Effect of Dermatan Sulfate Glycosaminoglycans on the Quasi-Static Material Properties of the Human Medial Collateral Ligament

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ABSTRACT: The glycosaminoglycan of decorin, dermatan sulfate (DS), has been suggested to contribute to the mechanical properties of soft connective tissues such as ligaments and tendons. This study investigated the mechanical function of DS in human medial collateral ligaments (MCL) using nondestructive shear and tensile material tests performed before and after targeted removal of DS with chondroitinase B (ChB). The quasi-static elastic material properties of human MCL were unchanged after DS removal. At peak deformation, tensile and shear stresses in ChB treated tissue were within 0.5% ($p > 0.70$) and 2.0% ($p > 0.30$) of pre-treatment values, respectively. From pre- to post-ChB treatment under tensile loading, the tensile tangent modulus went from 242 ± 64 to 233 ± 57 MPa ($p = 0.44$), and tissue strain went from $4.3 \pm 0.3\%$ to $4.4 \pm 0.3\%$ ($p = 0.54$). Tissue hysteresis was unaffected by DS removal for both tensile and shear loading. Biochemical analysis confirmed that 90% of DS was removed by ChB treatment when compared to control samples, and transmission electron microscopy (TEM) imaging further verified the degradation of DS by showing an 88% reduction ($p < .001$) of sulfated glycosaminoglycans in ChB treated tissue. These results demonstrate that DS in mature knee MCL tissue does not resist tensile or shear deformation under quasi-static loading conditions, challenging the theory that decorin proteoglycans contribute to the material behavior of ligament. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:1–11, 2007

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INTRODUCTION

The mechanical characteristics of ligament and tendon are a function of their composition and molecular organization. The major solid phase constituent of ligament and tendon is type I collagen, which represents over 70% of the total solid phase.[1,2] The remaining constituents include other collagens (III, V, VI, XI, XIV), extracellular matrix proteins (e.g., elastin) and proteoglycans.[3,4] The hierarchical organization of type I collagen in ligament has been extensively studied; type I collagen fibrils form parallel arrays of fibers that are the main contributors to ligament material properties.[5] However, there are very limited data on the mechanical influence of the non-collagenous components of ligament. Understanding the contributions of these components to connective tissue mechanics can clarify their roles and aid efforts to engineer replacement tissues.

The primary proteoglycan in ligament,[6,7] decorin, and its single sulfated glycosaminoglycan (GAG) have been the subject of numerous studies due to their respective proven and theoretical roles in tissue-level organization and mechanics at the molecular level. Dermatan sulfate (DS) is the GAG that associates with the decorin core protein in nearly all mature tissues[8,9] (Fig. 1A). The concave face of decorin has been modeled to straddle the D-period binding site of a single collagen triple helix[10,11] (Fig. 1B). Decorin appears to be excluded from the tightly tropocollagen packed fibril interior,[12] localizing to the surface of the collagen fibrils (Fig. 1C). The highly charged DS GAG

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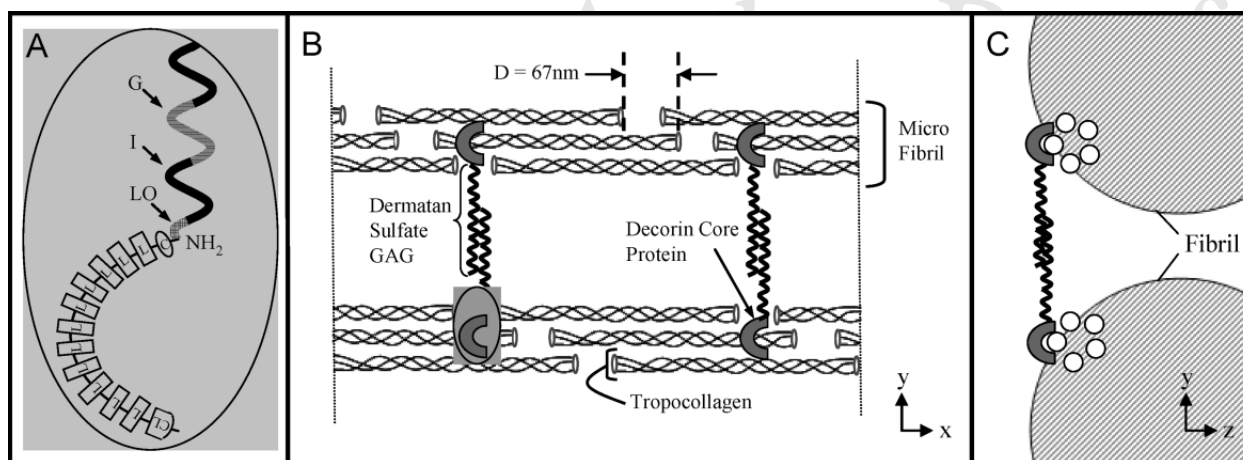
Figure 1. (A) Magnified schematic of decorin proteoglycan. The boxed domains "L" represent the leucine-rich repeats of the protein core. "C" represents the cysteine rich domain. The dermatan sulfate GAG is attached to the protein core near the amino (NH_2) terminus through a serine linked oligosaccharide ("LO"). On the macroscopic level, dermatan sulfate is made up of iduronic acid-containing regions "I" and glucuronic acid-containing regions "G." Chondroitinase B will degrade the iduronic acid regions mainly into disaccharides. (B) Frontal view of collagen fibril assembly. Five quarter staggered tropocollagen units construct a microfibril. Decorin core proteins likely bind to tropocollagen triple helix units at the D-period gap region of the microfibril. Although decorin binds to collagen, the exact mechanism of this attachment is debated.[61] (C) Cross-sectional view of collagen fibril assembly. A fibril consists of a variable number of microfibrils, however decorin is only able to bind to tropocollagen units on the outer surface of the fibril. The proposed interaction between collagen fibrils involves the association of at least two dermatan sulfate GAG side-chains bonded to decorin core proteins on adjacent collagen fibrils.

