Published in final edited form as:

Acta Biomater. 2015 October 1; 25: 304–312. doi:10.1016/j.actbio.2015.07.011.

Elastin Governs the Mechanical Response of Medial Collateral Ligament Under Shear and Transverse Tensile Loading

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Abstract

Elastin is a highly extensible structural protein network that provides near-elastic resistance to deformation in biological tissues. In ligament, elastin is localized between and along the collagen fibers and fascicles. When ligament is stretched along the primary collagen axis, elastin supports a relatively high percentage of load. We hypothesized that elastin may also provide significant load support under deformation transverse to the primary collagen axis. Quasi-static transverse tensile and simple shear material tests were performed to quantify the mechanical contributions of elastin during deformation of porcine medial collateral ligament. Dose response studies were conducted to determine the level of elastase enzymatic degradation required to produce a maximal change in the mechanical response. Maximal changes in peak stress occurred after 3 hours of treatment with 2 U/ml porcine pancreatic elastase. Elastin degradation resulted in a 60-70% reduction in peak stress and a 2-3 $\times$ reduction in modulus for both test protocols. These results demonstrate that elastin provides significant resistance to deformation transverse to the collagen axis while only constituting 4% of the tissue dry weight. The magnitudes of the elastin contribution to peak transverse and shear stress were approximately 0.03 MPa, as compared to 2 MPa for axial tensile tests, suggesting that elastin provides a highly anisotropic contribution to the mechanical response of ligament and is the dominant structural protein resisting transverse and shear deformation of the tissue.

Abstract

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The authors have no professional or financial conflicts of interest to disclose.

Disclosures: The authors have no conflicts of interest related to the content of this manuscript.

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Keywords

ligament; elastin; transverse tensile; shear; elastase

1. Introduction

Ligament and tendon are composed of up to 70% water, and the remaining constituents include type I collagen (~70% of dry weight), other collagens in significantly lesser amounts, proteoglycans and elastin, with lesser populations of fibrillin, fibrinogen, fibronectin and laminin [1]. The hierarchical structural organization and mechanical role of type I collagen in ligaments and other dense connective tissues is well appreciated, with its organization spanning multiple physical scales from tropocollagen molecules to fibrils, fibers, fascicles and ultimately the macro-scale tissue [2], where the mesoscale crimping and twisting of collagen leads to nonlinear material behavior [3]. Studies of the structure and function of normal ligament and tendon and alterations due to injury and disease have typically focused on the contributions of type I collagen due to its high percentage of tissue dry weight, highly aligned structure, stiffness and strength. However, the structure, organization and function of the remaining extracellular components of the matrix (the so-called “ground substance”) have received considerably less attention. In order to understand the structure-function relationships in these biologic materials and to interpret the alterations in organization and mechanical response of ECM components due to disease or injury, we must first characterize their contributions to normal tissue mechanics.

Elastin constitutes approximately 5% of the dry weight of ligaments [4-6]. Assembled in the extracellular space, it consists of an elastin core of tropoelastin molecules surrounded by a fibrillin-rich microfibril scaffold [4, 6]. Repeating α -helix segments composed of alanine and lysine oxidize to form highly stable covalent crosslinks between tropoelastin molecules [7, 8]. Elastin stretches and recoils through both entropic and hydrophobic mechanisms [4, 6]. We recently examined the role of elastin in ligament mechanics via selective degradation with elastase [9] and found that elastin provided a disproportionately high contribution during uniaxial tensile deformation along the primary collagen axis. Although elastin constituted only 4% of the tissue dry weight, it supported up to 30% of tensile stress under uniaxial strain [5]. In addition, elastin is localized between and along collagen fibers in cruciate ligaments [10]. These factors led us to hypothesize that elastin may also resist transverse and shear tissue deformation relative to the primary collagen axis.

Therefore, the purpose of this study was to quantify the contribution of elastin to the quasi-static mechanical response of ligament when tested in elongation transverse to the primary collagen fiber direction and in shear along the fiber direction. We hypothesized that both stress and stiffness would decrease after selective enzymatic treatment to degrade elastin for both test protocols. In combination with our previous study, the results of this study provide a multiaxial characterization of both native and elastin-degraded ligament. These results clarify our understanding of the mechanical function of elastin, provide the basis for formulating constitutive models that include elastin for both normal and pathological dense connective tissues, and in the future, these results will help to interpret tissue pathologies that involve elastin in disease and injury.

