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ARTICLES

Decorin Regulates Assembly of Collagen Fibrils and Acquisition of Biomechanical Properties During Tendon Development

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Abstract Tendon function involves the development of an organized hierarchy of collagen fibrils. Small leucine-rich proteoglycans have been implicated in the regulation of fibrillogenesis and decorin is the prototypic member of this family. Decorin-deficient mice demonstrate altered fibril structure and mechanical function in mature skin and tail tendons. However, the developmental role(s) of decorin needs to be elucidated. To define these role(s) during tendon development, tendons (flexor digitorum longus) were analyzed ultrastructurally from postnatal day 10 to 90. Decorin-deficient tendons developed abnormal, irregularly contoured fibrils. Finite mixture modeling estimated that the mature tendon was a three-subpopulation mixture of fibrils with characteristic diameter ranges. During development, in each subpopulation the mean diameter was consistently larger in mutant mice. Also, diameter distributions and the percentage of fibrils in each subpopulation were altered. Biomechanical analyses demonstrated that mature decorin-deficient tendons had significantly reduced strength and stiffness; however, there was no reduction in immature tendons. Expression of decorin and biglycan, a closely related family member, was analyzed during development. Decorin increased with development while biglycan decreased. Spatially, both had a comparable localization throughout the tendon. Biglycan expression increased substantially in decorin-deficient tendons suggesting a potential functional compensation. The accumulation of structural defects during fibril growth, a period associated with decorin expression and low biglycan expression, may be the cause of compromised mechanical function in the absence of decorin. Our findings indicate that decorin is a key regulatory molecule and that the temporal switch from biglycan to decorin is an important event in the coordinate regulation of fibrillogenesis and tendon development. J. Cell. Biochem. 98: 1436–1449, 2006.

Key words: collagen fibrillogenesis; tendon; development; decorin; biglycan; extracellular matrix

Tendons are uniaxial connective tissues that transmit mechanical forces. They are composed primarily of aligned columns of fibroblasts, collagen fibrils grouped as fibers, and an interfibrillar matrix [Benjamin and Ralphs, 2000; Kjaer, 2004; Zhang et al., 2005]. The structure and function of a mature tendon are determined by the tendon-specific assembly of the extracellular matrix. Fibrillogenesis and matrix assembly are multistep processes and each step is independently regulated during tendon development. Each step takes place in a unique compartment, both cytoplasmic and extracellular [Birk and Trelstad, 1985, 1986; Birk et al., 1989, 1990, 1995, 1996; Canty et al., 2004]. This provides a mechanism for compartmentalizing

Abbreviations used: FDL, flexor digitorum longus tendon; SLRP, small leucine-rich proteoglycan/protein; P, postnatal; Dcn, decorin; Bgn, biglycan.

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the regulatory interactions involving extracellular macromolecules, such as quantitatively minor fibrillar collagens, fibril-associated collagens, and small leucine-rich proteoglycans (SLRPs) [Iozzo, 1999; Ezura et al., 2000; Young et al., 2000; Ameye and Young, 2002; Wenstrup et al., 2004; Zhang et al., 2005].

The interactions of collagen fibrils with small leucine-rich proteoglycans have been implicated as important regulators of collagen fibrillogenesis. SLRPs are a family comprising structurally related, but genetically distinct proteoglycans/glycoproteins that can be grouped into three classes [Iozzo, 1999]. The members of each class have high protein sequence identity and show related biological roles. Decorin and biglycan (class I) and fibromodulin and lumican (class II), are expressed in tendon [Ezura et al., 2000; Corsi et al., 2002; Zhang et al., 2005]. These four SLRPs bind to fibrillar collagens via two distinct sites. Decorin and biglycan compete for the same site on collagen I and this locus is distinct from the fibromodulin/lumican-binding site [Rada et al., 1993; Svensson et al., 1995; Schonherr et al., 1995a,b]. Leucine-rich proteoglycans have a central domain with leucine-rich repeats flanked by two cysteine-rich domains [Iozzo, 1998, 1999]. The leucine-rich domain is a structural module involved in molecular recognition. Modeling of decorin, the prototypic SLRP, demonstrates an arch-shaped structure with dimensions that would permit the interaction with a collagen triple helix [Weber et al., 1996].

