C3H have a more uniformly distributed fibril radius. Histograms of the collagen fibril distribution for both groups. \( n \) is the total number of fibrils.

**GAG analysis**

The GAG assay indicated a statistically significant difference in GAG concentration (chondroitin sulfate and dermatan sulfate); for B6 \( 0.83 \pm 0.11 \, \mu g/mg \) and for C3H \( 0.60 \pm 0.07 \, \mu g/mg \) (\( p < 0.05 \)). Representative transmission electron micrographs of the longitudinal sections of the two inbred strain are shown in Figure 4.4.

**Biomechanical testing**

During mechanical testing, all tendons failed in the midsubstance. Video analysis revealed no slippage at the clamp. The mechanical parameters are summarized in Table 4.2. Both groups had a similar tendon length (5.5 ± 0.7 mm for the B6 and 5.5 ± 0.6 mm for the C3H), and cross-sectional area (0.49 ± 0.10 mm\(^2\) for the B6 and 0.54 ± 0.14 mm\(^2\) for the C3H). The C3H group exhibited a median E-modulus of 140 MPa while B6 was significantly lower; 123 MPa (\( p < 0.05 \)). No statistically significant difference in either mean failure strain (\( \epsilon_{B6} = 36 \pm 17 \% \), \( \epsilon_{C3H} = 33 \pm 9\% \)) or mean ultimate stress (\( \sigma_{B6} = 10 \pm 4 \, MPa \), \( \sigma_{C3H} = 13 \pm 4 \, MPa \)), was observed.
4.1 Collagen fibril morphology

Figure 4.4: Representative transmission electron micrograph of a longitudinal section of the Achilles tendon for (A) B6 and (B) C3H at a magnification of $\times53000$. The black lines between the fibrils are the stained GAGs. (C) Means and standard deviations of sulfated GAG content in the two groups. * statistically significant for $p$-value < 0.05.

Assessing structure and function

Table 4.1 and Table 4.2 summarize all the measured values of the two strains of inbred mice. The two groups had similar gross anatomical characteristics, with no statistical difference seen in cross-sectional area, or tendon length at the given pre-load. No differences were observed in stiffness, maximal force and max strain. Mechanically, the C3H showed a statistically higher elastic modulus than B6 ($p < 0.05$). Ultimate stresses were higher in the C3H, but this was not statistically significant.

Data demonstrated morphological differences at the collagen fibril level. The C3H group had significantly larger mean fibril radii and lower fibril perimeter than the B6, while the B6 showed a higher specific fibril surface ($p < 0.05$). Other morphological parameters such as collagen area fraction, interfibrillar distance and fibril contact area did not show statistical differences.

Thus, the B6 group tendons were similarly sized, but generally less stiff than the C3H tendons, which had fewer, larger collagen fibrils and smaller potential for lateral interactions between fibrils or between fibrils and the other components of the ECM.
Table 4.2: Summary of the measured mechanical parameters for B6 and C3H mice. The E-modulus is showed as a median (first quartile; third quartile).

<table>
<thead>
<tr>
<th>Groups</th>
<th>L₀ (mm)</th>
<th>Area (mm²)</th>
<th>Force max (N)</th>
<th>Failure strain (%)</th>
<th>Stiffness (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>5.5±0.7</td>
<td>0.54±0.14</td>
<td>6.0±2.3</td>
<td>36±17</td>
<td>52±36</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>5.5±0.6</td>
<td>0.49±0.10</td>
<td>6.4±1.8</td>
<td>33±9</td>
<td>75±28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ultimate stress (MPa)</th>
<th>E-modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>10.4±3.9</td>
<td>123 (52:203)*</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>13.0±3.7</td>
<td>140 (112:198)*</td>
</tr>
</tbody>
</table>

* Statistical significant for p < 0.05

Discussion

Tendon stiffness is central to its proper function [26] and how tendon structure relates to function is critical to understanding tendon pathology [27, 28, 29, 30] as well as in developing strategies for healing [3, 31]. In this work, we introduce a potentially useful comparative tendon structure-function model in two inbred mouse strains by describing the collagen morphology and the corresponding mechanical properties of the soleus tendon. The mechanical properties of this load bearing, elastic tendon were considered with respect to the observed morphology of the collagen fibrils, where it was found that tendons with larger diameter collagen fibrils and lower specific fibril surface also exhibited a higher elastic modulus.

Previous studies have investigated the morphology of collagen fibrils from cross-sectional area TEM images. Some have focused on the collagen radius in ruptured tendons [32, 27], some on the changes to the collagen during collagen assembly [33]. Others have focused on the collagen fibril diameters in different anatomical structures and their relationship to corresponding function [34]. In all of these studies fibril morphology has been described primarily in terms of fibril diameter. In the current study we introduce new morphological parameters that permit insight into potential fibril-fibril interactions and fibril-non-fibrillar matrix interactions such as the interfibrillar distance between the fibrils, the specific fibril surface, and direct fibril-fibril contact (see Figure 4.2, and Table 4.1). These parameters not only give a more thorough description of the collagen morphology, but may also offer better insight into structure-function relationships.

Studies analyzing structure-function in tendons usually separately consider the
4.1 Collagen fibril morphology

The functional relevance of collagen fibril morphology and proteoglycan content, and thus neglect potential functional interplay between collagen and PGs. By expanding our quantification of collagen fibril morphology to include descriptors relevant to fibril-fibril interactions, we have attempted here to better bridge conceptual treatment of these two components that are known to influence each other during tissue formation [10, 35, 36], and may influence each other with regard to mechanical function [37, 38, 39, 7, 40].

Our mechanical and morphological comparison between B6 and C3H showed that despite very similar collagen area fractions, C3H tendons are considerably stiffer than the B6. On one hand, this could be attributed to larger fibril size; the B6 mouse strain has Achilles tendons constituted by smaller fibrils. Alternatively, the lower modulus in the B6 mice may also be partly explained by greater collagen fibril surface area and fibril-hydrated ECM interactions; since collagen fibrils are densely linked to hydrophilic PG chains where their surface is in contact with the matrix, these chains may facilitate relative movement of the fibrils through the matrix [41].

Considering that the collagen fibrils are linked to PG chains where their surface is in contact with the matrix, we surmise that the amount of PGs present in the tendon is directly correlated to the relative fibril surface area. Our results described in Table 4.1 and Table 4.2 show that C3H mice, which have larger E modulus and lower total fibril surface area, also have a lower measured concentration of sulfated groups (DS and CS). Thus, less PG may be associated with a higher elastic modulus. As discussed below, this is in contrast with other studies that invoke the structure function model that the PGs may actually interconnect neighboring fibrils [42, 43, 14, 44], transfer forces between them [38, 45], and lead to stiffer tendons [39].

Given comparable macroscopic/microscopic tendon architecture in the two evaluated inbred strains, classic composite theory suggests that the observed similarity in collagen area fraction (e.g. effective collagen content) in both groups would yield a similar mechanical response in terms of elastic modulus and failure stress [46, 47, 41]. In fact, the C3H demonstrated significantly higher elastic modulus, but a similar failure behavior. This suggests that a more sophisticated model of tendon structure function is required.

More elaborate models of tendon structure-function have been introduced, taking into account fibril-fibril interactions through matrix cross-linking by proteoglycan GAG sidechains [48, 39]. In the present study, the close similarity in both mean interfibrillar distance and fibril-fibril contact area suggests that there would be no relative advantage in either group with regard to direct fibril-fibril interactions (lat-
eral force transfer between fibrils) that might promote a higher elastic modulus of the C3H tendons. In fact according to this model the higher relative GAG content in the B6 mice should confer stiffer tendon properties, which is in direct contrast to our experimental observations. Thus the present study suggests that tendon structure-function is also not adequately described by the theoretical framework of proteoglycan mediated collagen fibril load sharing. This lends support to our recent studies indicating that GAG side chains of small leucine rich proteoglycans do not play a dominant role in tensile mechanics of the tendon midsubstance [7, 49].