projects away from the decorin core protein,[13] generally orthogonally aligned to the fibril direction.[8]

Scanning electron and atomic force microscopy of connective tissues demonstrate that the majority of DS GAGs span the space between neighboring fibrils.[12,14] Several studies report that collagen fibrils in various species are short and discontinuous,[15–17] suggesting that a secondary microstructure could be involved in transferring axial force between contiguous fibrils. This finding led to an appealing hypothesis that decorin-based DS GAGs on adjacent fibril surfaces interact, creating functional "cross-links" that transfer forces between discontinuous fibrils[8,14,17–23] (Fig. 1C). Supporting this hypothesis, molecular dynamics predict that the summation of GAG interaction forces in collagen-decorin networks are capable of transferring interfibril stress,[14,24] and experiments confirm that DS does indeed self-associate.[25] The proposed fibril–fibril link would prevent fibril sliding and potentially contribute to quasi-static mechanical properties under both shear and tensile loading.

Using knockout mice and competitive peptides, previous studies have investigated the possible mechanical role of decorin indirectly. Knockout studies have reported that decorin deficiency decreases the tensile strength in the dermis,[26] does not affect tensile strength or modulus in tail tendon fascicles,[27,28] and increases modulus in the patellar tendon.[27,28] NKISK, a pentapeptide that inhibits the ability of decorin to bind to collagen, caused fibril to fibril disassociation,[15] and ultimately greater tissue laxity without reduction in material strength.[29] The mechanical behavior of connective tissues from knockout mice may be influenced by changes in tissue development due to the absence of decorin, such as irregular collagen structure[26] and regulatory activity that increases biglycan production as a compensatory mechanism.[30–32] Lack of biochemical analyses in the NKISK studies has left the molecular alterations in question. If NKISK specifically inhibits decorin binding, observed changes may be due to the absence of the decorin core protein and not necessarily the associated GAG. Altogether these studies have left the mechanical role of DS

under elastic tensile deformation unclear and controversial.

In this study, a new experimental model was developed to quantify the effects of DS GAGs on the elastic material behavior of mature ligament tissue. The objective of this study was to assess the role of DS in resisting quasi-static tensile and shear deformation along the fiber direction in human ligament by targeted removal of DS cross-links with enzymatic degradation. Understanding the elastic contribution of DS will clarify the structure-function relationships in ligament and address the validity of the decorin cross-linking theory.

METHODS

Experimental Design

The medial collateral ligament (MCL) of the human knee was chosen for study due to the extensive prior mechanical test data available[33–36] and our well-developed experimental protocols for mechanical characterization.[34,35,37] Knees were acquired fresh-frozen and were allowed 16 h to thaw prior to dissection. The freeze–thaw cycle does not influence the quasi-static-material properties of ligament.[38,39] Knees with surgical scars, ligament injury, or cartilage degeneration characteristic of osteoarthritis were eliminated. To account for material symmetry characteristics of ligament, two types of mechanical tests were performed— uniaxial tensile testing[34] and shear testing.[35] For tensile testing, 16 specimens were harvested from 4 unpaired human MCLs (donor age = 57 ± 5 years). For shear testing, 16 specimens were harvested from 5 unpaired human MCLs (donor age = 55 ± 8 years). The specimens were divided between two treatment groups: a control treatment group and a chondroitinase B (ChB) treatment group. ChB specifically degrades DS side chains of proteoglycans.[40] Mechanical testing was performed pre- and post-treatment on the same specimen.