Gene-targeting studies using mice deficient in decorin indicate that these SLRPs/glycoproteins are involved in determining the mature collagen fibril structural phenotype and tissue function [Danielson et al., 1997]. Decorin-deficient mice have defects in different connective tissues including dermis and tendon. The fibril profiles in the dermis and tail tendons are irregular relative to the wild-type controls, but tend toward circular. The fibrils from the deficient tissues also have larger diameters. Biomechanical studies demonstrate a decrease in tensile strength of the decorin-deficient dermis [Danielson et al., 1997] and comparable differences also are observed in periodontal ligaments [Hakkinen et al., 2000]. Biglycan-deficient mice have abnormal fibril structures, analogous to those seen in the decorin-deficient mice, in a variety of tissues including skin, bone, and tendons [Young et al., 2002]. Mice double deficient in both decorin and biglycan contain a population of fibrils that are markedly aberrant in structure, suggesting an interaction between these two closely related SLRPs [Corsi et al., 2002]. Mechanical studies indicate complementary roles for decorin and biglycan in different tendons [Robinson et al., 2005]. Structural and functional analyses of developing/maturing load-bearing tendons, for example, flexor digitorum longus, are required to address the regulatory roles of the proteoglycans.

The purpose of this study was to determine the functional role(s) of decorin in the development of the structural and functional properties of tendon. To this end, ultrastructural analyses of fibril structure and diameter distribution; analyses of decorin and biglycan expression; and evaluation of mechanical properties were conducted at multiple stages of tendon development, growth, and maturation in the flexor digitorum longus. In addition, the functional relationship between decorin and biglycan expression in tendon development was investigated, because complementary roles for decorin and biglycan have been suggested by previous structural [Corsi et al., 2002; Young et al., 2002] and mechanical [Robinson et al., 2005] studies. We hypothesized that decorin has a primary role in the regulation of tendon fibrillogenesis. However, decorin and biglycan may have coordinate regulatory functions in tendon development.

MATERIALS AND METHODS

Animals

Decorin-deficient mice were generated by targeted gene disruption as described [Danielson et al., 1997]. The flexor digitorum longus (FDL) tendons from hind limbs of male B1/Swiss animals were used in the study except for postnatal days (P) 1 and 10 where sex was not determined. In addition, the tail tendons from 7.5-month male mice were analyzed. Sections were taken from comparable regions in the mid tendons, in the case of the FDL in the mid region prior to its bifurcation to the digits.

Transmission Electron Microscopy

Transmission electron microscopy was done as previously described [Birk and Trelstad, 1986; Birk et al., 1997]. Briefly, tendons were dissected from both decorin$-/-$ and decorin$+/+$ mice at multiple developmental stages (P1, 10, 60, 90, and 7.5 months) and then fixed in 4%

paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, with 8.0 mM $CaCl_2$ followed by post-fixation with 1% osmium tetroxide, and enbloc staining with 2% uranyl acetate in 50% ethanol. After dehydration in an ethanol series followed by propylene oxide, the tendons were infiltrated and embedded in a mixture of EMbed 812, nadic methyl anhydride, dodecenylsuccinic anhydride and DMP-30 (Electron Microscopy Sciences, PA.). A Reichert UCT ultramicrotome and a diamond knife were used to prepare thin sections (90–100 nm). The sections were stained with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid, pH 3.2. Cross sections from the mid-plantar regions of FDL tendons were examined and photographed at 75 kV using a Hitachi 7000 transmission electron microscope.

Fibril Diameter Distribution

For each genotype, four to six different tendons from two to six different animals were analyzed. Micrographs (21–41/group) from non-overlapping regions of the central portion of the tendon were taken at 31,680X. Negatives were randomly chosen in a masked manner from the different groups, digitized, and diameters were measured using a RM Biometrics-Bioquant Image Analysis System (Nashville, TN). A region from a single photographic negative, containing 50–528 fibrils/field, was measured. Frequency of different diameter fibrils was analyzed in a total of 2,876–9,550 collagen fibrils/group. For each group, the number of animals, number of different fields (negatives) [Animals/negatives (fibril number, min–max)] is as follows: P1+/+, 5/25 (259–528); P1−/−, 5/25 (184–488); P10+/+, 5/38 (160–358); P10−/−, 6/41 (110–315); P60+/+, 2/21 (94–163); P60−/−, 2/22 (57–126); P90+/+, 5/37 (57–157); and P90−/−, 5/39 (50–176).