Until now, most studies investigating GAG mediated fibril-fibril interactions have relied upon either PG knock-out mice [40] or enzyme digestion of the PG side chains [50, 51, 7, 49]. Cross-sectional comparisons of structure and function in two different inbred wild type strains can complement such studies by avoiding problematic limitations inherent in these approaches. For instance, a “targeted” enzymatic digestion of the GAG component does not guarantee that another component will not be affected and show secondary effects, such as swelling of the collagen fibers [52]. Also in studies with knock-out mice, the lack of a certain gene does not ensure that redundant processes will not compensate its loss and restore function [53, 40].

Our choice of inbred mouse strains with regard to a structure-function baseline in tendon is rooted within a history of studies investigating bone morphology of different inbred strains to correlate them with their mechanical response [12, 15, 16, 17, 18]. B6 and C3H have been identified as a model to study the genetic factors in osteoporosis [54]. Since tendon to bone healing remains a pressing clinical challenge [55, 56], this model may be enlarged to permit a broader view of how these two connective tissues influence each other.

It should be noted that our previous studies have revealed that heterogeneous histological structure (e.g. tendon regions nearer to the bone and muscle insertions) has important implications with regard to the mechanical influence of proteoglycans on tensile mechanics [7]. The aim of the present work was to focus on a specific subregion of the tendon with more homogeneous tissue architecture, in hopes of reducing the parameters considered in our structure-function analysis. While we intentionally focused the scope of the present investigation to the tendon midsubstance, quantitative investigation of the transition between tendon and muscle or tendon and bone remains ground for future work.

To conclude, inbred C3H mice have higher elastic modulus, larger mean fibril radius, lower fibril surface area, lower GAG content, similar collagen area fractions, and similar direct fibril-fibril contact compared to B6 mice. This study thus establishes a useful baseline for further investigation of the influence of tendon morphology
on tendon mechanical properties in two inbred strain populations that are increas-
ingly used as models for orthopedic disease. This baseline forms a foundation for
future studies into the genetic regulation of tendon modeling and remodeling. It
will also aid in the interpretation of existing studies that have employed selective
chemical degradation of tendon ECM components, and transgenic animal models
used to understand how tendon derives its mechanical characteristics from its basic
architecture and biochemical composition.

Acknowledgements

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fold internal architecture design to match bone elastic properties with desired

deficiency in mice alters the ultrastructure, mechanical properties and compo-


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

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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.

Keywords:
collagen fibril, atomic force microscope, collagen network, tendon

Introduction

The mechanical properties of tendon must be optimized to avoid injury and efficiently transfer load from muscle to bone [1]. Tendon is hierarchically structured (Figure 4.5), 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 [2]. As the primary load-bearing protein in tendon, the nature of collagen load distribution has been widely investigated at multiple size scales [3, 4, 5, 6]. Particular attention has been directed toward understanding the behavior of the collagen fibrils themselves [7] as well as how the bundled collagen fibrils collectively respond to applied macroscopic loads [8].

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 [9, 10]. More complex anatomical structures composed of intertwined fibers have also been characterized [11, 12] and modeled [13, 14] 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 [8, 3, 7]. Although mechanical properties of single fibrils have been investigated by one-dimensional tensile testing using optical tweezers [15] and atomic force microscopy (AFM) [16, 17], the nature by which collagen fibrils interact within their network to accommodate applied tendon strains remains largely unknown [3]. Part of this uncertainty lay in the 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 (Figure 4.5). 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 [18, 11, 19]. The relative straining associated with collagen molecule stretch can be monitored by assessing d-period length changes [3, 7].

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 [20, 21, 22], AFM on isolated fibrils [16, 23], or scattering X-ray spectroscopy on tendon fibers [8, 3, 24, 7]. 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 [25, 26]. X-ray spectroscopy has been successfully used to infer collagen fibril strain in loaded tendon fibers [8, 3, 7], 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 [16, 23], 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.
Materials and Methods

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 [27, 28, 29], 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 [26], 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 (Figure 4.5) corresponding to reversible (non damaging) deformation at the fibril level [8] 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 hours until the structure was fixed [30].

The applied load and corresponding mechanical strain over eight hours were recorded for each tendon (see Figure 4.5). Afterwards the tissue was rinsed with PBS, embedded in Tissue-Tek (Tissue-Tek, 4583 Compound, Sakura Finetek Europe, Zoeterwoude, Netherlands) for at least 12 h at -20°C and sectioned in the longitudinal plane with a cryostatic microtome (SLEE, Mainz, Germany) at -20°C [31]. Twenty micrometer thick frozen sections were attached to a polylysine-coated glass slide (SuperFrost, Menzel-Gläser, Braunschweig, Germany) and dehydrated in an oven at 60°C. For each fixed tendon, 3 slices from the tendon midsubstance (central third) over a length of 60 µm were analyzed as described below.
4.2 Collagen fibrils’ role in tendons behavior

Figure 4.5: 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).

Atomic force microscopy measurements

Possible differences in the d-period between different parts of the same tendon were measured by comparing planar sections cut parallel to the load axis. AFM experiments were carried out with an Asylum Research MFP-3D-SA (USA), at room temperature, using titanium and platinum coated silicon AFM tips on a triangular cantilever with a nominal spring constant of 2.35 N/m (NSC11/Ti-Pt; MikroMasch, Madrid, Spain). Images of collagen fibril topography were acquired in contact mode at a scanning rate of 0.5 Hz in air on the dehydrated thin-sectioned samples.

From each slice, a minimum of five 2μm × 2μm regions of interest (ROI) were analyzed. All height images were flattened and equalized with WSxM 4.0 Develop 11.4 software (Nanotec Electronica S.L.) [32], as displayed in Figure 4.6a. For each ROI, between 5 and 10 fibrils were randomly identified for analysis. For each analyzed fibril, a line of analysis was selected on the long-axis of the fibril in the X-Y
imaging plane, and the two-dimensional profile in the z-axis was plotted (see Figure 4.6b). The d-period was then calculated from this plot using a Fourier transform to reveal the periodic banding frequency [23], such as that shown in Figure 4.6c. Reproducibility tests were performed by measuring the same fibril seven times starting from the capture of two-dimensional profile to the d-period measurement. The measurement of the d-period length for a single fibril was found to be reproducible within 0.3%. Intra-image (or intra-ROI) differences in the d-period length were measured to investigate differential responses among neighboring collagen fibrils. If the measured strains could not be fit with a normal distribution, they were considered as an inhomogeneous response to the applied load. Intra-tendon differences in mechanical response were also statistically tested to analyze strain differences in the anatomical regions (i.e. regions randomly distributed on the whole tendon area).

To compare across tendon strain conditions (0% 5% 10% strain groups), all d-period lengths of measured collagen fibrils within a given tendon were pooled. The reference d-period length in tendons fixed at 0N was determined and afterwards used as the control group for statistical comparison. All d-period lengths were accordingly normalized to the control d-period length to calculate the d-period strain.

Fibril diameter and spacing between neighboring fibrils was also measured in both unloaded controls and loaded test samples. Here the diameters of the fibrils were measured using the cross-sectional profile of the fibril as shown in Figure 4.7 [23, 33].
Only fibrils with clearly exposed surface contours were considered in the diametric analysis. The diameter was measured as the transverse distance (perpendicular to the long fibril axis) between the troughs in the tip-trace corresponding to the sides of a fibril profile.

