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# Proteoglycans and glycosaminoglycan fine structure in the mouse tail tendon fascicle

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**Abstract**

The isolated mouse tail tendon fascicle, a functional and homogenous volume of tendon extracellular matrix, was utilized as an experimental system to examine the structure–function relationships in tendon. Our previous work using this model system demonstrated relationships between mean collagen fibril diameter and fascicle mechanical properties in isolated tail tendon fascicles from three different groups of mice (3-week and 8-week control and 8-week Mov13 transgenic) K.A. Derwin, L.J. Soslowsky, J. Biomech. Eng. 121 (1999) 598–604. These groups of mice were chosen to obtain tendon tissues with varying collagen fibril structure and/or biochemistry, such that relationships with material properties could be investigated. To further investigate the molecular details of matrix composition and organization underlying tendon function, we report now on the preparation, characterization, and quantitation of fascicle PGs (proteoglycans) from these three groups. The chondroitin sulfate/dermatan sulfate (CS/DS)-substituted PGs, biglycan and decorin, which are the abundant proteoglycans of whole tendons, were also shown to be the predominant PGs in isolated fascicles. Furthermore, similar to the postnatal maturation changes in matrix composition previously reported for whole tendons, isolated fascicles from 8-week mice had lower CS/DS PG contents (both decorin and biglycan) and a higher collagen content than 3-week mice. In addition, CS/DS chains substituted on PGs from 8-week fascicles were shorter (based on a number average) and richer in disulfated disaccharide residues than chains from 3-week mice. Fascicles from 8-week Mov13 transgenic mice were found to contain similar amounts of total collagen and total CS/DS PG as age-matched controls, and CS/DS chain lengths and sulfation also appeared normal. However, both decorin and biglycan in Mov13 tissue migrated slightly faster on sodium dodecyl sulfate polyacrylamide gel electorphoresis (SDS-PAGE) than the corresponding species from 8-week control, and biglycan from the 8-week Mov13 fascicles appeared to migrate as a more polydisperse band, suggesting the presence of a unique PG population in the transgenic tissue. These observations, together with our biomechanical data [Derwin and Soslowsky, 1999] suggest that compensatory pathways of extracellular matrix assembly and maturation may exist, and that tissue mechanical properties may not be simply determined by the contents of individual matrix components or collagen fibril size. © 2001 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

## Introduction

Soft connective tissues such as tendon and ligament are composed of collagen fibrils embedded in a hydrated matrix [5]. It is generally accepted that collagen fibrils and their assembly into higher-ordered structures are critical to the mechanical function of the tissue. Consequently, 'structure–function' studies in tendon and ligament have primarily considered correlations between collagen fibril diameters and mechanical properties such as tensile strength and stiffness [2,29]. Whereas collagen fibril diameters in cross-sections of tendons and ligaments can be readily quantified, these determinations alone may be insufficient to define the tissue mechanical behavior. In the light of the evidence that individual collagen fibrils are discontinuous in extracellular matrices [43], to achieve mechanical integrity a molecular mechanism for providing interfibrillar connection has been postulated. It is proposed that non-collagenous macromolecules may play a role in forming such connections [27,42]. In particular, members of the leucine-rich small PGs, lumican, fibromodulin and decorin, have been implicated, as they can associate with collagen fibrils both in vitro and in vivo [7,17,24,25,32,39,46].

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Another member of this PG family, biglycan, is also present in soft connective tissues, but its relationship to fibrillar collagens is less well defined, as it has been mostly localized to the pericellular space [4,17,28] and does not readily associate with fibrils [7,28,31,37].

The glycosaminoglycan (GAG) chains of the collagen-binding PGs, in particular, may provide for the proposed interfibrillar connections. Evidence to support such a hypothesis comes from studies demonstrating a qualitative correlation between GAG type and the 'functional load bearing' of skin [18], a contribution of total GAGs to the material properties of developing rat tail tendon [22], and the capability of isolated dermatan sulfate for self-association in vitro [20]. It has been postulated that in the extracellular matrix GAGs from collagen bound decorin may extend away from the fibril surface [44] and interact with chains from neighboring fibrils to form 'interfibrillar bridges' [38]. In addition, PG or GAG networks formed in interfibrillar spaces or near cells could influence the overall tissue mechanics.

Despite a large number of elegant qualitative studies of matrix composition in tendons and ligaments [1,3,19,45], there have only been limited reports that quantify the PG and collagen components and relate these to tissue mechanical properties (e.g., [23]).This has, in part, been due to the complex hierarchical nature of the specimens utilized for biochemical analyses and biomechanical testing. For example, since most tendons or ligaments are comprised of multiple fascicles surrounded by connective tissue sheaths and their associated cells, the measured biochemical and mechanical data reflect the combined contributions of all these structures, rather than just a defined volume of extracellular matrix. Investigations of direct relationships between biochemical and mechanical properties in these "higher-ordered" composite samples are, therefore, difficult to interpret.