Uniaxial Tensile Testing

Four unpaired human MCLs were used for tensile testing. A hardened steel punch[34] was used to extract four samples from different locations in each superficial MCL between the tibial and femoral insertions, for a total of 16 tested samples. The punch shape included beveled ends for gripping and was oriented so that its long axis was aligned with visible fiber bundles. Sample geometry met ASTM requirements for fiber reinforced composite materials.[41] Samples were randomly divided between the control and ChB treatment groups and loaded in a clamp assembly. A 0.1 N preload was applied to establish a consistent reference length. Vertical position of the actuator (Tol-O-Matic, Hamel, MN; accuracy ± 1.0 μm) and high-resolution micrometer measurements (Newport, Irvine, CA; accuracy ± 0.5 μm) of the x-y table were logged so that the reference configuration could be reproduced after treatment. Sample position was further measured by tracking 4.75 mm dia acrylic white spheres adhered to each clamp and to a fixed reference with a digital camera (Pulnix TM-1040, $1024 \times 1024 \times 30$ fps, Sunnyvale, CA) and digital motion analysis software (DMAS, Spica Technology Corp, Maui, HI; accuracy ± 0.005 mm).[42] This protocol verified that post-treatment clamp positions were within 0.04 ± 0.01 mm of pre-treatment clamp positions.

Specimen dimensions were measured using digital calipers (Mitutoyo, San Jose, CA; accuracy ± 0.02 mm) by taking an average of three measurements (Table 1). Following 5 min of relaxation, a triangular displacement profile was applied for 10 cycles at a strain rate of 1.0%/s with a clamp-to-clamp strain amplitude of 8% of the specimen length. Clamp-to-clamp strain was based on substructural failure limits of ligament, reported to occur after 5% tissue strain.[43] Tissue–tissue strain[AQ1] is approximately half the clamp-to-clamp strain,[33] so an 8% clamp-to-clamp strain was chosen to avoid structural damage. The strain rate was selected to minimize viscoelastic and inertial effects.[34] Tissue strain was measured by tracking 1-mm diameter contrast markers on the specimen mid-substance using the DMAS system. Force and displacement were monitored continuously, with peak stress calculations based on the 10th cycle. Tangent modulus was calculated as the slope of the linear region of the stress-strain curve using local tissue strain, and was standardized for each case by using linear regression to fit the stress-strain data for the final 1% strain region. Hysteresis was determined from the area enclosed by the loading and unloading stress-strain curves from the last loading cycle.

[AQ1]

Shear Testing

Five unpaired human MCLs were used. A rectangular punch (10×25 mm) was used to extract up to four

Table 1. Physical Dimensions of the MCL Test Specimens[a]

Specimen type	Mean clamp-to-clamp length ± SD	Mean width ± SD	Mean thickness ± SD
Tensile	15.06 ± 1.84	1.81 ± 0.38	1.48 ± 0.58
Shear	6.93 ± 0.91	9.81 ± 0.93	1.84 ± 0.42

MCL, medical collateral ligaments.
[a]$n = 16$.

samples from each superficial[AQ2], for a total of 16 tested samples. Samples were randomly divided between the control and ChB treatment groups and loaded in a clamp assembly so that visible fibers were along the direction of displacement.[35] Load cells (Sensotec Inc, Columbus, OH; $1,000g$, accuracy $\pm 0.1g$) monitored both transverse and longitudinal clamp reaction forces. After the specimen was loaded and aligned in an unstrained state, a $1g$ preload was applied in the transverse direction. The vertical displacement was then set at a neutral position, defined as the inflection point of the force response resulting from small cyclic up-down clamp displacements. Clamp position and physical dimensions were recorded (Table 1) using methods identical to the tensile mechanical protocol. Following a 5-min equilibration period, a triangular displacement profile was applied for 10 cycles at a strain rate of 6.5%/s, with peak displacement based on $\tan(\theta) = $ (peak displacement)/(clamp-to-clamp length) $= 0.4$.[35] Strain rate was chosen to minimize viscoelastic and inertial effects, and peak displacement was selected to maximize loading without damaging the tissue. Transverse force, longitudinal force, and vertical displacement were monitored continuously, with peak stress calculations based on the 10th cycle. Shear modulus was defined as the slope of the stress-strain curve over the final 0.1 shear strain region. Hysteresis effect was measured using the previously mentioned methods.