Figure 4.7: (A) AFM height image of collagen fibrils taken in contact mode, (B) the two-dimensional profile of the dash line, and (C) schematic interpretation of fibril arrangement from the profile. Some fibrils can be easily recognized on the profile (e,f,g,h), while others have a more complicated arrangement and do not show a clear round profile. In (C) the 2D profile from inset (B) is displayed to show the correspondence with the collagen fibril diameter. On the fibrils the AFM trace is drawn to scale, while in the line above the trace is amplified 33 times. The pink semi-circles represent the tip in contact to the surface.

Statistical analysis

The d-period measurements collected for each ROI were analyzed with a Lilliefors test to examine homogeneity of fibril loading within the ROI. Homogeneity across different anatomical regions of a given tendon was similarly investigated using Lilliefors test to verify data normality. Finally, Lilliefors test was performed to verify
Table 4.3: Relative homogeneity of collagen fibril loading in tendon (indicated by % of normally distributed measures). While some heterogeneity was observed in isolated regions of analysis in loaded tendons, collagen fibrils strains were generally homogeneous.

<table>
<thead>
<tr>
<th>Tendon strain</th>
<th>Tendon (normally distributed)</th>
<th>ROI (normally distributed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>5%</td>
<td>100%</td>
<td>83.3%</td>
</tr>
<tr>
<td>10%</td>
<td>100%</td>
<td>87.5%</td>
</tr>
</tbody>
</table>

that data distributions within test groups (0%, 5%, 10% applied strain) appeared normally distributed. In this case, a pairwise $t$-test with Bonferroni adjustment was then used to examine between-strain-group differences in fibril strains and fibril diameters. A $p$-value < 0.05 was considered to be significant.

Results

Load-strain curves during tendon loading to a target strain were typical of native (non-fixed) tendons, indicating a physiological material response during initial loading to the target strain. Load-time and strain-time curves (Figure 4.5), indicated that additional strain of the tendon under constant load (i.e. creep) was largely arrested by glutaraldehyde fixation within the first 20 minutes, and reached a near plateau after approximately 5 hours. AFM analysis of collagen fibril strains in a given region of interest (randomly sampled 2µm x 2µm ROI, yielding measures of 5 to 10 individual fibrils) revealed that fibril strains appeared to be consistently (and normally) distributed both within individual ROI and across the various ROI’s from an individual tendon (Table 4.3). This indicates that the collagen fibril loading within a tendon was fairly uniform, and did not vary according to anatomical location. Further, fibril mechanical conditioning within the experimental groups was apparently uniform, with little variability in the d-period fibril distribution seen in tendons loaded to the same target strain (Figure 4.8).

In mechanically strained tendons, d-period length was observed to increase with increasing applied strain (Figure 4.8, Table 4.4). The mean d-period length of unloaded tendon was $l_{0\%} = 64.45 \pm 1.83$ nm. Collagen fibrils trended toward to an elongation of 0.9% ($l_{5\%} = 65.01 \pm 1.68$ nm, $p=0.15$) at an applied tendon strain of 5% — corresponding to the end of the toe region of the tendon material curve. Fibril elongation was only observed to statistically differ for applied strains of 10%
Table 4.4: Summary of fibril structure characterization. $A,B,C,D$ statistical significant for $p$-value < 0.05

<table>
<thead>
<tr>
<th>Target tendon strain</th>
<th>0%</th>
<th>5%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-period length (nm)</td>
<td>64.45±1.83$^A$</td>
<td>65.01±1.68$^B$</td>
<td>65.89±1.09$^A,B$</td>
</tr>
<tr>
<td>Intrafibril elongation</td>
<td>0.0%</td>
<td>0.9%</td>
<td>2.1%</td>
</tr>
<tr>
<td>Fibril diameter (nm)</td>
<td>193.63±35.52$^C$</td>
<td>195.58±32.25$^D$</td>
<td>175.34±27.68$^{C,D}$</td>
</tr>
</tbody>
</table>

($l_{10\%} = 65.89 \pm 1.09$ nm, $p < 0.05$), corresponding to the linear region of the tendon material curve. Progressive collagen fibril recruitment was indicated both by a shift toward larger fibril strains and a narrowing of the fibril strain distribution at higher tendon strain (10% applied strain).

In unloaded tendons, collagen fibrils generally appeared to lie parallel to each other and the D-periods were observed as wavy lines approximately perpendicular to the fibril axis (Figure 4.9a). In loaded tendons the D-periods appeared to straighten, assuming a skewed orientation to the respective fibril axes (Figure 4.9b).

Similar to the observed changes in fibril d-period elongation, collagen fibril diameter apparently decreased with applied load. The mean fibril diameter for the control group with 0N load and 0% strain was found to be to 193.6 ± 35.5 nm. At 5% applied strain the diameter was statistically unchanged (195.6 ± 32.2 nm), but was significantly lower at 10% applied strain (175.3 ± 27.7 nm, $p < 0.05$). Comparison between all groups of target strain level did not indicate different characteristics with respect to fibril spacing, intertwined fibrils or broken fibrils. Analyzing single fibrils within an individual tendon indicated no correlation between d-period length and fibril diameter; fibril diameter was uniformly distributed over the d-period range (data not shown).

**Discussion**

While many studies have investigated tendon structure-function at the ultrascale, much remains unclear regarding the precise ultrastructural mechanisms that underlie the versatile biomechanical behaviors of tendon such as elastic energy storage and recovery after creep [3]. These mechanisms include straining, relaxation, creep, and kinematic reorientation of loaded collagen fibrils in the extracellular matrix. Although collagen fibril morphology and non-collagen matrix composition are widely believed to dictate macroscopic behavior [10, 34, 35, 11, 36, 19], how these ultrastructural aspects translate to tissue function is poorly understood. This is due in
Material level

Figure 4.8: Probability distribution of the collagen fibril d-period length as a function of the measured strain on the tendon. The d-period length is increasing with increasing total strain of the tendon, whereas the variability of the d-period length is decreasing with increasing strain.

Figure 4.9: Height AFM images of collagen fibrils taken in contact mode for (A) an unloaded tendon, and (B) a tendon stretched to 10% strain. The z-range is indicated by grayscale bar with values in nm.
4.2 Collagen fibrils’ role in tendons behavior

Figure 4.10: Plot of the applied tension for the bulk tendon, the measured d-period length of the collagen fibrils and the mean collagen fibril diameter as a function of the target strain. At 10% strain, the increase in the d-period length and the decrease in fibril diameter is statistically significant (* indicates a $p$-value < 0.05). No significant difference was found between the 0% and 5% strain groups.

part to inherent methodological limitations of experimental approaches that have been applied to quantifying collagen fibril strains under load (scattering x-ray spectroscopy [7]; scanning electron microscopy [20]; longitudinal TEM [21, 22]; isolated fibril studies using AFM [16]); While each method has yielded valuable but partial insight into collagen fibril mechanics, none of these methods has proven capable to characterize heterogeneity in collagen fibril load distribution in the tendon extracellular matrix. Available methods therefore offer little to the study of collagen fibril load sharing.

In this study we attempted to implement AFM to visualize individual collagen fibrils in murine Achilles tendons under load. We expected that the high morphometric accuracy of AFM imaging, combined with an experimental approach permitting
Material level

a “snapshot” of the strained collagen fibrils within their network could yield unique insight into the nature of collagen fibril load bearing. To enable this characterization, we quantified the periodic banding and diameters of over 200 collagen fibrils in loaded and unloaded tendon, and analyzed them to respectively assess collagen fibril straining and thinning.