To overcome such limitations we have prepared individual fascicles from mouse tail tendons after removal of the vast majority of epitenon and paratenon sheaths by careful dissection [34]. Isolated fascicles give an essentially homogenous volume of tendon extracellular matrix [11,13]. We have demonstrated that stiffness and maximum load can be determined for individual fascicles and that these parameters are moderately correlated with the mean collagen fibril diameter [12]. We report here the identification and quantitation of the small PGs, decorin and biglycan, as well as fine structure analyses of substituent GAGs, in isolated tail tendon fascicles from three different groups of mice (3-week and 8-week control and 8-week Mov13 transgenic). Briefly, these groups of mice were used for our previous structure–function studies [12] and chosen to obtain tendon tissues with varying collagen fibril structure and/or biochemistry such that relationships with material properties could be investigated. In particular, the Mov13 mouse carries a provirus which prevents initiation of the transcription of one pro-$\alpha$1(I) collagen gene [35]. Type-I collagen analyzed from Mov13 skin appeared structurally normal in that migration of $\alpha$1(I) and $\alpha$2(I) chains on SDS-PAGE was indistinguishable from controls [6]. However, the mutation was shown to be associated with a 50% decrease in total collagen at the organ level in skin [6], which is consistent with our observations of reduced skin thickness and tendon mass in Mov13 (unpublished data). Data on the proteoglycans and glycosaminoglycans obtained from the tail tendon fascicles in this study are discussed in light of their implications for structure–function relationships in tendon.

## Methods

All chemicals used were of the highest purity grade available. For GAG fine structure and SDS-PAGE analysis: Papain, Q-sepharose fast flow ion exchange resin, cetylpyridinium chloride (CPC), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemicals, St. Louis, MO. Chondroitinases (protease-free ABC, ACII, and B) and unsaturated dermato- and hyaluro-disaccharide standards (ΔDi4S: 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDiB: 2-acetamido-2-deoxy-3-O-(2-O-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDiHA: 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose) were from Seikagaku America, Rocksville, MD. Non-reducing terminal standards (Di4S: 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfo-D-galactose; GalNAc4S: *N*-acetylgalactosamine-4-sulfate; GalNAc4,6S: *N*-acetylgalactosamine-4,6-di-sulfate) were prepared as described [30]. Healon® and the Superdex Peptide column were from Pharmacia, Piscataway, NJ. Precast gradient gels (4–20%) were from Jule Biotechnolgies, New Haven, CT. For Western blot analysis: Precast mini-gels (4–20%), SDS Sample and Running Buffers, Transfer Buffer, and poly(vinylidene difluoride) (PVDF) membranes, were from Novex™ – Novel Experimental Technology, San Diego, CA. Q-sepharose fast flow ion exchange resin, ECL™ Western blotting analysis system, and Hyperfilm™ ECL™ were from Amersham Pharmacia Biotech, Buckinghamshire, England.

### *Tissue retrieval*

Three-week control ($n = 24$), 8-week control ($n = 19$), and 8-week heterozygous Mov13 ($n = 18$) male mice were obtained from Jackson Laboratories, Bar Harbor, ME. The mean ± S.D. of body weight for the three groups was 9.0 ± 1.0 g (3-week control), 24.2 ± 1.2 g (8-week control), and 21.8 ± 0.9 g (8-week Mov13). All mice were of a C57 Bl/6 genetic background. Animals were sacrificed with a lethal dose of $CO_2$, tails were removed with the skin intact, immediately wrapped in saline wetted gauze (PBS, pH 7.5) and stored at −20°C until analyses.

### *Collagen content*

Fascicles from both ventral tail tendons from six mice in each group were dissected into sterile water at 4°C, blotted lightly, and dried at 60°C for 24 h. Tissue dry weights, determined using a microbalance, were 2.5–5.0 mg per animal. Pooled fascicles from each animal were hydrolyzed in 6 N HCl at 110°C for 16 h, acid evaporated, and the hydrolyzate dissolved in water. Small portions of each sample were removed for standard amino acid analyses (University of Michigan Protein and Carbohydrate Structure Facility) to confirm that the hydroxyproline:proline ratio was essentially identical for fascicles from the three experimental groups. Total hydroxyproline content was determined on the remainder of each sample [41], and collagen content was calculated based on the premise that hydroxyproline is solely derived from collagen, and that it represents 13% of this protein by weight [10].