Chondroitinase B Treatment Protocol

Following initial shear and tensile testing, each specimen was removed from the test machine while still mounted in the clamps. The entire specimen and clamp assembly was bathed for 1 h at room temperature in 15 ml of buffer solution (15 ml of 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$) with protease inhibitors (1 tablet of mini-complete per 10 ml of buffer). Samples were then bathed in 15 ml of either the control buffer solution or the ChB (ChB, 1.0 IU/mL) for 6 h at room temperature with gentle agitation using an orbital shaker. Preliminary tests confirmed that all DS was completely degraded using 0.25 IU/mL for 6 h (data not shown). Immediately after treatment, the clamp assembly was reattached to the test machine and returned to the original testing position. The sample was allowed to equilibrate for 5 min. Shear or tensile post-treatment testing was then performed with the same test parameters. For the tensile test specimens, failure tests were performed subsequently using our published protocol.[34] The sample was then removed from the clamps and placed in a stop buffer (20 mM Tris, pH 7.5, 150 mM sodium chloride, and 10 mM EDTA) to inhibit any further ChB activity. Samples were kept continuously moist by applying 0.9% saline solution through a nozzle.

Specificity of Chondroitinase B

A large quantity of ChB was required to treat all ligament specimens. ChB was cloned into a prokaryotic expression vector as previously described.[44] ChB degrades DS into disaccharides by cleaving regions that contain iduronic acid (Fig. 1A). One international unit (IU) of ChB activity corresponds to the amount of enzyme required to liberate 1 μM of unsaturated uronic acid from DS per min at 30°C [pH 7.5, (DS) = 2 mg/mL].

The specificity of ChB was determined by incubating ChB with various sulfated GAGs and then determining the GAG concentration remaining after digestion using the dimethylmethylene blue (DMB) assay.[45] Individual reactions (30 μl, $n = 6$ for each condition) were set up containing 1.0 IU/mL ChB and 500 μg/mL of sulfated GAG (DS, chondroitin sulfate A and C, heparan sulfate, or keratan sulfate). Control reactions contained GAGs and buffer only: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM CaCl$_2$. The reaction was allowed to proceed for 6 h at RT. Five microliters of each reaction (diluted twofold) was transferred to a 96-well plate in duplicate, along with GAG standards. Two hundred microliters of dimethylmethylene blue reagent were added to each well, and the plate was immediately read in a plate reader at 530 and 590 nm. GAG concentrations were expressed as a percentage of control reactions.

Verification of ChB Activity and Removal of DS

Following mechanical testing, the ligament tissue between the clamps was isolated with a scalpel and cut into three pieces for GAG quantification, decorin Western blot analysis, and transmission electron microscopy (TEM). GAG quantification followed established guidelines,[46] which involved taking DMB assays from digested papain extracts. These guidelines permitted DS content in control and ChB treated samples to be calculated by subtracting the amount of GAG present in the papain extract treated with additional ChB from the papain extract that was treated with additional buffer only.

Western blot analysis examined the glycosylation state of decorin from extracted proteoglycans. Wet weights were obtained and samples were then frozen in liquid nitrogen and pulverized. Proteoglycans were extracted twice over 24 h at 4°C in a total of 25 volumes of 4 M GuHCl, 50 mM acetate buffer pH 6.0 plus protease inhibitors. Two-hundred microliters of extract were precipitated overnight by the addition of 1 ml of 100% ethanol. Following centrifugation at $14,100g$, the protein pellet was washed with 70% ethanol and resuspended in 20 μl of 8 M urea, followed by an additional 140 μl of 10 mM Tris pH 7.4. Twenty-five microliters of this extract were treated with and without additional ChB, as described above. Fifteen microliters of each reaction was resolved by size on 4%–16% gradient SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes and blocked in 3% BSA in 1X TBST (Tris-buffered saline plus 0.1% Tween 20) for 1 h at RT. Blots were incubated with 0.2 μg/ml biotinylated anti-human decorin antibody (BAF140, R&D Systems, Minneapolis, MN) for 3 h at RT on a

nutator. Blots were washed and incubated with 0.8 µg/ml with Neutravidin-HRP (Pierce, Rockford, IL) for 1 h at RT. Blots were developed using a chemiluminescent substrate (Bio-Rad, Hercules, CA) and imaged with a gel documentation system with an integrated 12-bit camera.

TEM was used to investigate the presence of sulfated GAGs in the five control and ChB treated MCL specimens that were tested in shear. Ligament sections were fixed in 4% paraformaldehyde/2% glutaraldehyde at 4°C overnight. Samples were sectioned to 20 µm (cryostat, Leica CM3050S, Exton PA), mounted on slides, and stained with Cupromeronic Blue.[8,47] Ultrathin sections, approximately 70 nm, were obtained via ultramicrotome (Leica Ultracut UCT) and viewed using a Hitachi[AQ3] H7100 TEM with a LAB6 filament. Digital images of a minimum of four fields of view were obtained from each examined MCL. An image processing program was used to determine the number of stained GAGs per image. All images from a sample were averaged to produce a representative number of detected objects for each sample.