Statistical Analyses of Fibril Diameter Subpopulations

The distributions of fibril diameters in microscopic fields from P1 tendons were generally symmetric and unimodal. Therefore, the data from each microscopic field in P1 animals were first modeled as one normally distributed population. In the statistical analyses of the P10 and P90 data, the fibril diameters in each field were assumed to be a heterogeneous mixture of a few normal components/subpopulations, so that each component represents a normally distributed subpopulation of fibrils. This approach is called finite mixture modeling [McLachlan and Peel, 2000]. The data from each field was analyzed separately using the software package Emmix 2.0 (Emmix 2.0 (2000) D. Peel and G.J.McLachlan, University of Queensland, St. Lucia, Queensland, Australia). By fitting a mixture model, the means and standard deviations of these components as well as their mixing proportions were estimated. To determine the smallest adequate number of components in the mixture, the Schwarz's Bayesian Information Criterion (BIC) was considered for a range of components from one to four.

In the data from P10 tendons, 74% of microscopic fields required at least 2 components, 16% required at least 3 components, and 10% required at least 1 component in the finite mixture model. Thus, the fibril diameter distribution in each microscopic field from P10 animals was modeled as a two-component mixture of normal distributions. In the P90 data, 48% of microscopic fields (44% and 52% in wild-type and mutant tendons) required at least two components and 52% (56% in wild-type and 48% in the mutant) required at least three components in the finite mixture model. Therefore, the fibril diameter distribution in each microscopic field was modeled as a three-component mixture of normal distributions.

The means, standard deviations, and mixing proportions of normal components fitted for each microscopic field were assumed to depend on genotype, age, and weight of animal, and also randomly vary somewhat from animal to animal and from field to field. This dependency was modeled in a linear mixed effects model that allows the incorporation of animal-to-animal and field-to-field variability [Vonesh and Chinchilli, 1997]. Separate linear mixed effects models were fitted for the estimates of means, standard deviations, and mixing parameters for each developmental stage examined. In addition, one common model for the means from all ages was used. Standardized residuals were examined to validate assumptions of the linear mixed effects model, and outliers compromising these assumptions were excluded.

Biomechanics

FDL tendons were dissected from seven to nine mice per group, marked with stain lines for optical strain measures [Derwin et al., 1994] and thickness and width were measured as previously described [Soslowsky et al., 1994, 2000]. Tendons were held by compressive grips

at points just distal to where the tendon wrapped around the calcaneus and just proximal to where the tendon broadens and sends branches to each phalange. Each tendon underwent the following protocol: preload to 0.02 N, preconditioning for 10 cycles from 0.02 to 0.05 N at a rate of 0.1%/s, hold for 300 s, stress relaxation experiment to a strain of 5% at a rate of 25%/s, relaxation for 600 s, and finally a ramp to failure at a rate of 0.1%/s [Robinson et al., 2004, 2005]. To test for differences in biomechanical properties between decorin-deficient and wild-type FDL tendons, two separate one-way ANOVAs were performed at each stage of development (P60 and P150), with statistical significance set at 0.05.

Semi-Quantitative RT-PCR

Semi-quantitative two-step RT-PCR was done as previously described [Young et al., 2000; Zhang et al., 2003]. Total RNA preparations were isolated with TriZol (Invitrogen) from tendons of decorin-deficient (P30) and wild-type (P1, P4, P10, P30, and P90) mice. cDNAs were prepared by reverse transcription of total RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems). The decorin forward primer is: 5′-AGG CAT TCA AAC CTC TCG TG-3′, reverse primer: 5′-CCG CCC AGT TCT ATG ACA AG-3′ yielding an 181 bp product. The biglycan forward primer is: 5′-GAC AAC CGT ATC CGC AAA GT-3′ and reverse primer is: 5′-GTG GTC CAG GTG AAG TTC GT-3′ producing a 213 bp band.