*Sulfated glycosaminoglycan (S-GAG) content and fine structure*

For determination of the total chondroitin/dermatan sulfate (CS/DS) content, fascicles from both ventral tail tendons of an additional six mice in each group were dissected into phosphate buffered saline (PBS) at 4°C and dried. Tissues were digested with papain (20 μg enzyme/60 μl of 50 mM sodium acetate, 0.2 mM CysHCl, pH 6.0, per mg dry weight) at 60°C for 16 h, insoluble material removed by centrifugation, and GAGs quantitatively precipitated from the supernatants after the addition of 20 μg Healon®, using CPC [21]. Pellets were dissolved in 200 μl 0.1 M ammonium acetate, pH 7.3, divided into two equal-sized portions, and digested with 100 mU of either Chondroitinase ACII or ABC (16 h at 37°C). Products were chromatographed on Superdex Peptide eluted in 0.1 M ammonium acetate, pH 7.3, and elutant monitored by absorbance at 232 nm to detect Δdisaccharide products. The total unsaturated chondroitin lyase disaccharide content (ΔDiS) of each sample was determined from the Chondroitinase ABC digestions, and quantitated relative to the absorbance at 232 nm of 2 μg of ΔDi4S. To verify GAG recoveries and completion of chondroitinase digestions, 10 μg of purified bovine aggrecan (A1D1) was routinely taken through each of the steps described above. Approximately 90% of the aggrecan CS were recovered as Δdisaccharide products for all experiments.

Portions of Chondroitinase ABC digestion products (~5 μg) described above were aminated with 2-aminopyridine/borane-dimethylamine, and sulfated Δdisaccharides and non-reducing terminal residues were separated and quantitated by fluorescence-based ion exchange liquid chromatography (HPLC) [30]. From this analysis, the relative proportions of the various sulfated products were determined. Further, the number average chain length of the CS/DS chains in the tissue was estimated from the ratio of the primary non-reducing chain termini (GalNAc4S, GalNAc4,6S) to the total internal sulfated products (ΔDiS). Termini co-eluting with the standard Di4S were detectable in only two of the mice across all groups and were not included in the present analysis.

*Proteoglycan content and composition*

Fascicles from the ventral tail tendons of 3-week control ($n = 10$), 8-week control ($n = 6$), and 8-week Mov13 ($n = 5$) mice were dissected into sterile PBS (4°C) containing 0.5 mM PMSF, 10 mM *N*-ethylmaleimide and 5 mM benzamidine HCl. Fascicles were pooled with others from the same group, yielding three samples, each ~60–75 mg (wet weight). Tissues were blotted dry, homogenized in 2.5 ml 4M GdnHCl, 50 mM sodium acetate, pH 6.0, containing 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M 6-aminohexanoic acid, 1 mM PMSF, 10 mM *N*-ethylmaleimide, 5 mM benzamidine HCl, and then extracted for 18–24 h at 4°C on a rocking platform. Extracts were then clarified by centrifugation and the supernatants exchanged into 8 M urea, 0.3 M NaCl, 50 mM sodium acetate, 0.5% (v/v) Triton X-100, pH 6.0, by G-50 chromatography. Material collected in the void volume was mixed with 1 ml packed bed volume of Q-Sepharose resin (equilibrated in 8 M urea, 0.3 M NaCl, 50 mM sodium acetate, 0.5% (v/v) Triton X-100, pH 6.0) and incubated for 3 h at 4°C on a rocking platform. Resin was then transferred to an empty 10 ml column and washed with 10 ml of 8 M urea, 0.3 M NaCl, 50 mM sodium acetate, 0.5% (v/v) Triton X-100, pH 6.0. Bound material, containing the proteoglycans, was recovered with 4 ml of 8 M urea, 1 M NaCl, 50 mM sodium acetate, 0.5% (v/v) Triton X-100, pH 6.0.

To maximize recovery of the purified PGs, salt and detergent concentrations were adjusted to 0.0023 M urea, 0.58 mM tris-acetate, pH 7.3, and 0.116% (v/v) Triton X-100 by dialysis first against 8 M urea, 50 mM NaAc, pH 6.0, and sufficient Triton X-100 to reduce sample Triton concentration to 0.116% (5 mg per sample). This was followed by a second dialysis against water, tris-acetate and Triton X-100 to reduce the sample urea concentration to 0.0023 M and add tris-acetate to 0.58 mM. Dialyzed samples were lyophilized and resuspended in 100 μl distilled water to give approximately 0.1 M urea, 25 mM tris-acetate, pH 7.3, and 5% (v/v) Triton X-100. Twenty-five microliter aliquots of each sample were incubated with or without 0.01 U protease-free Chondroitinase ABC, ACII or B for 3 h at 37°C. Samples were brought to 4 M urea, 2% SDS, 10-20 mM dithiothreitol, and 0.002% bromphenol blue, heated at 100°C for 10 min and electrophoresed in 0.025 M Tris, 0.25 M glycine, 0.1% SDS, pH 8.8 [33] on a 4–20% gradient gel (16 × 18 cm, 1.5 mm thick). The total sample loaded in each gel lane was adjusted to represent equivalent weights of collagen (~500 μg hydroxyproline), and electrophoretic separation was carried out at a constant current of 10 mA for 1 h and 3 mA for 16 h. Separated PGs and their core proteins were visualized with Coomassie Blue (0.1% w/v in $H_2O$) and Alcian Blue (0.05% w/v in $H_2O$), and the staining intensity was measured by laser densitometric scanning.