Statistical Analysis

The effects of control and ChB treatment on tensile and shear peak stresses, tensile and shear tangent moduli, and peak tissue strain were assessed using paired t-tests to measure significance between pre- and post-treatment. Control versus ChB treatment effect upon hysteresis was assessed using ANCOVA, controlling for pre-treatment results, with Bonferroni adjustment for multiple comparisons. TEM and biochemistry results were assessed using independent t-tests to measure control versus ChB treatment effect. A power analysis demonstrated that a sample size of eight was sufficient to detect a 10% change with 80% confidence for all measured quasi-static mechanical results (Power = 0.80). Unless otherwise noted, all results are reported with standard error.

RESULTS

ChB and control treatments had no significant effect on tensile (Fig. 2) or shear (Fig. 3) stress-strain response. At peak deformation, there were no significant differences in the tensile peak stress due to control or ChB treatments [percent decreases of $0.4 \pm 1.3\%$ ($p = 0.75$) and $0.4 \pm 0.9\%$ ($p = 0.718$) due to control and ChB treatments, respectively]. Similarly, there were no significant differences in the shear peak stress due to control or ChB treatments [percent increases of $5.6 \pm 3.3\%$ ($p = 0.13$) and $1.7 \pm 1.6\%$ ($p = 0.32$) due to control and ChB treatments, respectively]. [AQ3]

Tensile tangent modulus, shear modulus, and tissue strain were unaffected by ChB and control treatments. For tensile tests, an 8.0% clamp strain equated to $5.1 \pm 0.4\%$ tissue strain. Between time stages, tensile control specimens went from $5.8 \pm 0.5\%$ to $5.7 \pm 0.6\%$ ($p = 0.58$) tissue strain, while tensile ChB treated specimens went from $4.3 \pm 0.3\%$ to $4.4 \pm 0.3\%$ ($p = 0.54$) tissue strain. The pre- and post-treatment tensile moduli from the 8% cyclic tests were, respectively, 160 ± 56 and 159 ± 55 MPa for the control ($p = 0.79$), 242 ± 64 and 233 ± 57 MPa for the ChB treated ($p = 0.37$). The tensile tangent modulus data calculated from the 8% cyclic strain tests (198 ± 28 MPa) had a high correlation with the tensile tangent modulus data calculated from the failure tests (202 ± 37 MPa) ($r^2 = 0.8$). The pre- and post-treatment shear moduli were, respectively, 133 ± 11 and 142 ± 14 KPa for the control ($p = 0.08$), 146 ± 39 and 151 ± 41 KPa for the ChB treated ($p = 0.08$).
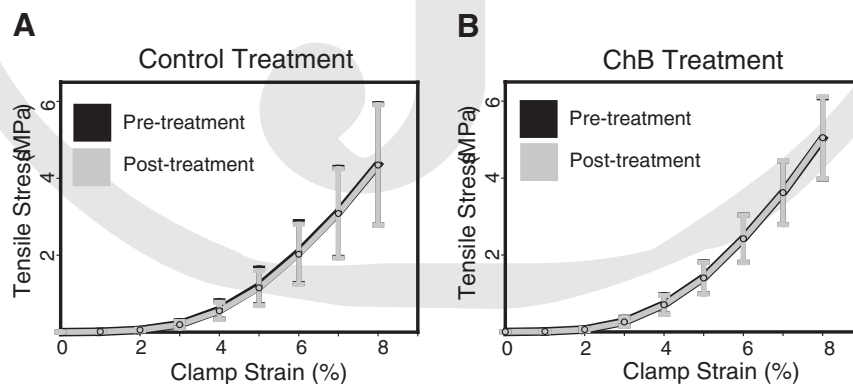

Figure 2. Stress-strain curves for tensile tests of control ($n = 8$) and chondroitinase B ($n = 8$) treated ligament samples. (A) Tensile mechanical response immediately after dissection (pre-treatment) and after 6 h of control treatment (post-treatment). (B) Tensile mechanical response immediately after dissection (pre-treatment) and after 6 h of chondroitinase B treatment (post-treatment). Bars = SE.

A

Control Treatment
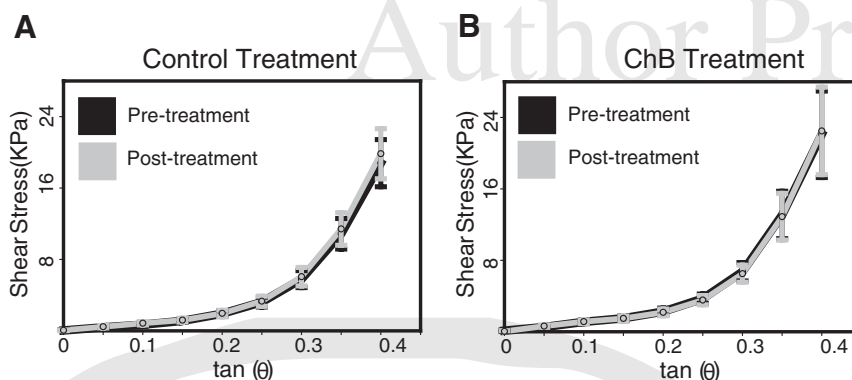

B

ChB Treatment

Figure 3. Stress-strain curves for shear tests of control ($n = 8$) and chondroitinase B ($n = 8$) treated ligament samples. (A) Shear mechanical response immediately after dissection (pre-treatment) and after 6 h of control treatment (post-treatment). (B) Shear mechanical response immediately after dissection (pre-treatment) and after 6 h of chondroitinase B treatment (post-treatment). Bars = SE.