2. Materials and methods

2.1. Experimental Design

Forty-three porcine medial collateral ligaments (MCLs, age 5-8 mo., mixed sex) (Innovative Medical Device Solutions, Logan, UT) were harvested and frozen until testing. Porcine MCL was chosen as it is a readily available planar ligament that is large enough to allow isolation of multiple rectangular test specimens, and its native material properties are similar to human MCL [5, 11]. The tissue was thawed and the ligament was fine dissected to remove overlying fascia. Tissue was kept moist with phosphate buffered saline (PBS) throughout dissection and experimentation. Tissue was refrozen to -70°C and two rectangular specimens were punched from each ligament perpendicular to the primary collagen axis (Fig. 1) [11, 12]. Specimen dimensions were 10 mm long $\times$ 14-20 mm wide (nearly the full width of the ligament). The width and thickness of each specimen were then recorded with a digital caliper (Mitutoyo, CA, accuracy ± 0.02 mm) for calculation of cross-sectional area. One of the two specimens was randomly selected to serve as a control and the other was treated with elastase enzyme to degrade elastin.

Elastin haploinsufficient [13-15] and human elastin knock-in [16] animal models have been developed to study the contributions of elastin to tissue development and mechanics, but these models cannot fully remove the influence of elastin. Animal viability suffers when total elastin levels drop below 30% of wildtype [16]. Alternatively, selective degradation of tropoelastin allows the study of tissue that has undergone normal development. Elastase enzymes cleave tropoelastin but leave the desmosine and isodesmosine crosslinks intact (Fig. 2) [9], resulting in a fragmented network that rapidly loses mechanical integrity as the level of elastin degradation increases [5].

Dose-response experiments were undertaken with transverse tensile tests to determine the elastase concentration and treatment time required to effect a maximal change in peak stress between control and treated specimens [5]. Fifteen specimens from 15 ligaments were used to test the influence of elastase concentration: 3 specimens treated for 3 hours each at 0 (control), 0.1, 1, 2, and 10 U/ml elastase. Twelve pairs of specimens from 12 ligaments were used to examine the effects of treatment time: 3 pairs treated with control buffer or 2 U/ml elastase for 0.5, 1, 3, and 6 hours. Eight pairs of specimens from 8 ligaments were used in both a transverse tensile and simple shear protocol (16 pairs total) once the optimal dose-response was determined.

2.2. Strip Biaxial Tensile Testing

The transverse tensile testing protocol was adapted from a prior study [12] where specimens were aligned with the primary collagen axis perpendicular to the test axis. Given the limited width of the porcine MCL (Fig. 1), harvesting a “dogbone” shaped specimen with at least an 8:1 aspect ratio (excluding tissue within the clamps) resulted in extremely thin, fragile specimens. Instead, a strip biaxial specimen shape was used to increase the cross sectional area of viable tissue and consequently the magnitude of the resisting force. The strip biaxial test utilizes specimens where the test axis is significantly shorter than the width thus minimizing lateral contraction during elongation [17].

Samples were clamped and then equilibrated in control buffer for 2 hours at room temperature with the tissue in a stress-free state. Clamps were then mounted in the test machine and a pre-stress of 0.005 MPa was applied, based on the harvest cross sectional area. The clamps were then locked together, removed from the test machine, and the tissue/clamp assembly was bathed in either control buffer or buffer containing elastase. After treatment, the clamps were returned to the test machine and the transverse tensile protocol was initiated. The custom materials testing system consisted of a servo-driven positioning stage (Tol-O-Matic, MN, accuracy $\pm 0.1 \mu\text{m}$) that applied displacement while a load cell (LSB200, 2 lb., Futek, CA, accuracy $\pm 0.1\%$ FS) monitored the force response. The tissue underwent 10 cycles of triangle wave displacement to 10% clamp-to-clamp strain at 1%/sec. Outcome metrics were extracted from the 10th cycle, including transverse tensile stress versus clamp strain, stiffness and hysteresis. Stiffness was defined by a linear regression of the stress over the last 1% clamp strain. Hysteresis was defined as the area between the loading and unloading curves and was expressed as a percentage of the control.

2.3. Simple Shear Testing

The simple shear testing protocol was adapted from our prior studies [11, 18]. Specimens were aligned with the primary collagen axis parallel to the shear test axis (Fig. 1). Samples were clamped and then equilibrated in control buffer for 2 hours at room temperature with the tissue in a stress-free state. Clamps were then mounted in the test machine and a transverse pre-stress of 0.005 MPa was applied based on the harvest cross sectional area. Vertical displacement was then set in a neutral position, defined as the inflection point of the force response resulting from small cyclic up-down clamp displacements. The clamps were then locked together, removed from the test machine, and the tissue/clamp assembly was bathed in either control buffer or buffer containing elastase. After treatment, the clamps were returned to the test machine and the shear protocol was initiated. The tissue underwent 10 cycles of triangle wave displacement to a shear angle of $\tan(\theta) = 0.4$ at a rate of 0.05 Hz. Outcome metrics were extracted from the 10th cycle, including shear stress versus shear angle curves, stiffness and hysteresis.