*Western blot analysis*

To confirm the identity of the products separated by SDS-PAGE, proteoglycans from the ventral tail tendon fascicles of additional 3-week control ($n = 2$), 8-week control ($n = 1$), and 8-week Mov13 ($n = 1$) mice were isolated essentially as described above, but PGs were purified using the Q-Sepharose ion exchange resin and recovered in 5 ml 8 M urea, 1.2 M NaCl, 50 mM sodium acetate, 0.5% (v/v) CHAPS, pH 6.0. Samples were concentrated to 200 μl using a Centricon-30 microconcentrator (Amicon).

A portion (50 μl) of each purified sample was diluted 10-fold with 0.1 M $NH_4Ac$, pH 7.0, and digested with Chondroitinase ABC (100 mU/ml) for 3 h at 37°C. Digested samples were then freeze-dried until their volumes were each approximately 40 μl, and then brought back to their original 50 μl volumes with water. Twenty microliters of this digested aliquot was used to quantify the amount of ΔDi4S in each sample using FACE analysis [8,9]. The remaining portions of the digested and undigested fractions of each sample were analyzed on SDS-PAGE under reducing conditions. Fractions to be probed with decorin antisera were first diluted 1:5 with 8 M urea buffer, while fractions to be probed with biglycan antisera were used directly. Both fractions were then diluted with an equal volume of 2× Tris–glycine SDS sample buffer such that the final samples contained 63 mM Tris, 10% glycerol, 2% SDS, 0.0025% Bromophenol Blue, and 50 mM dithiothreitol. All samples were heated at 100°C for 3 min and electrophoresed in running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3, on a Tris–glycine 4–20% mini-gel (1 mm thick), in a Novex™ Xcell II Mini-Cell. The total sample volume loaded in each gel lane (digested and undigested) was adjusted to represent ~35 ng ΔDi4S in lanes to be probed with decorin antisera, and ~175 ng ΔDi4S in lanes to be probed with biglycan antisera. Electrophoretic separation was carried out at a constant voltage of 125 V for 90 min.

The resolved PGs and their core proteins were electrophoretically transferred to pre-cut 0.2 μm PVDF (poly(vinylidene difluoride)) membranes in a Novex™ Blot module at a constant voltage of 25 V for 2 h in a buffer containing 12 mM Tris, 96 mM glycine, 5% methanol. The blots were blocked for 1 h in Tris buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% dry milk, rinsed, and then incubated for 1 h with primary antiserum in Tris buffer containing 5% dry milk. The primary antisera were as follows: murine decorin (1:8000, LF-113, mouse domain analogous to Human LF-30) and murine biglycan (1:1000, LF-106, mouse domain analogous to Human LF-15) [15,16]). After rinsing, the blots were incubated for 1 h with peroxidase conjugated donkey anti-rabbit immunoglobulin G (1:5000, Amersham Pharmacia Biotech, Buckinghamshire, England) in Tris buffer containing 5% dry milk. Immunoreactive PGs and core proteins were visualized using the ECL™ Western blotting analysis system and the film was exposed for 10–60 s.

## Results

*Collagen content*

The collagen content of fascicles for the three experimental groups is shown in Table 1. Hydroxyproline content per dry weight of fascicle from control animals was significantly lower at 3-weeks than at 8-weeks ($P < 0.001$, analysis of variance with post hoc Tukey test), whereas the content in fascicles from Mov13 mice was almost identical to the age-matched controls.

Table 1
Collagen and GAG contents of mouse tail tendon fascicles (mean ± S.D.)

| | Hydroxyproline (μg/mg dry wt.) ($n = 6$) | Collagen[a] (μg/mg dry wt.) | CS/DS (ng/μg OH-pro[b]) ($n = 6$) | CS/DS/Collagen (μg/mg) |
|---|---|---|---|---|
| 3-week control | 108.1 ± 4.6*,# | 831 | 29.8 ± 3.2*,# | 3.9 |
| 8-week control | 129.6 ± 6.1* | 997 | 12.4 ± 1.3* | 1.6 |
| 8-week Mov13 | 130.5 ± 10.2# | 1004 | 15.1 ± 2.5# | 2.0 |

*,# $P < 0.001$.
[a] Estimated from the hydroxyproline content as described in Methods.
[b] Determined from an aliquot of the total papain digest, before CPC precipitation (see Methods).