There were no significant differences in percent hysteresis between control and ChB treatments for tensile ($p = 0.16$) and shear ($p = 0.79$) material tests. The pre- and post-treatment percent hysteresis for tensile specimens were, respectively, $18.8 \pm 0.9\%$ and $20.4 \pm 0.9\%$ for control treated, and $20.0 \pm 0.7\%$ and $20.8 \pm 0.9\%$ for ChB treated. The pre- and post-treatment percent hysteresis for shear specimens were, respectively, $21.2 \pm 1.8\%$ and $27.5 \pm 1.5\%$ for control, and $21.2 \pm 1.1\%$ and $28.0 \pm 1.9\%$ for ChB treated.

ChB reduced the concentration of the DS standard by 78% (Fig. 4). Concentrations of chondroitin sulfates A and C, heparan sulfate, and keratan sulfate were unaffected by ChB
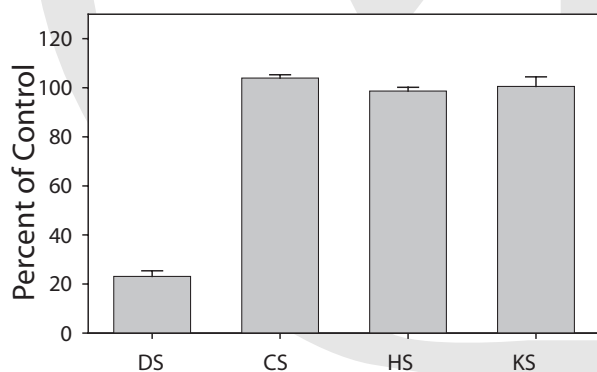

Figure 4. Specificity of chondroitinase B. Chondroitinase B (1 U/ml) was incubated with glycosaminoglycans (500 µg/ml) for 6 h. GAG concentration after 6 h was determined using the DMB assay. Concentrations were normalized to control reactions which did not contain chondroitinase B. DS, dermatan sulfate; CS, equal mixture of chondroitin sulfates A and C; HS, heparan sulfate; KS, keratan sulfate. $N = 6$. Error bars = SD.

treatment. A likely reason that the specificity test did not show a complete degradation of DS is due to the presence of DMB detectable glucuronic acid-containing regions in DS, which are not digested by ChB.[48,49] Biochemical analysis of the mechanically tested samples found that over 90% of the DS in ChB treated ligament samples (0.1 ± 0.2 µg DS/mg dry tissue) were eliminated when compared to control specimens (1.5 ± 0.5 µg DS/mg dry tissue) ($p < 0.001$). DS accounted for $44 \pm 4\%$ of all sulfated GAGs (3.6 ± 0.6 µg sulfated GAGs/mg dry tissue) in the mechanically tested controls.

Western blot results for three control and three treated specimens are shown in Figure 5, but all shear samples and random tensile samples were tested and yielded similar results. Control specimens (lanes 1, 3, and 5) had two predominant species, a band at ~43 kDa and a smear from 50 to 100 kDa. When extracts were treated with ChB (lanes 2, 4, and 6) the smear was eliminated and there were two major bands at 45 and 90 kDa. The species are consistent with published results in the literature—the 45 kDa band, which appears as a doublet with shorter exposure times, represents decorin that has lost its glycosaminoglycan side chain, and the 90 kDa band is a dimeric form of decorin.[50,51] Protein extracts obtained from ChB treated ligament samples (lanes 7, 9, and 11) did not show the smear pattern between 50 and 100 kDa, suggesting that decorin obtained from these samples has already lost its DS side chain. Additional ChB treatment of these extracts did not cause any further changes (lanes 8, 10, and 12).