2.4. Treatment protocol

The specimen treatment followed the protocol utilized in a prior study of elastin in porcine MCL [5]. The control buffer consisted of 15 ml of PBS with 0.1 mg/ml soybean trypsin inhibitor (SBTI). The treatment buffer consisted of control buffer + porcine pancreatic elastase (trypsin-free, EC134, Elastin Products Co., MO). Elastase concentration (U/ml) was based on manufacturer-stated activity of $>9 \text{ U/mg}$ of protein on the substrate Suc-Ala-Ala-Ala-pNA, where 1 U will hydrolyze 1 mmol of substrate/min at pH 8.3 and 25°C. The elastase concentration and treatment time were varied as described for the dose-response testing. Conditions for the final 8 pairs in each test protocol were based on the results of dose-response testing.

2.5. Visualizing Elastin in MCL

Two photon laser scanning microscopy was used to visualize the spatial distribution of elastin in porcine MCL and to assess the effects of elastase treatment on elastase distribution and collagen structure. Neighboring full thickness mid-substance ligament specimens ($\sim 3 \times 3$

mm) were treated with control buffer or elastase and then sectioned along the primary collagen axis to 100 μ m on a cryostat (Leica CM3050S, Exton PA) at -25°C. After sectioning, the specimens were affixed to gelatin coated slides. Of note, elastase treated sections were extremely fragile and a high percentage of preparations failed due to disruption of the tissue during sectioning. A gasket of Parafilm (Bemis, Oshkosh, WI) was placed around the sections to support a cover slip and prevent compression of the specimens. A drop of Fluoromount-G (Southern Biotech, Birmingham, AL) was placed on each section before the cover slip was laid down, and then the coverslip was sealed around the perimeter with clear nail polish.

Imaging was performed with a FV1000MPE multiphoton laser scanning microscope on a BX61 upright frame (Olympus, Center Valley, PA) using a 25 $\times$ water immersion objective. Collagen was visualized with second harmonic generation (SHG) using 860 nm excitation and emission bandpass filtered at 420-460 nm [19, 20]. Elastin autofluorescence was captured with 800 nm excitation and a 495-540 nm bandpass filter. Twelve bit images were collected at 1024 $\times$ 1024 resolution over a 253 $\times$ 253 μ m field of view (at 2 $\times$ optical zoom).

2.6. Elastase Effects on Collagen and Glycosaminoglycans

Cleavage of structural collagen fibrils/fibers or other matrix molecules by elastase could affect the mechanical properties of the ligament and confound the results of the present study. Therefore, experiments were undertaken to quantify any enzymatic activity of elastase against collagen and glycosaminoglycan content of the tissue. First, a hydroxyproline assay was performed on buffer surrounding control collagen gels (Dulbecco's PBS (DPBS)) and those treated with elastase (2 U/ml elastase in DPBS) or collagenase (2 U/ml collagenase in DPBS, where 1 U = 125 collagen digestive units/mg, Product C0130, Sigma, St. Louis, MO) as a positive control for collagen degradation [21]. Each buffer contained 0.1 mg/ml SBTI, and all treatments were performed for 6 hr at room temperature. Collagen gels were used in lieu of ligament so as not to confound measures of hydroxyproline originating in collagen with that originating in elastin [22].

Collagen gels (5 mg/ml, 1 ml final volume, No. 354249, Corning/BD Biosciences, San Jose, CA) were polymerized over 4 hours and then washed in DPBS before treatment with sodium borohydride to crosslink stabilize the collagen. For reduction, 500 μ l of 1 mg/ml sodium borohydride (26.4 mM [23]) treated each gel for 1 hr during gentle agitation for a final 1:10 ratio of sodium borohydride to collagen [24]. Gels were then washed 3 $\times$ for 20 minutes with DPBS during gentle agitation. After control or enzyme treatment, the supernatant was isolated and aliquots were taken in triplicate for assay of free hydroxyproline content. A 100 μ g/ml stock of hydroxyproline was prepared, and serial dilutions were performed to create a standard curve of hydroxyproline content. A colorimetric assay for hydroxyproline content [25, 26] was then performed in a plate reader (Synergy HT, BioTek, Winooski, VT) and hydroxyproline content was normalized to the volume of supernatant aliquot. This protocol (collagen gels, treatment, assay) was performed 3 $\times$ on a total of 9 collagen gels. Assays of each buffer (no gel treatment, N=3) were also analyzed to determine if a background signal was present.