We explored several hypotheses regarding an expected mechanical loading heterogeneity in the collagen network. For instance, we hypothesized that a multi-modal distribution of collagen fibril strains would be observed, and that this could be further related as a function of fibril diameter. Numerous authors (including ourselves) have conjectured that the bimodal distribution of large and small diameter fibrils may respectively enable load and creep resistance [37, 9, 25, 26, 38]. This hypothesis was not born out by our data, which indicated that the collagen network stretches in a very uniform manner, both locally and across various anatomic tendon subregions, without obvious differences in mechanical strains between neighboring fibrils or between fibrils of different diameters. In this sense our results were rather unexpected, and indicate that a refined view of diameter biased fibril recruitment is required.

Based on previous reports [3, 7, 5], the expected behavior of the collagen fibrils was of a statistically longer d-period in the loaded tendon compared to the unloaded tendons. Our study confirms these findings. We further confirm the reported discrepancy between applied macroscopic tendon strain and the resultant collagen fibril strain where overall strain of the tendon fiber appeared always larger than the strain in individual fibrils [8, 24, 7]. In the “toe region” of the material curve, tendon strain is characterized by no corresponding collagen strain. This implies that the mechanical resistance derives mostly from inter-fibril movements within the matrix, and that strain of the collagen fibrils itself is a likely a protective mechanism against overstrain. Fratzl et al attributed this behavior as the elongation of the macroscopic “crimp” that disappears after approximately 5% fiber strain [8]. After 5% strain the entire structure is then under load and the fibrils begin to actively bear incrementally applied load. For strains larger than 5%, the d-period elongation has been reported to contribute approximately 50% of the total measured fiber elongation [7].

Our own data (Figure 4.2) also indicate that fibrils start to significantly elongate only above 5% tendon strain, but that this increase in d-period strain amounted to only approximately 20% of the incrementally applied tendon strain. We attribute this difference to the fact that the tendons we assessed presented a more complex structure than those assessed in earlier studies on isolated tendon fascicles [3, 39]. Additional work will be required to clearly establish how the differences in mechanical strain between whole tendon, tendon fascicles/fibers, and collagen fibrils relate
4.2 Collagen fibrils’ role in tendons behavior

to the structural organization of the tendon.

Similar to the observed trends in fibril elongation, collagen fibril diameters also appeared unchanged at 5% strain and then statistically differed at 10% applied tendon strain. The observed decrease in mean fibril diameter corroborates an earlier study indicating smaller diameter fibrils in loaded tendons [25].

Although this study offers additional insight into the distribution of load among collagen fibrils, it does have some limitations. First, because destructive processing steps were required to gain visual access to the tendon core, AFM imaging of native (unfixed) tendon structures was not possible. Glutaraldehyde fixation was thus used to preserve native morphology of the mechanically loaded collagen fibrils. Glutaraldehyde fixation has long been relied upon as a fixative in morphological studies of collagen using electron microscopy (e.g. [40]), and this approach has more recently been utilized to fix native collagen morphology under load [41, 42, 30]. We thus believe that 8 hours of glutaraldehyde fixation of fresh (native) tendons under load adequately captured and preserved the native morphology of the loaded collagen fibrils. It should be noted that the amount of creep occurring over the course of chemical fixation was uncontrolled [30]. However, the majority of creep strain was small relative to the applied target strain (Figure 4.5), occurred mostly within the first ten minutes of fixation, and was generally consistent from sample to sample. While time dependent effects are secondary to the mechanical response of the fibers and fibril within the first 20 seconds of an applied load [3], creep loading and uncontrolled dynamics of chemical fixation may have nonetheless influenced the resultant fibril morphology. While we made an effort to minimize such effects by mechanical preconditioning, quantifying effects of and differences between creep and relaxation remain ground for future work.

As a separate limitation, the preparation of high quality slices for AFM analysis was experimentally challenging. Before sectioning, all samples were embedded in a medium containing polyvinyl alcohol which is known to form thin films. Some samples, or sample subregions, showed poor image quality (i.e. smoother “edges” of the fibrils), possibly due to surface residues, and these had to be excluded for morphometric analysis. While we have confidence in the fidelity of the image data we did not exclude the possibility that film residues may have adversely affected morphometric accuracy of the subsequent d-period and diameter measures.

In conclusion, we have presented a novel and potentially useful method for investigating the mechanical response of collagen fibrils in their parent tissue. Using this method, we have produced visual, quantitative evidence that mechanical loads in the murine Achilles tendon are approximately uniformly distributed among the

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resident collagen fibrils, apparently independent of collagen fibril diameter. In providing an accurate analysis of loaded collagen fibril strains in response to applied stress or strain, this method may provide a superior means of investigating complex structure-function hypotheses in tendon.

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References


4.3 Tendon glycosaminoglycan proteoglycan sidechains promote collagen fibril sliding — AFM observations at the nanoscale

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Abstract:
The extracellular matrix of tendon is mainly composed of discontinuous Type-I collagen fibrils and small leucine rich proteoglycans (PG). Macroscopic tendon behaviors like stiffness and strength are determined by the ultrastructural arrangement of these components. When a tendon is submitted to load, the collagen fibrils both elongate and slide relative to their neighboring fibrils. The role of PG glycosaminoglycan (GAG) side chains in mediating inter-fibril load sharing remains controversial, with competing structure-function theories suggesting that PGs may mechanically couple neighboring collagen fibrils (cross-linking them to facilitate fibril stretch) or alternatively isolating them (promoting fibril gliding). In this study we sought to clarify the functional role of GAGs in tensile tendon mechanics by directly investigating the mechanical response of individual collagen fibrils within their collagen network in both native and GAG depleted tendons. A control group of Achilles tendons from adult mice was compared with tendons in which GAGs were enzymatically depleted using chondroitinase ABC. Tendons were loaded to specific target strains, chemically fixed under constant load, and later sectioned for morphological analysis by an atomic force microscope (AFM). Increases in periodic banding
of the collagen fibrils (d-period) or decreases in fibril diameter were considered
to be representative of collagen fibril elongation and the mechanical contribution
of GAGs at the ultrascale was quantified on this basis. At high levels of applied
tendon strain (10%), GAG depleted tendons showed increased collagen stretch (less
fibril sliding). We conclude that the hydrophilic GAGs seem thus not to act as
mechanical cross-links but rather act to promote collagen fibril sliding under tension.

**Keywords:**
Tendon, Collagen proteoglycan interaction, atomic force microscopy, molecular me-
chanics, glycosaminoglycans

**Introduction**

Collagen and proteoglycans (PGs) are the principal constituents of tendon by dry
weight [1]. At the ultrascale, tendon collagen fibrils are structural elements with
diameters between 10 and 400 nm that can self-assemble until reaching hundreds of
micrometers in length [2]. The fibrils are typically aligned to the functional axis of
tendon, bringing tensile stiffness and strength to the tissue [3]. The small leucine
rich PGs in tendon (primarily decorin and biglycan) are known to regulate the mod-
ulation of growth factor activity and to facilitate organization of the extracellular
matrix during fibrillogenesis (ECM) architecture [?, 4, 5]. PGs are constituted of a
core protein to which one or more secondary glycosaminoglycan (GAG) sidechains
are covalently bonded. The GAGs found in tendons are typically dermatan sulfate
(DS), chondroitin sulfate (CS) and keratan sulfate (KS). These hydrophilic chains
play a large role in osmotic balancing of hydrostatic pressures, and thus provide
tendon with mechanical resistance to compressive loads [6, 7]. On the other hand,
the mechanical contribution of decorin other PGs under tensile load remains a point
of contention. Based on experimental observations, numerous researchers have sug-
gested that PGs may mechanically link adjacent collagen fibrils and thus play a
direct role in inter-fibril load sharing [8, 9, 10, 11, 12, 4, 13]. Theoretical models
have been further introduced that verify the mechanical plausibility of this theory
[14, 15, 16]. These models conceptualize tendon as a composite material of stiff fibers
embedded within a softer matrix, with a remarkably large cumulative mechanical
effect of weak individual PG binding forces. This substantial cumulative effect stems
from the extremely large length to diameter ratios of collagen fibrils, which provides
an enormous amount of surface area for inter-fibril forces to act. These models thus
adopt the classic theory of “shear-lag” which assumes that shear forces distributed
4.3 PGs’ mechanical role in tendon behavior

along the entire length of the fiber act through the surrounding matrix to transfer loads between adjacent fibers [17, 18, 19].