Semi-quantitative PCR was done using the QuantunRNA™ Classic II 18S internal standard (Ambion). To normalize the samples, 18S rRNA was used as an internal control. The control's linear range was attenuated by mixing the 18S primers with competimers that anneal to the 18S cDNA, but can not amplify it. This allowed analyses of the target RNA and the internal standard in the same linear range. The classic II 18S internal standard was used at a primer pair to competimer ratio of 2:13 and yielded a 324 bp product. The PCR program was: 94°C 2 min followed by cycles of 94°C 15 s, 60°C 25 s, 72°C 40 s (20 cycles for decorin and 21 cycles for biglycan for the analyses of different developmental stages; and 25 cycles for decorin and 27 cycles for biglycan for the analyses comparing wild-type and mutant samples), and a final extension at 72°C for 10 min. The density of the PCR product bands was analyzed using Quantity One software (BioRad).

Immuno-Blotting Analyses

Tendons from decorin-deficient (P30) and wild-type (P4, P10, and P30) mice were dissected, rinsed in PBS, and homogenized in 10–20-fold excess (weight/volume) of extraction buffer (4 M guanidine-HCl, 50 mM sodium acetate, pH 5.8) with proteinase inhibitor (Roche). Proteoglycans were extracted at 4°C for 48 h with stirring followed by clarification by centrifugation. After dialysis against 150 mM Tris-HCl pH 7.3, 150 mM NaCl, the samples were digested with chondroitinase ABC for 24 h at 37°C. Total protein content in the samples was determined using a BCA Protein Assay Kit (Pierce).

For semi-quantitative analysis of decorin and biglycan in normal tendons, 10 μg of total protein from each preparation was loaded. In the analyses of wild-type and mutant tendons at P30, 20, 10, and 5 μg of total protein from both decorin-deficient and wild-type mice were loaded. After transfer, the gels were stained with Gel Code Blue Stain Reagent (Pierce) to monitor the collagen type I loaded. Immuno-blotting was done as previously described [Young et al., 2002]. Anti-decorin (LF113) was used at 1:400 and anti-biglycan (LF159) at 1:200. The antibodies were from Dr. L. Fisher, NIH-NICDR [Fisher et al., 1995]. Goat anti-rabbit IgG-peroxidase (Sigma) was used as secondary antibody at 1:3,000 with an ECL (Amersham) detection system.

Immunofluorescence Microscopy

Tendons from P30 decorin-deficient and P4, P10, P30, and P90 wild-type mice were dissected, mounted in OCT, and quickly frozen in an ethanol-dry ice bath. Cryostat sections were prepared and stored at −80°C. Immunofluorescence staining was done as previously described [Zhang et al., 2003]. Anti-mouse decorin antibodies (LF113) were used at 1:200 and anti-mouse biglycan antibodies (LF159) at 1:100. The secondary antibody was anti-rabbit IgG-Alexa Fluor 568 (Molecular Probes) used at 1:400. DAPI was present in the mounting medium (Vector Lab) for nuclear localization.

RESULTS

Abnormal Regulation of Fibril Growth in Decorin-Deficient Mice

Collagen fibril ultrastructure was analyzed from cross sections of FDL tendons from decorin-deficient and wild-type mice at postnatal

day 1–90 (P1, P10, P60, and P90). These analyses revealed fibrils with increased diameters in the decorin-deficient tendons compared to the wild-type controls. Irregular fibril profiles were a prominent feature of fibrils from the decorin-deficient tendons at P10 to P90. In contrast to the uniformly circular outline of the fibrils in wild-type mice, individual fibrils in the decorin-deficient mouse tendons demonstrated irregular profiles and abnormal lateral association with adjacent fibrils, indicating abnormal lateral fusion or molecular rearrangement. The irregular shaped fibrils increased in frequency as the mutant tendons developed from P10 to P90 (Fig. 1).