Next, an SDS-PAGE was performed to quantify the size of collagen degradation products after treatment with elastase. An elastase activity assay[27] was first performed to determine the optimal concentration of elastase-specific inhibitor (Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone[28], M0398, Sigma, St. Louis, MO) required to quench elastase activity. Inhibited elastase acted as a negative control for the presence of elastase in the collagen preparations during SDS-PAGE. Utilizing the elastase concentration determined from dose response testing (2 U/ml), increasing concentrations of inhibitor were tested until activity against the substrate Suc-Ala-Ala-Ala-pNA was <1% of control activity. As a factor of safety, subsequent experiments utilized 2× the determined inhibitor concentration.

For the SDS-PAGE, 30 µg of soluble collagen (Corning) was prepared in 25 µl of control buffer (DPBS), elastase buffer (2 U/ml in DPBS), inhibited elastase buffer, and collagenase buffer (2 U/ml in DPBS). All buffers contained 0.1 mg/ml SBTI, and treatments were performed for 6 hr at room temperature. Each 25 µl preparation was then mixed with 9 µl of 4× Bolt LDS sample buffer and 4 µl of 10× Bolt reducing agent (LifeTechnologies, Grand Island, NY), centrifuged for 10 seconds, heated to 70°C for 10 minutes, and then centrifuged again for 5 min at 10k rpm. Twenty microliters of each preparation were run in duplicate (Bolt 4-12% Bis-Tris Plus 12-well Gel, LifeTechnologies). A SeeBlue Plus 2 prestained protein standard (LifeTechnologies) was also run for quantification of band migration. Three gels were processed on 3 separate days using the described protocol, and 8 MP JPEG images were collected in a gel imaging station. Protein band densitometry was performed in ImageJ [29].

Finally, a dimethylmethylene blue (DMMB) assay[11, 30] was performed to determine if elastase affected the amount sulfated glycosaminoglycans (GAGs) in ligament. Elastase (specifically EC134) should not degrade the polysaccharide chain of GAGs since it preferentially cleaves proteins after Ala residues [31]. Decorin and biglycan in porcine ligament contain 3-5% Ala versus >21% in elastin (<http://www.ncbi.nlm.nih.gov/protein/>, <http://web.expasy.org/cgi-bin/protparam>). Therefore, limited elastase activity against the proteoglycan core protein could liberate the GAGs and serve as a surrogate to measure elastase activity against proteoglycans in ligament. Three specimens from the same ligament were prepared in control, elastase treated (2 U/ml, 6 hr), and chondroitinase B (ChB from *Flavobacterium heparinum*, 2 U/ml, 6hr, Sigma Aldrich, St. Louis, MO) treated groups. ChB treated tissue served as a positive control for GAG digestion as ChB is specific to degradation of dermatan sulfate, the primary sulfated GAG in ligament[11]. After control or treatment, tissue was digested with papain prior performing the colorimetric DMMB assay on a plate reader. The DMMB protocol was performed on three separate ligaments for a total of 9 samples.

2.7. Statistical Analysis

All statistical comparisons were carried out with paired t-tests for samples from the same ligament (e.g. time dose, transverse tensile, shear), or independent t-tests for samples from different ligaments (e.g. concentration dose vs. control). Intra-group comparisons in dose-response testing were corrected with the Holm's step-down procedure to adjust for multiple

comparisons [32]. Significance was set at $p = 0.05$ for all tests. Data are presented as mean $\pm$ SD.

3. Results

3.1. Dose-Response Testing

As the enzyme concentration increased, peak stress decreased (Fig. 3A). The changes in peak stress plateaued at concentrations of elastase above 1 U/ml, where peak stress for 2 U/ml and 10 U/ml concentrations were significantly smaller than control ($p = 0.012$). Otherwise, no differences were detected between groups.

Peak stress in control tissue decreased as treatment time increased (Fig. 3B). A pilot study determined that this resulted from swelling of the tissue in buffer, independent of treatment. This decay was amplified after elastase treatment, where the peak stress decreased $\sim 70\%$ between control and treated specimens as treatment time increased (significant at 1 and 3 hours, $p = 0.020$). The peak stress plateaued for both groups of specimens above 1 hour of treatment; otherwise no differences were detected between groups.