Despite the attractiveness of the ultrastructure-function theory of PG mediated collagen load sharing, experimental results on murine tendon from decorin and biglycan knock-out models have inconclusively linked absence of these PGs with altered tendon mechanical properties [11]. Similarly disputing this theory, GAG content and mechanical properties were found to be inversely correlated when comparing Achilles tendons between strains of inbred mice [20]. Finally, more direct studies comparing native tendon with GAG-depleted tendon (enzymatically depleted using chondroitinase) indicated minimal, if any, PG contribution to tensile properties of the tissue [21, 14, 22]. Similar enzymatic GAG depletion studies in ligament reinforce these findings [23, 24]. These studies at the tissue level thus bring into doubt the primacy of PGs in mediating collagen load sharing.

The key difference between those studies supporting PG mediated collagen load sharing and those studies rejecting it, is the size scale of the experiments themselves. Nearly all of the studies supporting the theory have been based on observational (semi-quantitative) electron microscopy imaging studies that focused exclusively on collagen-proteoglycan interactions and treated the tendon as a mechanical continuum at this level. On the other hand, the cited studies that collectively refute PG mediated collagen load sharing all employ experiments at the tissue level. While tissue level experiments provide relevant insight into the impact of PGs at the organism level, such experiments include potentially confounding effects from each of the intervening levels of structural hierarchy [25, 26, 27]. To bridge this gap, we present here what we believe to be the first quantitative ultrascale study of collagen fibril load bearing (and sharing) as a function of PG GAG content.

In the present study we utilize a novel method for characterizing effects of mechanical load on in situ single collagen fibril morphology [28]. This method employs atomic force microscopy (AFM) to quantify load dependent changes in fibril banding periodicity (d-period) and diameter, which both can be used as a measure of collagen fibril stretch [3, 29, 30, 28]. We used this direct imaging approach to test the hypothesis that enzymatic depletion of GAG, PG sidechains, would alter collagen fibril load distributions. More specifically, we presumed that tendon inter-fibril load sharing is dependent on PG cross-linking, and that disrupting GAG interconnections would lead to decreased transmission of collagen shear forces under load. At the ultrascale, this removal of fibril-coupling should translate to increased collagen fibril sliding and decreased collagen stretch compared to native tendon at the same applied tissue strain (see Figure 4.11).
The aim of this study was therefore to clarify the still controversial role of PGs in collagen fibril load sharing. We implement a direct functional imaging approach that combines mechanical loading of whole tendon with ultrascale imaging analysis using AFM. Loaded collagen fibril morphology of native tendons and GAG-depleted tendons, specifically d-period and fibril diameter, were quantified and compared. According to our hypothesis, a statistically shorter d-period in GAG depleted groups would support a direct contribution of PGs to tensile tendon mechanics.

Figure 4.11: Schema of the mechanical behavior of collagen fibrils connected by PG cross-links. (top) Fibrils in a high cross-linked network slide and elongate homogeneously to bear the load. (bottom) For GAG-depleted tendons, we hypothesize that the fibrils are loosely coupled showing a lower fibril strain and a higher inter-fibril gliding than a native tendon. If the fibrils are tightly coupled, the total fibril strain would increase and the inter-fibril gliding decreases.
Materials and Methods

Sample preparation

All animal experiments were reviewed and approved by local and state authorities (Kantonales Veterinäramt Zürich, Zürich, Switzerland). The soleus tendons were harvested from the Achilles tendons of female 19-week-old C57BL/6 inbred (wild type) mice. A control group of 15 native tendons (left leg) was incubated for 12h at 37°C in Tris Hydrochloride 0.1M Sodium Acetate 0.1M buffer (pH 8), while a GAG-depleted group of 15 tendons (right leg) was incubated for 12h in Tris buffer + 0.15U/ml chondroitinase ABC (ChABC, Sigma-Aldrich, Switzerland), pH 8; in order to reduce the number of PG GAG sidechains [21, 31, 22].

The tendons of each group were divided into 3 subgroups each of 5 tendons that were then stretched to different target strains (0%, 5% and 15% nominal strain). The tendons were held under constant force and fixed with glutaraldehyde as previously described [28] (Figure 4.12a). To separate potentially confounding effects of dehydration associated with GAG depletion, two additional groups from both native and the digested tendons were measured at 0% strain. In these experiments, the control group consisted of tendons that were incubated in buffer (with and without ChABC) and chemically fixed in glutaraldehyde, the dehydrated group included tendons that were dissected, incubated in buffer (with and without ChABC) and then successively soaked for 1.5h in a freshly prepared solution of 8% PEG in buffer, in order to dehydrate the tissue [21]. The relative water gain/loss of these tendons during the different preparation steps was recorded by measuring the wet weight of tendons after each processing step. The tendons were then fixed at 0% strain in 1.25% glutaraldehyde using the protocol described above.

GAG analysis

After weighing, two additional groups of 5 tendons were treated according to the native and GAG depleted protocols, and were characterized for GAG content using the Farndale method [32]. Tendons were digested for 16h with 500 U/ml papain solution in buffer (0.1M disodium hydrogen phosphate, 0.01M EDTA disodium salt, 14.4mM L-cysteine) at 60°C. GAG content was determined spectrophotometrically (Cary 50, Varian, Zug, Switzerland) at 525 nm following binding to dimethylmethylene blue dye (DMMB) using CS as standard.
Figure 4.12: Experimental set-up of the tendon preparation and analysis. A) The tendon is clamped in a tensile test machine under constant load to reach a defined target strain. The chamber is filled with 1.25% glutaraldehyde to fix the tendon chemically while submitted to constant load. B) Representative AFM height image of a tendon fascicle showing collagen fibrils characterized by the repeated gaps forming the d-period. C) Two dimensional height profile of the black line in the image B, and D) result graph obtained after Fourier function transform analysis of the two-dimensional profile from Figure 4.12c. The highest peak corresponds to the inverse of the length of the most frequent periodicity.
Image analysis

After chemical fixation all tendons were embedded at -20°C in Tissue-Tek (Tissue-Tek, 4583 Compound, Sakura Finetek Europe, Zoeterwoude, Netherlands) for at least 12h. Each frozen tendon was then cut with a cryostatic microtome (SLEE, Mainz, Germany) at -20°C into three 20 µm thick slices and left to dry on a polylysine-coated glass slide (SuperFrost, Menzel-Gläser, Braunschweig, Germany) at 60°C for another 12h.

AFM experiments were then performed with an Asylum Research MFP-3D-SA (Germany). Images of collagen fibril topography were acquired in contact mode at a scanning rate of 0.5 Hz in air on the dehydrated thin-sectioned samples (Figure 4.12b). All measurements were carried out at room temperature. Titanium and platinum-coated, silicon AFM tips on triangular cantilever with a nominal spring constant of 2.35 N/m (NSC11/Ti-Pt; MikroMasch, Madrid, Spain) were used. In each slide five region of interest (ROI) were analyzed, which contained between 5 and 10 fibrils on the surface that were randomly identified and measured.