Moreover, collagen accounted for ~100% of the fascicle dry weight in both 8-week control and Mov13 mice, but was significantly lower ~(83%) in the 3-week control fascicles. Such differences in tissue composition may, in part, be explained by a higher contribution of cellular [12] and non-collagenous matrix proteins to tissue mass at 3-weeks of age.

*S-GAG content and fine structure*

Total GAGs were prepared from fascicles of all three experimental groups for quantitative and qualitative analyses using chondroitinases. GAGs from all fascicles were poorly digested with Chondroitinase ACII (Fig. 1, left panels), but completely digested by Chondroitinase ABC as detected by a significant increase in the ΔDiS products (Fig. 1, right panels). This result is consistent with an abundance of DS-substituted PGs in the tendon extracellular matrix [40]. The CS/DS content, calculated from the total ΔDiS produced by Chondroitinase ABC (Fig. 1, right panels) normalized by sample collagen content, was ~3.9, 1.6 and 2.0 μg/mg collagen for 3-week control, 8-week control and 8-week Mov13 fascicles, respectively (Table 1). These data were in good agreement with the fascicle GAG contents as determined by DMMB assay [14] (data not shown) and indicated that the majority of S-GAGs in these tissues are accounted for by CS/DS. An analysis of variance with post hoc Tukey test demonstrated the CS/DS content to be significantly different between 3- and 8-week fascicles ($P < 0.001$) but not between 8-week control and Mov13 (Table 1).

Fluorescence-based ion exchange chromatography was used to determine the Δdisaccharide sulfated isomers and the number average chain length of CS/DS in the fascicles (Fig. 2, left panel and Table 2). Based on the

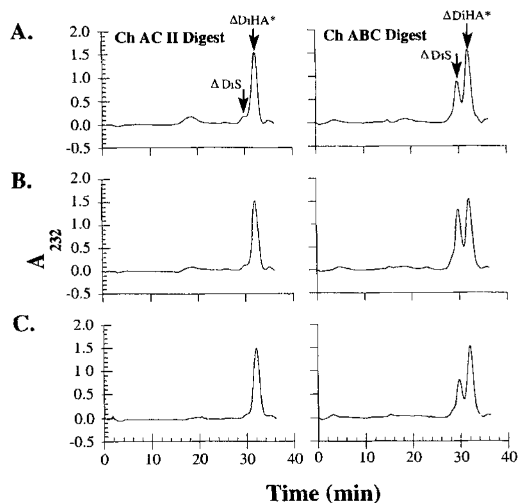


Fig. 1. Superdex peptide gel chromatography of chondroitinase ACII and ABC digestion products. Representative chromatograms of (A) 3-week control, (B) 8-week control, and (C) 8-week mov13 samples are shown (after subtracting the respective enzyme/buffer background data). Sulfated ΔDiS eluted at ~29 min, and the peaks at ~32.5 min represent ΔDiHA. Products have not been normalized by hydroxyproline content of the original sample. Calibration of the column demonstrates that 2 μg ΔDi4S = 0.216 $A_{232}$. Abbreviations: ΔDiS – unsaturated chondroitin lyase disaccharide product. * – Added as an internal standard (see Methods).

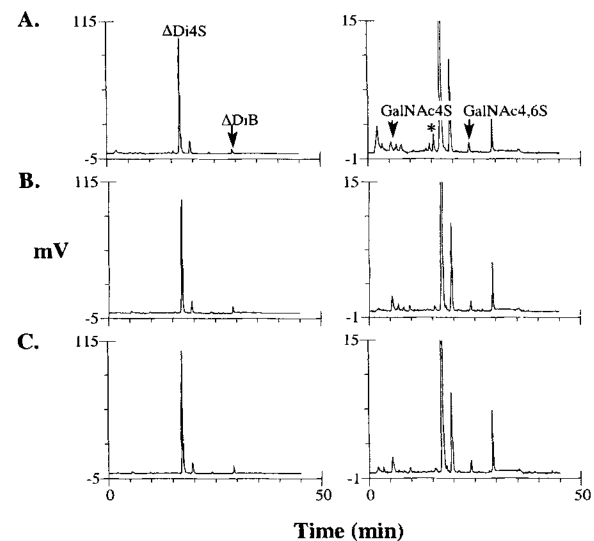


Fig. 2. Fluorescence AS4A ion exchange HPLC characterization of chondroitinase ABC digestion products of fascicles from (A) 3-week control, (B) 8-week control, and (C) 8-week Mov13 groups. For each group, the complete chromatogram is given on the left, and a scaled view (7.5×) of the same chromatogram on the right. Samples contained ~0.5 μg CS. * – A peak co-eluting with the standard Di4S was detectable in only two of the mice across all groups and was not included in the present analysis. GalNAc4S: terminal *N*-acetylgalactosamine-4-sulfate; GalNAc4,6S: terminal *N*-acetylgalactosamine-4,6-di-sulfate.