Stiffness followed the same trends as peak stress for both dose-response tests. Stiffness significantly decreased versus control tissue for each treatment containing elastase ($p = 0.046$) with the exception of the 0.5 hr time point ($p = 0.592$). For example, control stiffness was 1.01 ± 0.04 MPa, whereas the stiffness after treatment with 10 U/ml elastase was 0.41 ± 0.14 MPa. There were no differences in stiffness between elastase treatment groups. Hysteresis was unaffected by any treatment group in the dose-response test, with a global average of $30.1 \pm 9.1\%$. Based on these findings, the final tests for each mechanical protocol were carried out with 2 U/ml elastase for 3 hours in the treatment groups. There were no differences in specimen width (9.1 ± 1.2 mm), thickness (1.40 ± 0.3 mm) or cross sectional area (13.2 ± 3.6 mm²) for any of the dose-response groups ($p = 0.790$).

3.2. Influence of Elastin on the Transverse Tensile Response

The transverse stress-strain curve exhibited a nonlinear, upwardly concave shape in control tissues, and the peak stress decreased significantly for the elastase treated group ($p = 0.023$) (Fig. 4A). Peak stress decreased $70.5 \pm 11.5\%$ after elastin digestion ($p < 0.001$). Stiffness decreased significantly from 0.84 ± 0.22 MPa to 0.32 ± 0.10 MPa in control and elastase specimens, respectively ($p < 0.001$). While hysteresis was not significantly different between groups ($p = 0.230$), samples treated with elastase tended to exhibit more energy dissipation ($24.5 \pm 6.1\%$ vs. $37.1 \pm 23.0\%$). There were no differences in specimen width (9.3 ± 1.0 mm), thickness (1.5 ± 0.3 mm) or cross sectional area (13.8 ± 3.3 mm²) between control and elastase treated specimens ($p = 0.143$).

3.3. Influence of Elastin on the Shear Response

The shear stress-strain curve exhibited a nonlinear, upwardly concave shape as reported in previous studies [11, 18], and the peak stress decreased significantly for the elastase treated group ($p = 0.037$) (Fig. 4B). Peak stress decreased $62.0 \pm 24.6\%$ after elastin digestion ($p = 0.002$). Shear stiffness decreased from 0.66 ± 0.37 MPa to 0.33 ± 0.47 MPa in control and

elastase specimens, respectively ($p = 0.013$). Hysteresis did not differ between groups for control ($69.8 \pm 7.0\%$) and elastase treated ($62.3 \pm 9.6\%$) specimens ($p = 0.063$). There were no differences in specimen width (8.7 ± 1.3 mm), thickness (1.6 ± 0.3 mm) or cross sectional area (13.8 ± 3.2 mm²) between control and elastase treated specimens ($p = 0.471$).

3.4. Visualizing Elastin in MCL

Collagen SHG imaging revealed the typical periodic crimp waveform of type I collagen in ligament (Fig. 6A). The elastin autofluorescence signal revealed that elastin was localized between and along the collagen fibers in ligament, following in register with the crimp waveform (Fig. 6B). In sections treated with elastase, the elastin autofluorescence signal was severely disrupted and residual elastin appeared as granulated deposits placed periodically throughout the image field of view (Fig. 6C). The collagen crimp waveform was also disrupted and was qualitatively less organized than in control tissues.

3.5. Elastase Effects on Collagen and Glycosaminoglycans

The background signal of the buffers in the hydroxyproline assay was 1.6 ± 0.7 µg/µl. Low levels of free hydroxyproline were present in the supernatant liquid of control collagen gels (6.0 ± 0.5 µg/µl). After digestion with elastase, free hydroxyproline increased to 11.8 ± 0.6 µg/µl. By comparison, collagenase digestion liberated significant amounts of hydroxyproline (333.6 ± 3.0 µg/µl). The buffer-only signal was significantly lower than all other groups ($p = 0.001$). There were significant differences between all collagen gel groups for total hydroxyproline content ($p = 0.001$).

Over 60 minutes, elastase activity decreased linearly for the control assay (Fig. 7, slope: -0.0064 , intercept: 1.10 , $R^2 = 0.88$). The addition of 0.75 U/ml of inhibitor significantly decreased elastase activity (slope: -0.0019 , intercept: 0.18 , $R^2 = 0.29$) while 1.0 U/ml of inhibitor completely suppressed the elastase activity (slope: -0.0002 , intercept: 0.01 , $R^2 = 0.05$).

In SDS-PAGE (Fig. 8), soluble collagen migrated into doublets localized around 215 - 235 and 115 - 130 kDa. Treatment with elastase or inhibited elastase had no apparent effect on the migration of the soluble collagen as measured with densitometry. The elastase and inhibited lanes contained 97.5 ± 31.1 and $91.4 \pm 2.4\%$ of control collagen for the 115 - 130 kDa bands and 101.6 ± 38.3 and $110.4 \pm 23.4\%$ of control for the 215 - 235 kDa bands. There were no significant differences between the elastase or inhibited groups and control ($p = 0.802$), or between each other ($p = 0.664$) for either doublet. Collagen migration bands were completely absent in lanes containing collagenase, where collagenase lanes contained $1.6 \pm 0.3\%$ of control collagen ($p = 0.032$).