TEM images further verified degradation of sulfated GAGs. ChB treated samples showed a significant reduction in the number of sulfated
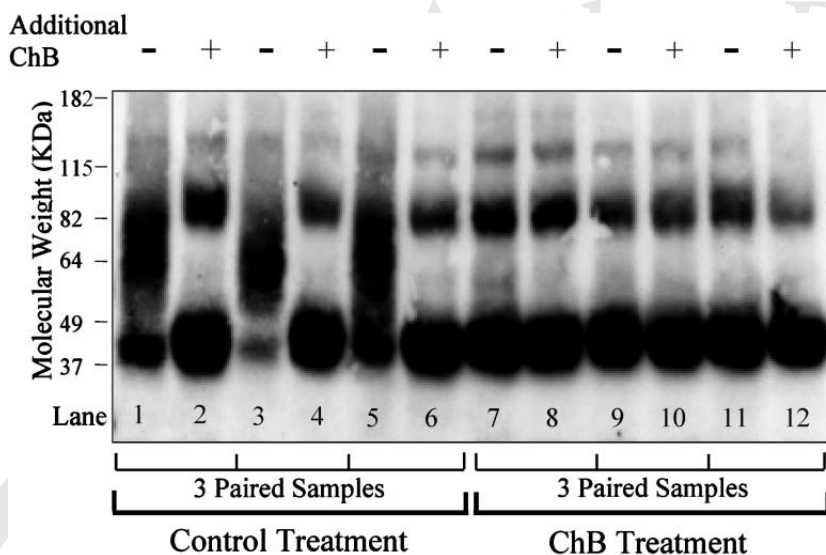
Figure 5. Decorin Western blot of proteins extracted from three control and three ChB treated ligament samples. Each lane represents protein extracted from approximately 375 µg of wet tissue. Two tests were performed on each sample. They were either given an additional control buffer treatment (−) or given an additional treatment of ChB (+). Decorin core protein migrated as a doublet at ∼45 kDa. Decorin containing its dermatan sulfate side-chain migrated as a smear from ∼55 to 120 kDa.

GAGs when compared to controls (88% reduction, $p < 0.001$). The quantity of stained GAGs was reasonably uniform within groups having 47 ± 13 and 350 ± 40 stained GAGs per field for ChB treated and control images, respectively. Stained GAGs remaining after ChB treatment were preferentially aligned with the local collagen fibrils and generally larger than the GAGs that were removed (Fig. 6).
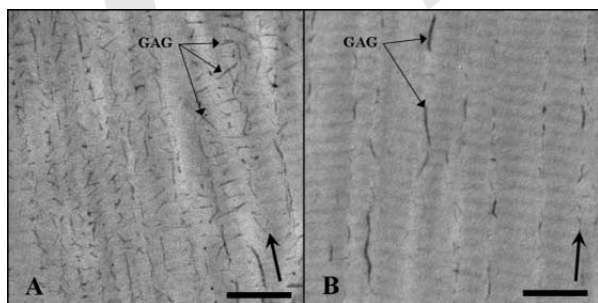

Figure 6. Representative TEM images of tissue stained with Cupromeronic Blue after mechanical testing. Large arrows indicate collagen fibril direction; small arrows indicate darkly stained sulfated GAGs. (A) Control treated specimen. (B) ChB treated specimen with 88% reduction in sulfated GAGs. Note the decrease in the overall number of fibril spanning GAGs and the preferred alignment of remaining sulfated GAGs along the collagen fibril direction. Bar = 200 nm.

DISCUSSION

The DS GAG of decorin has been implicated as a contributor to the material behavior of connective tissue.[26–28] Our present findings do not support the theory that sulfated GAG cross-links influence continuum-level mechanical behavior during quasi-static tensile loading.[8,14,17–22] Shear testing was conducted to determine whether DS contributes to the mechanical behavior of mature human MCL when fibrils are sheared relative to each other.[27,52] This hypothesis was similarly rejected. Therefore, DS in mature human medial collateral ligament does not resist quasi-static tensile or shear loads.

Mathematical studies have hypothesized that GAG cross-links contribute to the resistance of loads along the fiber direction by transferring force between discontinuous fibrils. For instance, Redaelli et al.[22] used a molecular dynamics model to explore the ability of decorin GAG cross-links to prevent sliding between discontinuous 100 µm fibrils. Results suggested that interfibrillar GAGs could transfer force between adjacent fibrils, with maximum load transfer and GAG strain occurring at 5% tissue strain. The current study demonstrated that even near 5% tissue strain, interfibrillar GAG cross-links did not contribute to the tensile material response of mature human MCL. This result does not negate the theory that DS may

resist fibril motion, but it does confirm that any interfibrillar resistance from DS interaction does not affect quasi-static mechanics at the macro scale. Past studies support this conclusion. Cribb and Scott[8] demonstrated that the orientation of sulfated GAGs does not change under tensile stretch along the direction of the fibrils, indicating that there was no relative sliding of the fibrils. Further, the mathematical model used by Redaelli et al.[22] assumed fibril length to be discontinuous in 100 μm segments. Fibril discontinuity is debated in the literature, with many investigators reporting that mature fibrils are continuous structures,[52–55] with tapered ends only present on embryonic and in vitro fibrils.[18,56,57] It should be noted that non-DS molecules, including other sulfated GAGs, may transfer forces between shorter discontinuous fibrils or contribute to the mechanical properties of ligaments in other test configurations.