Table 2
Chain length and sulfation of CS/DS chains

| | Number average chain length[a] (No. of ΔDiS) | ΔDi4S (ΔGlcA[b]-GalNAc4S[c]) per chain | ΔDiB[d](ΔGlcA2S[e]-GalNAc4S[c]) per chain |
|---|---|---|---|
| 3-week control | 50 | 48.3 | 1.7 |
| 8-week control | 36 | 33.7 | 2.3 |
| 8-week Mov13 | 36 | 33.5 | 2.5 |

[a] Number average chain length was estimated from the ratio of non-reducing chain termini (GalNAc4S, GalNAc4,6S) to the total internal sulfated fluorescent products (ΔDiS).
[b] Glucuronic acid.
[c] N-acetylgalactosamine-4-sulfate.
[d] Parameter was determined in fascicles from only $n = 1$ or 2 mice in each group.
[e] Glucuronic acid-2-sulfate.

ratio of the primary non-reducing chain termini (GalNAc4S, GalNAc4,6S) to the total internal sulfated fluorescent products ΔDiS), chains were estimated to contain an average of ~50 repeating disaccharides in immature fascicles, but were shorter (~36 repeating disaccharides) in both the adult control and Mov13 tissues. In all three groups, ΔDiS were almost exclusively recovered as the 4-sulfated isomer, consistent with the high abundance of DS chains. In addition, between 3% and 7% of the disaccharides were present in a peak coeluting with the standard ΔDiB, the disulfated disaccharide, GlcA2S-GalNAc4S. Assuming that such disulfated residues are evenly distributed among all chains, it was calculated that immature fascicles contain ~1.7 disulfated residues per chain, while chains in adult control and Mov13 contain ~2.3 and 2.5 disulfated residues, respectively (Table 2). Thus, differences in the fine structure of DS chains were only evident between mature and immature animals. There were no detected differences in either the number average chain length or the sulfation isomer composition of the CD/DS chains between adult control and genetically altered Mov13 fascicles.

*Proteoglycan content and composition of isolated fascicles*

The CS/DS chains isolated from the fascicle matrix are likely derived from a number of proteoglycans, and this was further examined by purification of intact PGs and their subsequent separation by SDS-PAGE (Fig. 3). Two distinct bands, migrating at 65–100 and 150–240 kDa, respectively, were apparently the major CS/DS PGs in fascicles from all three groups (Fig. 3, lanes 1, 5, 9). These were identified by Western blot analysis, using PG specific antisera, as decorin (65–100 kDa) and biglycan (150–240 kDa) (Fig. 4, panels A and B). In keeping with the total GAG analyses shown in Fig. 1, the core proteins of both decorin and biglycan were largely substituted with DS, as only Chondroitinases ABC and B, but not ACII quantitatively generated the 45 kDa core proteins (Fig. 3, lanes 2–4, 6–8, 10–12). It was noted that both decorin and biglycan from immature fascicles migrated slower than the corresponding species from 8-week control fascicles and those in Mov13 tissues migrated slightly faster than the age-matched controls (Fig. 4, panels A and B).

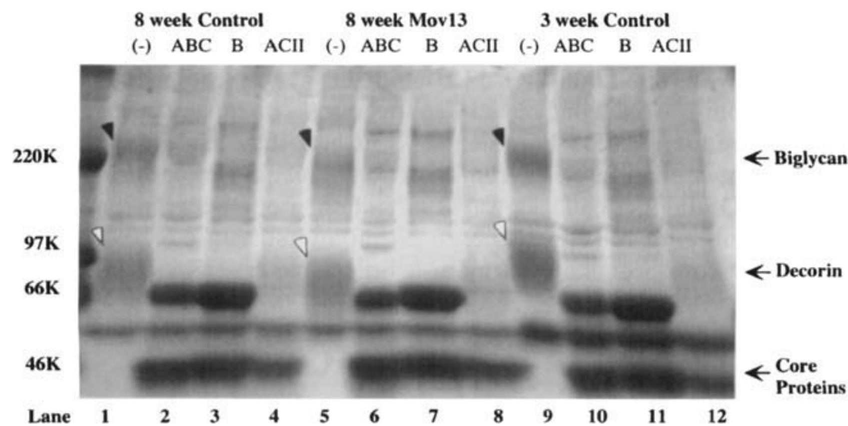


Fig. 3. SDS-PAGE analysis of PGs of tail tendon fascicles from 8-week control, 8-week Mov13, and 3-week control groups. For each group, lanes are designated: (–), (ABC), (B), and (ACII), which denote intact and chondroitinase ABC, B, and ACII digested PG, respectively. PG products in all lanes represent equivalent quantities of fascicular collagen (~500 μg OH-pro each). Gels were stained with Coomassie and Alcian blue. In the appropriate lanes, the area migration of biglycan is indicated by the black arrowhead and that of decorin by the white arrowhead. The Coomassie stained band at ~66 K is bovine serum albumin.