The DMMB assay showed some loss of sulfated GAGs after elastase digestion, with the total GAG content decreased to $82.6 \pm 1.0\%$ of untreated ligament. Tissue treated with ChB had only $18.3 \pm 0.6\%$ of the GAG content with respect to control ligament [11]. Both groups were significantly lower than the GAG content of control tissue ($p = 0.002$).

4. Discussion

The results of this study demonstrate that elastin is the dominant non-collagenous structural protein in ligament, providing up to 70% of load support during transverse tensile and shear deformation of the tissue. The findings confirmed the hypothesis that both stress and stiffness significantly decreased 2-3 $\times$ after elastin degradation. Dose-response curves determined that the elastin network was rapidly degraded at short treatment times with low elastase enzyme concentrations (Fig. 3). This result is in agreement with our prior result for longitudinal tensile tests [5]. Using a 3 hr treatment with 2 U/ml elastase, both transverse tensile and simple shear protocols exhibited significant decreases of 60-70% in peak stress after elastin degradation.

Although elastin constitutes only 4% of the dry weight of porcine MCL [5] (similar to connective tissue like skin [33], tendon and palmar aponeuroses [34, 35] and slightly lower than spinal ligament [36]), the percent load supported by elastin is much larger. During longitudinal tensile testing of porcine MCL, elastin supported up to 30% of the tensile stress but it did not affect the stiffness of the material at peak deformation [5] (Fig. 5A). This suggests elastin provided supplementary load support for collagen, while collagen ultimately controlled the stiffness of the tissue along its functional axis. To the contrary, the present study showed that in transverse tensile and shear deformations the percentage of elastin load support increased dramatically to 60-70% while the stiffness decreased 2-3 $\times$ (Fig. 5 B,C). This disproportionate contribution of elastin establishes its role as the primary structural ECM protein in ligament after collagen, dominating the multiaxial mechanical response during deformations deviating from the primary axis of the tissue.

When compared to the longitudinal tensile data (Fig. 5A, [5]), the magnitude of the transverse tensile and shear stress and stiffness are two orders of magnitude smaller (Fig. 5 B,C). Elastin supported nearly 2.0 MPa of applied stress along the collagen axis, but only 0.03 MPa in transverse or shear deformation. Similarly, elastase digested tissue had a longitudinal stiffness of 293 MPa (based on tissue strain) whereas the transverse and shear stiffness in the present study were approximately 0.33 MPa for each test (based on clamp strain). This suggests that any bonds or adhesions between elastin and neighboring collagen are trivial in strength compared to the axial recoil mechanisms of the intact elastin network. Given that specimens in both studies underwent similar preparation and treatment protocols, these data highlight the significantly anisotropic contribution of elastin to the multiaxial material behavior of ligament.

Unlike our prior study, we were unable to utilize a repeated testing protocol in this study. A significant amount of tissue swelling transverse to the collagen axis resulted in an effective softening of the tissue over time even without treatment, as exhibited by the 50% decrease in peak stress for control tissue as a function of treatment time (Fig. 3B). Similarly, pilot work demonstrated that transverse tensile and simple shear testing of porcine MCL resulted in permanent tissue damage at considerably lower strains and stresses than longitudinal tensile experiments (data not shown). To control for these factors, paired specimens were taken from each ligament. This ensured that paired samples underwent the same sequence of

equilibration, pre-stress application, treatment, and mechanical deformation while avoiding data corruption due to swelling and non-recoverable deformation.

The stress and strain distributions during the transverse tensile (strip biaxial) and shear testing in this study are inhomogeneous [18, 37, 38]. Therefore, the results reported for stress should be considered the “effective” or “average” stress over the specimen cross section at the clamped end, and other components of the stress tensor are not necessarily zero at the clamped ends or within the sample. Small tissue specimen size, low loads, and plastic deformation each prohibited use of specimens with a large enough aspect ratio to provide homogeneous stress distributions. Despite these limitations, the results of this study can be used to develop constitutive relationships between the primary collagen fiber family, elastin, and non-elastin ECM to represent the contributions of elastin to ligament mechanics. Parameter optimizations using the experimental data, unique specimen geometry, and finite element simulations of the experimental loading conditions [39] will allow us to determine anisotropic material coefficients required to model the complex multiaxial behavior of the elastin network.