The d-period of individual collagen fibrils within their collagen network was measured as described earlier [28]. Briefly, analyzed fibrils in each image were characterized by a two-dimensional height profile (Figure 4.12c). A Fourier transform was then applied to calculate the inverse of the d-period length corresponding to the highest peak in Figure 4.12d. For each analyzed fibril, diameter was measured from the two-dimensional height profile of taken approximately perpendicular to the fibril long axis.

Statistical analysis

Measured wet-weights for the control and treated groups (with and without ChABC) were tested with Bonferroni correction for multiple comparison with significance level set at $p$-value < 0.05. For the fibril structural quantification, the d-period lengths and diameters of analyzed collagen fibrils were pooled together within experimental groups according to target strain. After applying a Lilliefors test to verify whether distributions could be approximated by a normal distribution, ANOVA tests were used to test for differences in d-period length and diameter between treated and control groups at similar applied strain levels. A $p$-value < 0.05 was considered to be significant.
Material level

Results

Assays of GAG content in native and ChABC treated groups indicated a 60% reduction in GAG content in the treated tendons (2.6 ± 1.1 µg/mg vs. 6.1 ± 0.4 µg/mg, p-value <0.05). The effects of incubation (swelling and the relative dehydrating effect of GAG depletion) on d-period length were analyzed at 0% strain. Two groups of tendons were dehydrated with PEG according to previous studies [21] showing a statistical decrease in water content of about 15% for both native and digested groups (p<0.05). At the ultrastructural level, no significant differences in d-period lengths were found after incubation and dehydration (65.61 ± 1.61 nm) compared to native controls (65.28 ± 1.32 nm) (p=0.08). Similarly, no differences were observed in D-period lengths of GAG-depleted tendons at 0% applied strain as a function of PEG dehydration (65.00 ± 1.89 nm incubation only; 65.75 ± 2.26 nm incubation and PEG dehydration; p=0.22). Collagen fibril diameter was not apparently affected by GAG-depletion, and neither control nor GAG-depleted tendon fibril diameters were significantly affected by swelling (Table 4.5, Figure 4.13).

After verifying against potential confounding effects of dehydration due to the treatment protocol, we probed for differences in mechanical response in the native and the digested groups in terms of fibril d-period and diameter (Figure 4.14). No differences in collagen morphology were detectable at applied tendon strains corresponding to the “heel” region of the material curve (5%) for either GAG-depleted or native tendons. For higher tendon strains corresponding to the linear region of the material curve (15%), there was a significantly increased d-period length in both groups compared to corresponding low-strain groups. Moreover, d-period length was found to be statistically longer in GAG-depleted tendons compared to native tendons at similar target strains (p <0.05). Measured collagen fibril diameter was 19% lower in highly strained tendons (15%) than unstrained controls (p <0.005), but with no detectable difference between native and GAG digested groups at similar target strains. Comparison of d-period length and fibril diameter distributions in native and GAG-depleted tendons indicated that GAG-depleted fibrils had generally more morphological variability than corresponding controls.

Discussion

Collagen load bearing is by far the most important factor in tendon mechanics [26, 33, 12, 27]. At the ultrastructural level, tendon elongation is enabled by the mechanisms of collagen fibril sliding and collagen fibril elongation that occur simultaneously and in a critical balance. Tendon proteoglycans have been implicated in regulating this
balance [26, 34]. Given their ubiquity and regular spacing (64-68 nm intervals) along the sides of collagen fibrils [13], small leucine rich PGs such as decorin and biglycan have been suggested to possibly transfer forces between collagen fibrils by mechanically coupling them in the structure [8, 10, 15, 16]. Our previous quantitative analysis of structure and function have indicated that the mechanical contribution of PGs to tendon tensile mechanics is probably minimal, but potentially complex [21, 22, 28]. In the present study, we sought to more directly investigate how GAG sidechains of small leucine rich proteoglycans may mediate collagen fibril load sharing.

To this end, we employed a quantitative ultrascale analysis of collagen fibril mechanics using a recently developed AFM-based functional imaging approach [28]. Tendons were stretched to different target strains and their structural response at the ultrascale was quantified by analyzing collagen fibril d-period length [35] and diameter [36]. This functional imaging approach is able to capture a “snapshot” of the collagen fibrils within the network, but is limited in some aspects. The effective time that glutaraldehyde needs to totally fix the tendon under tension could not be precisely defined. Although consistent between the samples, some creep that might have been occurred in the structure could not be defined. Moreover, to prepare the structure slices for the AFM observation, all tendons were embedded in a medium containing polyvinyl alcohol, which might form an additional thin film on the structure that lead sometimes to image artifacts: reduction of the structural details or surface residues. We used this approach to elucidate mechanical contribution of GAGs on collagen mechanical load transmission by enzymatic GAG depletion by ChABC. We decided to use ChABC instead of knock-out mice, because the lack of specific genes that assure a lower PG concentration do not ensures that other processes compensate this lost to restore the same function in the body [11, 37]. Although ChABC digestion might affect the tendon structure by collagen fibrils swelling [31] or alteration of other components [38, 23], we attempted to preclude

Table 4.5: Incubation effect on Type I collagen’s d-period and fibril diameter at 0% strain. The control group was incubated in PBS and successively chemically fixed, the dehydrated group was incubated in PBS, in PEG and successively chemically fixed and the digested group was incubated in PBS with ChABC and chemically fixed.

<table>
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<th>Dehydrated</th>
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<tr>
<td>D-period (nm)</td>
<td>65.28±1.32</td>
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</tr>
<tr>
<td>Diameter (nm)</td>
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<td>209.07±32.10</td>
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<td>194.96±38.27</td>
</tr>
</tbody>
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confounding morphological changes associated with incubation, dehydration or enzymatic digestion by ChABC, with visual observation at 0% strain indicating no measurable artifacts due to the experimental protocols (Table 4.5). The morphological differences we observed between treatment groups in relation to the mechanical response were therefore attributed to functional differences due to GAG content (here measured to be 60% lower for ChABC treated tendons).

Consistent with our previous AFM functional imaging study of loaded tendon collagen structures, native d-period elongated in accordance with applied tendon strain (Figure 4.14), while collagen fibril diameter decreased at higher strains [28]. The GAG depleted group showed similar trends, with longer d-periods and smaller collagen fibril diameters at 15% applied strain compared to 0% and 5% strain groups (p-value <0.005 with 0% strain and p-value <0.05 with 5% strain). In both native and GAG-depleted tendons, no d-period lengthening was observed until 5% applied strain, which at the macroscale corresponds to loss of collagen fiber crimping [30]. However, a direct comparison between native and GAG-depleted samples at 15%
4.3 PGs’ mechanical role in tendon behavior

Figure 4.14: Mean fibril diameter and d-period length versus total measured target strain in the tendon for native and digested tendons. In the native tendon the d-period length is statistically longer for 15% in comparison to the 5% strain group. The digested tendon shows a regular increase in the d-period length that is significant at 15% strain with both the 0% and 5% target strain group. At 15% strain a statistical difference is showed between the native and digested tendons. For both native and digested tendon group the diameter is gradually decreasing and is statistically smaller over 5%.

applied strain showed higher levels of fibril strains in the GAG digested tendons ($p$-value <0.05). Simultaneously, the collagen fibrils diameter of the digested group was smaller than in native tendons, although not significantly. It thus appears that GAG content in native tendon is related to lower collagen strains (reduced collagen fibril stretching, with an implied corresponding increase in collagen fibril sliding).