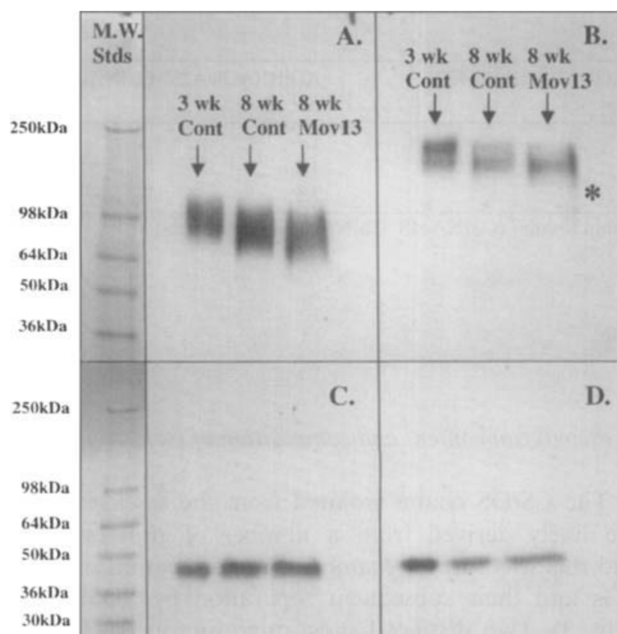


Fig. 4. Total purified proteoglycans (panels A, B) and chondroitinase ABC generated core proteins (panels C, D) were western blotted after electrophoretic separation and visualized with specific antisera to decorin (A, C) or biglycan (B, D). Migration positions of molecular weight standards are shown in the left-hand panels. * – Immunoreactive biglycan from 8-week Mov13 fascicles appeared to migrate as a more polydisperse band, with a small portion slightly overlapping the size range expected of decorin.

Since the total sample loaded in each gel lane was adjusted to represent equivalent weights of collagen, the proportional amounts of decorin and biglycan could be estimated by densitometric scanning of Alcian Blue and Coomassie Blue stained PGs (Table 3). Decorin was clearly the predominant CS/DS-substituted PG and accounted for 63, 66 and 55% of the total Alcian/Coomassie Blue staining in 3-week control, 8-week control and 8-week Mov13 samples, respectively. Further, the data suggest that the ratio of decorin to biglycan was approximately 2:1 in control fascicles, but closer to 1:1 in Mov13 tissue. In keeping with the total S-GAG contents (Table 1), fascicles from 3-week control animals contained appreciably more total CS/DS PG (i.e., decorin + biglycan PG) per collagen than fascicles from mature animals, while total CS/DS PG per collagen was similar between 8-week control and Mov13 tissues (Table 3). Quantification of the core proteins was not possible at this time due to oversaturation of these bands.

## Discussion

We have utilized the isolated mouse tail tendon fascicle as a functional and homogenous volume of tendon extracellular matrix suitable for structure–function investigations [12]. Biomechanical tests on isolated fascicles harvested from 3- and 8-week control mice and from 8-week Mov13 demonstrated a moderate correlation between mechanical properties such as stiffness with parameters of matrix organization such as mean fibril diameter [12]. To more fully define 'molecular' structure–function relationships of the tendon ECM, we have now developed and applied a detailed procedure for biochemical analyses of the proteoglycan components of the isolated mouse tail tendon fascicle.

The analyses of fascicles from 3- and 8-week control mice showed that the maturation related increases in collagen fibril diameters [12] are accompanied by an increase in collagen content and a corresponding decrease in both CS/DS GAG content and CS/DS PG content (decorin and biglycan). This is similar to what has been reported for maturing rat tail tendon [40]. Interestingly, an approximate 2:1 ratio of decorin to bigylcan is maintained throughout this postnatal maturation period, suggesting that the proportion of these small PGs may be interrelated in normal tendon. Moreover, CS/DS chains substituted on PGs in the fascicles from the mature animals were shorter (based on a number average chain size, Table 2), and this might in part explain the differential migrations of decorin and biglycan on SDS-PAGE (Figs. 3 and 4). Finally, this study demonstrates that CS/DS chains in the fascicles become richer in disulfated disaccharide residues with maturation. These observations clearly confirm that the documented postnatal maturation changes in the matrix composition and organization of whole tendons [10,26] are also evident in isolated fascicles. Further, they lend support for the existence of functional relationships during growth and maturation of tendon, both between parameters of matrix composition and structure (e.g., GAG content and collagen fibril diameter) [18,40], as well as parameters of matrix