Material characterization of ligament and tendon typically focuses on the structural collagen given its high tissue content, strength and stiffness, but under multiaxial loading tensile collagen is not the primary load bearing structure. The present study establishes elastin as the primary contributor to these off-axis loading scenarios. Prior studies have tested the ability of other ECM constituents to tissue resist transverse and shear deformation but no dominant molecular species was previously identified. The decorin proteoglycan controls collagen diameter during tissue development [40, 41], but the effects on mechanical integrity of the tissue are still debated [40-44]. The glycosaminoglycan side chains of decorin were thought to bridge neighboring collagen [45], but contributions to the tensile and shear responses were not detectable at the continuum level in normally developed tissues [11, 46, 47]. Collagen crosslinks may resist relative movement between adjacent fibers [48] and influence tissue stiffness [49], but experiments suggest that this phenomena may not alter tissue mechanics under high loads [50]. Fibronectin, laminin and fibrinogen serve as ECM organizers, fibrillin and fibulin contribute to elastic fiber development and organization, and tenascins and thrombospondin are implicated in tissue repair and inflammatory responses [1]. By testing in orientations where the contributions of collagen were minimized, this study has demonstrated that elastin is the mechanically dominant ECM structural protein during shear and transverse deformation. Potential collagen interweaving, other ECM molecules, and crosslinking therefore likely provide the residual 40% of the total load support during multiaxial loading.

Both multiphoton microscopy and biochemical assays confirmed that while elastin was digested, collagen remained relatively unaffected. This is in agreement with our previous results where the modulus of ligament along the primary collagen axis was unchanged after elastin digestion.[5] Elastin autofluorescence confirmed that elastin was localized between and along the collagen fibers in porcine MCL (Fig. 6). After elastase degradation, the elastin signal was severely disrupted with little apparent changed to the collagen structure aside from an apparent increase in the crimp waveform period. Since the tissue was treated with

elastase prior to cryostat sectioning, the differences in collagen morphology could be due to either elastase treatment or sectioning artifacts.

Regarding the potential for elastase to act against structural collagen, the hydroxyproline assay confirmed an increase in free hydroxyproline after elastase digestion of collagen gels. It is possible that there is some small degree of proteolytic activity against collagen, even when gels were stabilized with sodium borohydride, given that the increase was twofold that of the control measurements of free hydroxyproline. It is therefore also possible that degradation of elastase mobilized collagen in the tissue. By comparison, the 30× increase in hydroxyproline content after collagenase digestion puts the relative scale of elastase activity into perspective as a minor contributor to degradation of structural collagen. These results were confirmed in the SDS-PAGE, where elastase had no appreciable effect on the migration of soluble collagen in comparison to collagenase, which completely eliminated all migration bands.

Finally, the DMMB assay confirmed that elastase also had limited activity against decorin and biglycan via measurement of their associated sulfated GAGs, specifically dermatan sulfate, which is the primary GAG component of ligament [11]. Elastin may have targeted the Ala residues in the core protein and therefore liberated the GAG sidechains, but elastase had a relatively minor effect when compared to ChB, which is a dermatan sulfate-specific enzyme. GAGs and proteoglycans have previously been implicated in the altered mechanical properties of connective tissues in knock out models [40, 42, 44] and in the compressive properties of ligament[51]. To the contrary, GAG degradation has shown no effect on the continuum level tensile or shear properties of normally developed ligament or tendon [11, 46, 47]. The mechanical implications of proteoglycans are currently unknown given the difficulty in selectively disrupting the core protein in normally developed tissues.

Several limitations of the study warrant discussion. Unlike our previous studies [5, 11, 47], a repeated measures protocol could not be used in the current study due to technical challenges arising from tissue fragility and swelling. It is possible that smaller deformations could have been used to limit the degree of plastic deformation, but resolution of the differences in peak load and stiffness would have been significantly more difficult to detect. Second, molecular fragments of degraded elastin trapped in the tissue could have contributed to the mechanical response after elastase digestion. The plateau in the dose-response curves (Fig. 3) provides confidence that the contributions of elastin were fully muted considering the same phenomena was seen in a prior study where elastin content continued to decrease even after changes in the mechanical response had ceased [5]. Finally, in enzymatic degradation studies, there is always the potential that the enzyme may have had proteolytic activity against other molecular populations. Previous studies using elastase have reported such concerns, but elastase in those studies could have been contaminated with trypsin, resulting in SBTI blocking activity against collagen [33-35]. The study presented herein used chromatographically purified trypsin-free EC134 elastase. Elastase has previously been used to isolate collagen monomers [52, 53], but degradation of lysyl oxidase bonds between the collagen and elastin was the likely mechanism of action, not direct degradation of structural collagen. The results of the hydroxyproline assay, SDS-PAGE, and DMMB assay confirm a small degree of elastase activity against collagen and

proteoglycans, but it is possible that lesser molecular species with Ala residues may have been degraded and contributed to the mechanical changes. Ultimately, the majority of the differences detected in this study are likely directly attributable to the degradation of the elastin network.