Our findings aid the interpretation of results from studies using knockout mice and competitive peptide techniques. Results of this study suggest that material alterations reported for tendons from decorin knockouts[26] are likely due to genetic compensation or developmental abnormalities inherent in decorin deficiency. In another study, pentapeptide NKISK treatment[29] disrupted the decorin-collagen interaction in mouse tendon, resulting in increased strain behavior. Since the current study found that DS degradation did not influence strain behavior, another factor may have influenced tissue mechanics in the NKISK study. Biochemical analyses are needed to understand how NKISK alters molecular composition.

Since negatively charged GAGs have an affinity for water molecules, we investigated hysteresis during both shear and tensile tests. There were no significant changes in hysteresis following the control and ChB treatments. However, DS may still contribute to the viscoelastic response when other loading rates or protocols are used. In studies of decorin-deficient knockout mice, decorin content correlated with strain-rate sensitivity[28] and stress-relaxation behavior.[58] As stated previously, knockout models are unable to directly measure DS contribution. Thus to completely characterize the possible role of DS in ligament mechanical behavior, viscoelastic testing in both tensile and compressive configurations is necessary.

To have confidence in our results, the experiments needed to be performed in a reproducible manner that provided data consistent with the literature. Ligament mechanical behavior can vary between donors, and this can be especially problematic in studies using cadaveric tissues. Measured mechanical response can also be influenced by tissue heterogeneity, clamping, and specimen alignment in the material testing system. These variables were controlled with the repeated measures design of the experiments. Measured mechanical properties were also in good agreement with past research. Peak shear stress for this experiment was 22 KPa, while published peak shear stress using similar shear strains and loading configurations was 25 KPa.[35] Past studies measured an average tangent modulus of 332 MPa in human MCL specimens taken along the anterior portion of the superficial MCL between the tibial insertion and the meniscal attachment.[34] Samples taken from this same region in the current study had a similar average tangent modulus of 330 MPa.

Biochemical and TEM analysis verified selective degradation of DS and elimination of interfibrillar cross-links. Samples treated with ChB had 90% less DS than control samples and an absence of the DS GAG side chain commonly associated with decorin. TEM results demonstrated that ChB treatment removed the vast majority of GAGs. Since the sulfated GAGs that remained after ChB treatment were oriented along the fibrils, DS represented the majority of fibril-spanning sulfated GAGs in the mature human MCL.

Since ChB degrades all DS chains, it likely degraded DS side chains on biglycan, a small leucine rich proteoglycan that accounts for less than 10% of all proteoglycans in ligament.[6] Biglycan has two associated GAG side chains and a core protein that binds to collagen[59] and is highly homologous to decorin. Biglycan is another proteoglycan that could contribute to the mechanical properties of tendon.[27] However, since biglycan side chains are predominantly DS, and DS was eliminated via ChB treatment, biglycan proteoglycans similarly did not contribute to quasi-static mechanical behavior of the human MCL.

Several limitations of this research must be mentioned. A quasi-static loading rate was used and thus DS depletion could have an effect if mechanical testing was performed using faster loading rates. Further, DS may contribute to ligament mechanical properties in other test configurations such as compressive loading. Results from this study are based on in vitro tests and long-term effects of DS depletion were not considered. The human MCL tissue used in this study came from relatively old donors with several different causes of death. The exact time from death to postmortem freezing was unknown but less than 24 h. Although the experimental design utilized

pre-and post-treatment testing of the same sample (repeated measures design), which controls for the effect of initial differences between samples, the postmortem conditions and differences in cause of death may have affected the starting concentration of DS in the tissues. Similar to previous studies that used a repeated measures design to assess treatment,[58,60] multiple samples were taken from each donor. However, it should be noted that the measured quasi-static mechanical parameters and GAG content had as much variability between samples from a specific donor as between donors. Had mechanical test data from each donor been averaged $(n = 4)$ to focus on variability between donors, an acceptable change of 20% would be detected with 80% confidence for all measured quasi-static mechanical results.

In summary, mechanical tests on the same mature human MCL specimens before and after targeted DS removal demonstrated that DS does not contribute to the quasi-static tensile or shear material properties of mature human MCL. Future studies should examine how DS contributes to viscoelasticity, and if other GAGs influence tissue mechanics. By continuing to examine microstructural influence on mechanical properties of tissue, the origins of tissue material response can be elucidated and applied to the improvement of tissue engineering and wound healing methods. Studies seeking to engineer replacement tissues can use the results of this study and others like it to understand the contribution of specific molecules to the mechanics of the tissue that is being replaced.

ACKNOWLEDGMENTS

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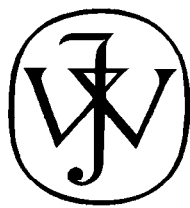
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