This finding is inconsistent with the concept that GAG sidechains of PGs act as mechanical cross-links that transfer shear force between neighboring collagen fibrils in the collagen network. That tendon behavior depends on GAG mediated shear transfer between collagen fibrils is directly contradicted by the fact that removing GAG “connectivity” between the fibrils and matrix leads to a higher fibril strains. In contrast, these data suggest that the highly hydrophilic GAGs act to mechanically isolate individual fibrils, facilitating sliding and perhaps “protecting” collagen fibrils from overstrain. This conclusion is further supported by our earlier observations in murine Achilles tendons positively relating higher collagen fibril surface area and increased GAG concentration with a reduced mechanical stiffness [20].

These findings thus complement (and perhaps complete) our previous studies
using mechanical testing on GAG-depleted tendons to demonstrate that tensile mechanics of native tendon is not heavily dependent on GAG content [14, 21, 22, 20, 28]. The current study does however introduce a subtle, and perhaps important, mechanical role that GAGs play in promoting fibril sliding. This may play a key role in “slow” viscoelastic mechanical processes such as creep or relaxation [21], and GAGs could plausibly enable creep recovery under cellular tension of the matrix. In any case, GAGs play an essential role in collagen fibrillogenesis and are essential to the attainment of proper mechanical properties in tendon development and healing [39, 40]. Further, aberrant levels of PGs and their associated GAG chains is correlated to poor functional recovery after injury. So although we can now confidently conclude that tendon PGs do not play a “collagen cross-linker” role, the small leucine rich proteoglycans continue to lie at the center of tendon structure and function.

Acknowledgements

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References


Chapter 5

Synthesis

Tendon is a passive connective tissue that transfers muscle forces to the skeleton, enabling minimal energy loss during load transfer while allowing enough extension to prevent injury in the compliant muscle connected to the rigid bone [1]. Although the gross mechanical characteristics of tendon are well documented, the role of the structure in facilitating and controlling the mechanical behavior is less clear [2]. Tendon injuries are associated with enormous costs in the work place [3] and account for 30% to 50% of all sports injuries [4, 5]. Despite decades of research and increasing clinical attention to these injuries, their clinical outcome remains unpredictable [6, 7, 8, 6, 9]. A comprehensive understanding of the physiological and biomechanical processes that underlie tendon function and pathology is essential to improve clinical diagnosis and treatment of tendon pathology.

Collagen structure-function

The mechanical integrity of tendon was historically attributed to collagen fibrils that are predominantly arranged into longitudinal fibrils with diameters ranging between 20 and 400 nm. Fibrils are the stiffer component of tendon located in the extracellular matrix (ECM) in close proximity to their neighbors, with radial separations of several orders of magnitude less than fibril length. Several studies characterized the fibril morphology and mechanical properties [10, 11, 12, 13, 14]. Others compared the tendon to a composite material in an effort to better understand how the collagen influences the overall mechanical response [15, 16, 17, 18]. In general, all these studies focused on one specific hierarchical level and its mechanical response. More recent studies that focus on the collagen structure-function are considering the phenomena that occur simultaneously at different scale levels of tendon throughout the hierarchy [19, 2, 20]. When a tendon is under load there are several phenomena that occur simultaneously at different scale levels. The collagen molecules extend and there is a sliding between the collagen fibrils and fibers as well [2]. The sliding
occurring in the structure is probably controlled by the surrounding PG matrix, which is the other main component of tendon [21, 22].

**Potential role of proteoglycans**

PGs were widely studied over the last decades; they are thought to principally transfer force between the fibrils under tension [23], or to be responsible for the viscoelastic nature of tendon as a consequence to their high affinity with water [24]. Theoretical models have been introduced on the basis that PGs act as elastic bonds between the collagen fibrils that transfer forces between them [25, 26]. Experimentally, Liao supported this theory by showing the skewness angle of PGs between the fibrils changes with applied load [23], however it is unclear whether this change is directly caused by the increasing load or is just a reorganization of the PGs. Conflicting studies found no influence in the mechanical response that can be attributed to PGs [27, 28, 29]. Other studies focused on the viscoelastic properties of the tendon to model its mechanical response [30, 31]. Microstructural modeling studies suggested that the inter-fibrillar matrix is important for controlling the viscoelastic behavior [32, 33]. This latter proposal is supported by experimental studies which indicated sliding between fibers during quasi-static tissue loading [21, 2].

**Into the big picture**

Despite a dearth of quantitative evidence regarding the precise roles of the ultrastructural tendon components, the tendon structure-function relationship has been widely speculated upon. This relationship has been partly supported by experimental studies that have indicated correlations between tendon failure strength and collagen content [10, 14, 34] as well as correlations between collagen fibril number and size with tendon stiffness [35, 36]. However, many of these experiments have yielded conflicting and/or counterintuitive results indicating that basic assumptions regarding how ultrastructure relates to function need to be reevaluated.

This thesis aimed to illuminate some of these aspects by providing a new approach to analyze the influence of the tendon mechanical components in the whole tensile response. In this work, tendons were mechanically investigated at the tissue-organ level in relation to the PG concentration and the collagen fibril diameter. Their structure was further analyzed at the microscale in order to elucidate the mechanical contribution at a smaller hierarchical level.

The major aim of Chapter 3 was to examine the possible functional role of PGs and their related secondary chain — *i.e.* the glycosaminoglycans (GAGs) — in the
tensile mechanics of murine Achilles tendon. Previous studies showed that the concentration of PG is not equally distributed in the tendon [37]. In Achilles tendon, the tensile region contains mainly small PGs, while the compressed region towards the bone insertion have more aggrecans [38]. Thus, a novel experimental set-up was first developed and validated in order to investigate the regional variation of the tendon elongation in tension depending on PG concentration. The total tendon mechanical response was analyzed mechanically with a universal tensile machine that recorded elongation under load; simultaneously frames of the posterior view of the tendon were collected with an external camera. The optical acquisition of the mechanical response of the tendon was used to regionally analyze three regions equally distributed on the longitudinal axis. From the recorded video it was possible to see that murine Achilles tendons were formed from intertwined primary fiber bundles that twisted in a coordinated fashion under load. These bundles had unique functional axes, and were surrounded and interconnected by fasciae. The combination of both the mechanical and the optical measuring systems of the overall mechanical response and the regional elongation of the Achilles tendon, showed that tendons do not behave homogeneously along their tension axis. Furthermore, a digestion of the GAGs in the tendon induced a softening at the bone insertion, which has naturally the highest concentration of PG [39]. Variations in PG concentration did not alter the intrinsic mechanical response in the midsubstance of the tendon (the tensile region), as supported by later studies investigating the mechanical influence of PG concentration in soft tissues [28, 27]. This first study emphasized the complicated role of PG in tension, and then in the following chapter an investigation at a lower hierarchical scale was made in order to elucidate the phenomena occurring further in the structure.

In Chapter 4.1 the local mechanical analysis of the tendon mechanics was related to detailed collagen fibril morphology of two inbred mouse strains. In tensile load the mouse strain exhibiting the higher E-modulus (C3H) was found to have collagen fibrils with a larger mean diameter and smaller specific fibril surface (the surface surrounding the fibril that is in contact with the non-collagenous part of the ECM). Furthermore, GAG analysis in both groups showed that the stiffer-tendon mouse group (C3H) had a lower GAG concentration in the tissue. This result concurs with the well-known property of PGs to modulate the formation and final size of collagen fibrils [40, 41], and other studies that show large fibril size in a lower GAG concentration milieu. In contrast to previous studies, this particular structure-function analysis of tendons considered the functional relevance of collagen fibril morphology and GAG content, thus focusing on the potential interplay between collagen and
PGs. The goal was to better understand the mechanical contributions of these two components that are known to influence each other during tissue formation [42, 40]. This study attempted to correlate the collagen fibril morphology to the tendon mechanical response. If the tendon is compared to a composite material made of stiff fibers embedded within a softer matrix, from a simple equation of the composite material (see equation 2.2), for equal fibril and matrix E-modulus, the fibril area fraction should directly influence the total E-modulus. The results found in this study show that there is not a significant correlation between the fibril density and the tendon E-modulus. However, the E-modulus was found to be significantly larger for a smaller specific fibril surface (the surrounding area of the fibril). Moreover the GAG concentration in the tendon was positively correlated with the specific fibril surface, which is believed to be the contact area of the PG chains [33]. GAG content could be related to promoted fibril sliding, which one would expect to reduce E-modulus [24]. This statistical difference in the GAG concentration for different E-moduli re-opened the question of the GAG’s role in mechanics since the two first studies at both tissue-organ and material level gave conflicting results. This difference might be solely a consequence of fibril morphology since they constitute 60% of tendon composition. Another plausible explanation of this difference could be that the chemical digestion of the GAGs altered the structure and increased the biological variability in such a way as to reduce the mechanical capacity of the tendon.