5. Conclusions

In summary, elastin is the primary structural ECM protein responsible for resisting transverse tensile and shear deformation of ligament. When considered with prior data for longitudinal deformation of MCL, elastin provides a highly anisotropic contribution to ligament integrity. While the magnitude of the load support is significantly less than tensile deformation along the primary axis of the structural collagen, elastase degradation showed that stress support decreased up to 70% and tissue stiffness decreased 50-60% as compared to native tissue. Given that elastin comprises only 4% of the tissue dry weight, it provides a disproportionately high degree of mechanical integrity in the tissue during multiaxial deformation.

Acknowledgments

Financial support from NIH #AR047369 is gratefully acknowledged. The authors would like to thank S. Joshua Romney (LifeTechnologies / Thermo Fisher Scientific) for assistance in experimental design and analysis related to elastase activity against collagen and proteoglycans.

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Figure 1.

Schematic of specimen harvest locations in porcine MCL. Two neighboring rectangular specimens were harvested at the midpoint of the ligament (punch footprint shown in red). A representative transverse tensile and shear specimen (blue) are shown as the area between the clamps with respect to the overall punch dimensions. Red arrows denote the axes of deformation relative to the collagen fibers. The shear specimen height was reduced to ensure the area between the clamps was nearly square, allowing for adequate tissue to be gripped in the clamps. Note that within a ligament both specimens were harvested for the same test protocol. Shear and transverse tensile are shown here in the same ligament for illustration only.

Figure 2.

Elastin structure and degradation products. **A:** Elastin recoils due to hydrophobic and entropic forces, but extends under applied force (F). Desmosine and (iso)desmosine crosslinks resist network deformation. **B:** Elastase degrades elastin via cleavage of tropoelastin, leaving fragments with crosslinks intact.

Figure 3.

Dose-response curves for transverse tensile tests. **A:** Peak stress decreased rapidly and fit an exponential decay function as the concentration of elastase increased (3 hr treatment for all specimens). The trend stabilized beyond 2 U/ml elastase. (*) significant difference with respect to control (0 U/ml). **B:** Peak stress decreased rapidly and fit an exponential decay function for both control and elastase treated pairs (2 U/ml elastase for treated specimens). Decay in the control samples is representative of tissue swelling in the buffer, which stabilized after 1 hr of treatment. The difference between control and treated samples also stabilized above 1 hr treatment. (*) significant difference between matched pairs of control and treated samples.

Figure 4.

The stress-strain response of transverse tensile (**A**) and simple shear (**B**) specimens can be described by exponential growth curves. In both tests, stress and stiffness of the tissue significantly decreased after elastase degraded the elastin (*).

Figure 5.

Complete quasi-static material characterization of control and elastase digested porcine MCL in (A) longitudinal tensile, (B) transverse tensile and (C) simple shear deformations. Note that (B) and (C) are on a stress scale two orders of magnitude smaller than (A), highlighting the anisotropic nature of the tissue construction. While elastin is the primary structural protein that supports nearly 70% of transverse and shear stress, the relative magnitude of the contribution is minute in comparison to that in the longitudinal deformation. Figure 5A [5] reproduced with permission (Wiley #3474980233721).

Figure 6.

Multiphoton laser scanning microscopy images of porcine MCL. A: Collagen second harmonic generation signal collected at 860 nm excitation, emission filtered to 420-460 nm. Arrow denotes collagen axis. B: Elastin autofluorescence signal collected at 800 nm excitation, emission filtered to 495-540 nm. Elastin is localized between and along neighboring collagen fibers. C: Image of the elastin autofluorescence signal in tissue treated with elastase. Note the granulated appearance of the elastin and the lack of elastin interweaving the collagen.

Figure 7.

Elastase activity assay for control and inhibited assays. A concentration of 1 U/ml of elastase inhibitor was found to quench the equivalent activity of 2 U/ml elastase as used in the mechanical tests.

Figure 8. SDS-PAGE of soluble collagen in the presence of enzymes. Elastase and inhibited elastase had no appreciable effect on collagen migration bands. Collagenase significantly degraded collagen as exhibited by the lack of collagen banding in the collagenase lanes.