Table 3
Densitometric scanning intensity of Alcian/Coomassie blue stained CS/DS PGs (A)

| | Decorin PG[a]/collagen[b] | Biglycan PG[a]/collagen[b] | (Decorin + Biglycan) PG[a]/collagen[b] |
|---|---|---|---|
| 3-week control | 1467 (63%) | 860 (37%) | 2327 |
| 8-week control | 1084 (66%) | 550 (34%) | 1634 |
| 8-week Mov13 | 785 (55%) | 631 (45%) | 1416 |

[a] Biglycan and decorin PGs determined as Alcian/Coomassie Blue stained bands migrating between ~65–240 kDa (see Fig. 3).
[b] All lanes were normalized to represent ~500 μg of hydroxyproline.

composition and mechanics (e.g., GAG or collagen content and tissue modulus) [23].

However, our data on matrix organization and composition in the Mov13 transgenic fascicles showed that alternate pathways of extracellular matrix maturation could exist. In mature Mov13 mice, we showed that individual fascicles from the tail tendons of transgenic animals had a reduced cross-sectional area compared to age-matched controls and contained an increased number of small diameter fibrils, similar to the collagen profile seen in the immature control animals [12]. At the molecular level, collagen content, total PG (decorin + biglycan), and S-GAG content per collagen showed no differences between 8-week control and Mov13 fascicles, and CS/DS sulfation patterns and number average chain length were identical. However, both decorin and biglycan in Mov13 tissue appear to migrate slightly faster on SDS-PAGE than the corresponding species from 8-week control fascicles. As all core proteins detected by Western blots were similar in size (45 kDa) between groups (Fig. 4), the difference in migration of the intact PGs is likely related to their substituted GAG chains.

Western blot analyses of biglycan and decorin from Mov13 fascicles showed that compared to age-matched controls, both PGs contained an additional population of faster migrating molecules. This could suggest the presence of uniquely glycosylated PGs [36], perhaps substituted with shorter chains. Although the average length of all CS/DS in Mov13 fascicles was not notably different from age-matched controls (Table 2), it cannot be excluded from the current study that specific alterations in chains derived from decorin and biglycan may be masked if a large CS PG such as versican [47] contributes appreciably to the disaccharide analysis (Fig. 3). Alternately, differences in iduronate and charge density distributions may cause the intact PG molecules to migrate differently on SDS-PAGE. Furthermore, the observation that immunoreactive biglycan from the 8-week Mov13 fascicles appeared to migrate as a more polydisperse band slightly overlapping the size range expected of decorin suggests the presence of a unique PG population in the transgenic tissue (Fig. 4, panel B). This may suggest that a small proportion of biglycan in Mov13 fascicles is substituted with a single CS/DS chain [37]. Additional studies are necessary to investigate these and other possible explanations for our findings as well as the functional consequences of altered proteoglycan synthesis.

Between 3- and 8-weeks of age, the decorin:collagen ratio in control fascicles was reduced in association with a significant increase in collagen fibril diameters [12], as has been shown previously in maturing tendon [40]. Interestingly, this ratio was even lower in (mature) Mov13 fascicles, albeit they had a significantly higher abundance of small diameter collagen fibrils than age-matched controls [12]. In other words, the presence of small fibrils in Mov13 does not appear to be associated with an elevated decorin:collagen ratio as it is in the immature tissue, but could result from a greater abundance of other matrix components. Alternately, the reduced fibril size in Mov13 may be predominantly influenced by the genetically altered collagen production. The results of this study together with our previous observations that the material properties of modulus and maximum stress in Mov13 fascicles are 2–5 times greater than 3-week controls and 20–25% greater than age-matched controls [12], however, do suggest that neither individual matrix components nor collagen fibril size are the sole determinant of tissue mechanics. Investigation of these and other potential adaptive pathways for matrix construction are the focus of our ongoing structure–function studies.

In summary, our ability to examine both the biochemical composition and biomechanical properties of isolated mouse tail tendon fascicles will allow us to investigate the extent to which other experimental paradigms, such as immobilization, overuse, and specific genetic alterations in matrix molecules may influence the structure–function relationships in this tissue. Such detailed and integrated studies on this well defined tissue/extracellular matrix model system should not only yield novel insights into molecular structures, interactions, and mechanical functions of tendons, but may also reveal adaptive pathways that can be activated during the treatment of clinical conditions affecting these and similar types of soft connective tissues.

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