A complementary investigation at this scale level was therefore critical to elucidate this issue.

With this last statement, Chapter 4.2 and 4.3 focused on the interactions between the tendon components in their native network at the nanoscale with the overall mechanical response. A new experimental approach combining macroscopic mechanical loading of tendon with a morphometric ultrascale assessment of longitudinal and cross-sectional collagen fibril deformations was developed and validated in Chapter 4.2. Complex anatomical structures composed of intertwined fascicles are constantly modeled [43, 44] in efforts to understand the interaction between these structures under load. The motivation for this analysis was the lack of experimental studies that relate the nature by which collagen fibrils interact within their network to accommodate applied tendon strains. The collagen molecule extension was monitored by assessing d-period length and diameter changes with atomic force microscopy (AFM). The innovation of this method was the ability to mechanically load a entire tendon and to analyze how the collagen fibrils behave in their network that was not altered. The measurements showed that the mean collagen fibril elongation measured in the network is smaller than the whole strain displayed by
the tendon. The new method was consistent with published reports performed with different techniques [45, 2, 21], which also report a discrepancy between the total strain measured at the macroscale and at the ultrascale. However, the analyses in these studies were made with isolated fascicle or fibers and were limited to discerning in situ strains in individual collagen fibers. As soon as a tissue structure is altered to isolate a part of it, all the network movement and the influence of the neighbors can not be measured. The advantage of the approach presented in Chapter 4.2 is that the structure was not altered before the mechanical response occurred. Achilles tendons were stretched and chemically fixed with their native structure remaining untouched, so the whole mechanical response was the sum of contribution of each component. With this new approach the influence of the PG chain to the mechanical response was measured in the following study.

Finally, in Chapter 4.3 the collagen fibril mechanical response in tension in relation to different PG concentration was characterized with the AFM. With the novel method developed and described in the previous subchapter the mechanical contribution of PGs at the ultrascale was quantified by comparing the difference in the d-period length and the collagen diameter between native and GAG-depleted tendon. No change in collagen diameter was observed between the groups, however the fibrils from the group with a lower concentration of GAG presented a greater elongation for equal tendon strain. This implies that in tendons with a lower GAG concentration the collagen fibrils were more stressed under tensile load. This surprising outcome was in contrast with a common assumption that the PGs are linked to the collagen fibrils and transfer the load between them [46]. Such a network of cross-links could facilitate the load transfer in the matrix and provide stiffness to the tissue if they act simultaneously. A depletion of GAG was originally thought as a malfunctioning network where not all the fibrils can be loaded as schematized in Figure 4.11. However, in fact a lack of GAG resulted in a higher strain in the fibrils implying a highly coupled structure that bears all the load. Since PGs are highly hydrophilic [42], this suggests that the PGs are implied in the mechanical response of the tendon as cushions that may supply mobility between the collagen fibrils as introduced in previous studies [31, 24].

Limitations

Although providing additional insight into tendon mechanics, these studies do have some limitations. The optical measurement of soft tissue strains proposed in Chapter 3 improved the analytical precision in examining the substructures; however these measurements were limited by the assumption that surface strains are indica-
Synthesis

tive of the strains inside the various tendon subregions. These measurements were not sufficiently sensitive to detect fiber uncrimping [47] or to determine whether inter-fascicle slippage occurred [48, 49]. Finally, the complex hierarchical collagen structures of tendon do not generally represent the response of a continuum material. Despite these limitations, useful insight into local tendon mechanics could be extracted; the softening of the bone-tendon-muscle complex was identified as an alteration of the bone insertion, solely, rather than in all three tissues.

For the analysis of the fibril morphology presented in chapter 4.1, a limitation that one could argue is that the sample preparation protocol does not allow morphological analysis in the same tendon that was mechanically tested to failure. In this study it was critical to analyze the morphology of the tendon structure in the tensile region which would not have been possible after rupture of the tendon. Despite this, the results revealed a significant trend that relates PG and collagen morphometry together with tendon mechanical behavior in tension.

The two AFM studies presented also have some limitations. First, the amount of creep experienced during chemical fixation of the tissue with glutaraldehyde [23] was relatively uncontrollable (although generally consistent from sample to sample). However, the majority of the creep deformation occurs within the first minute and is limited afterward, and further, in relaxation experiments it was shown that the largest effects at the fiber and fibril levels occur within 20 s of the applied load [2]. Creep loading and uncontrolled dynamics of chemical fixation could therefore affect the resultant fibril structure, although whether and how this may influence the results is difficult to predict. While we made an effort to minimize such effects by mechanical preconditioning, effects of and differences between creep and relaxation remain ground for future work. As a second limitation, the preparation of high quality slices for AFM analysis was experimentally challenging. Before sectioning, all samples were embedded in a medium containing polyvinyl alcohol which is known to form thin films and can leave surface residues. While we have confidence in the fidelity of the image data we did not exclude the possibility that film residues may have adversely affected morphometric accuracy of the subsequent d-period and diameter measures.

**Conclusion and future work**

In conclusion, tendon is a complex material that can not be modeled as a simple composite material. Fibril density is not directly related to tendon stiffness and the PGs do not appear to transfer load between the collagen fibrils. GAG concentration does not significantly influence the overall tendon mechanical response, most likely
due to mechanisms that compensate this lack. However, at the microscale GAGs are responsible for changes in collagen fibril elongation, which increases at lower concentrations. An absence of GAGs corresponds to a lower hydration in the tissue and a higher condensation of the collagen fibrils that are closer to each other. The higher proximity of the fibrils combined with the common cross-section reduction in tensile load, might therefore couple them instead of allowing them to slide. The “mechanical role” of GAG does not appear to be linking the collagen fibrils in order to transfer forces between them in tension, but rather hydrating the ECM to induce a viscoelastic deformation that prevents fibril damage possibly by reduced sliding. In the future, viscoelastic tests could be performed to confirm this theory by proving that PGs actually provide sliding.

In conjunction with previous studies [2, 21, 45], the measured fibril elongation is smaller than the tendon strain. Further investigation in the multiscale transitions should be performed to understand whether there is lost energy, or if it is a consequence of the fibril sliding.

References


### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2D</td>
<td>two-dimensional</td>
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<td>3D</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
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<td>BEC</td>
<td>BEST Engineering Cluster</td>
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<td>BEST</td>
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<td>elastic modulus</td>
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<td>ETH</td>
<td>Eidgenössische Technische Hochschule</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<td>Growth/Differentiation Factor</td>
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<td>scanning electron microscopy</td>
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<td>transmission electron microscopy</td>
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List of Figures

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2.10 Schema of the similar function in tension of a tendon and a composite material. The tendon structure is composed of collagen fibrils connected with PGs within the ECM and the composite material is composed of a stiff fiber embedded in a softer matrix that follow the shear-lag theory.

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