Collagen fibril diameter distributions were analyzed development (Fig. 2). Postnatal day 1 (P1) was representative of a developmental period characterized by the assembly of immature fibril intermediates. Postnatal day 10 (P10) and 60 (P60) represent stages characterized by the linear and lateral growth of fibrils from immature intermediates. The 3 month tendon (P90) is a structurally and functionally mature tendon. At all developmental stages analyzed (P1, P10, P30, and P90) there was a population of larger diameter fibrils in the decorin-deficient tendons. These abnormal, large diameter fibrils were not present in the wild-type tendons. At P1 and P10 there was a broadening of the distribution with the number of largest diameter fibrils increased in the deficient tendons. At P60 and P90, in addition to the population of larger diameter fibrils in the mutant tendons, there was a decrease in the number of 20–200 nm diameter fibrils compared to the control tendons. During fibril growth, the decorin-deficient fibril intermediates progressed through the multiple fibril growth steps more rapidly and this was associated with the assembly of an abnormal population of large diameter fibrils. These data suggest a less regulated assembly of fibril intermediates in the absence of decorin.

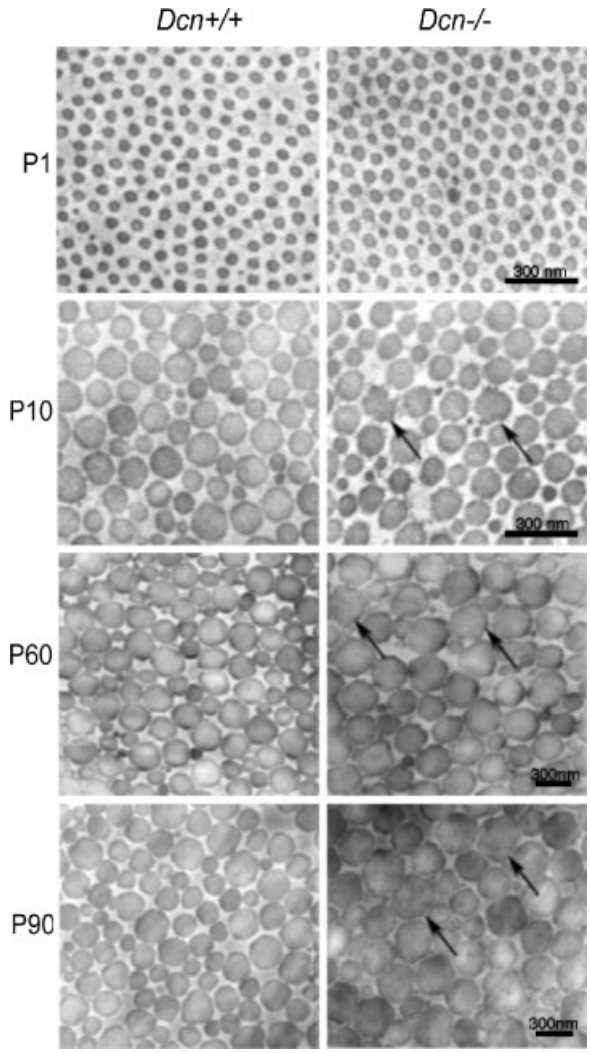

Fig. 1. Abnormal fibril structure in decorin-deficient tendons. In decorin-deficient FDL tendons (*Dcn−/−*), irregular shaped collagen fibrils increased in frequency (arrows) and the fibril diameters were consistently larger, compared with wild-type tendons (*Dcn+/+*). This indicates that decorin-deficiency results in abnormal fibril growth. The P1 tendons were composed of fibrils with circular profiles. The increased diameter and abnormal fibril structure was associated with lateral fibril growth stages (P10, P60) and the mature tendon (P90). The magnification in these transmission electron micrographs of P1 and P10 tendons is approximately twice that for the P60 and P90 tendons.

Analysis of Distinct Steps in Fibril Assembly and Growth Using Statistical Modeling

The analyses of the wild-type fibril diameter distributions at P1 through P90 indicated that fibril subpopulations were added as tendon development progressed (Fig. 2). At P1 the distribution was unimodal, additional fibril subpopulations were acquired during fibril growth (P10–P60). The multiple fibril diameter subpopulations were maintained in the mature tendon (P90). The resultant tendon fibril population is an irregular, non-Gaussian distribution pattern. Thus, to analyze the assembly of the initial fibril intermediates, fibril growth from these intermediates and the fibrillar composition of the mature tendon as well as the effects of decorin on these processes, statistical models to fit the fibril diameter data were developed using finite element analysis.

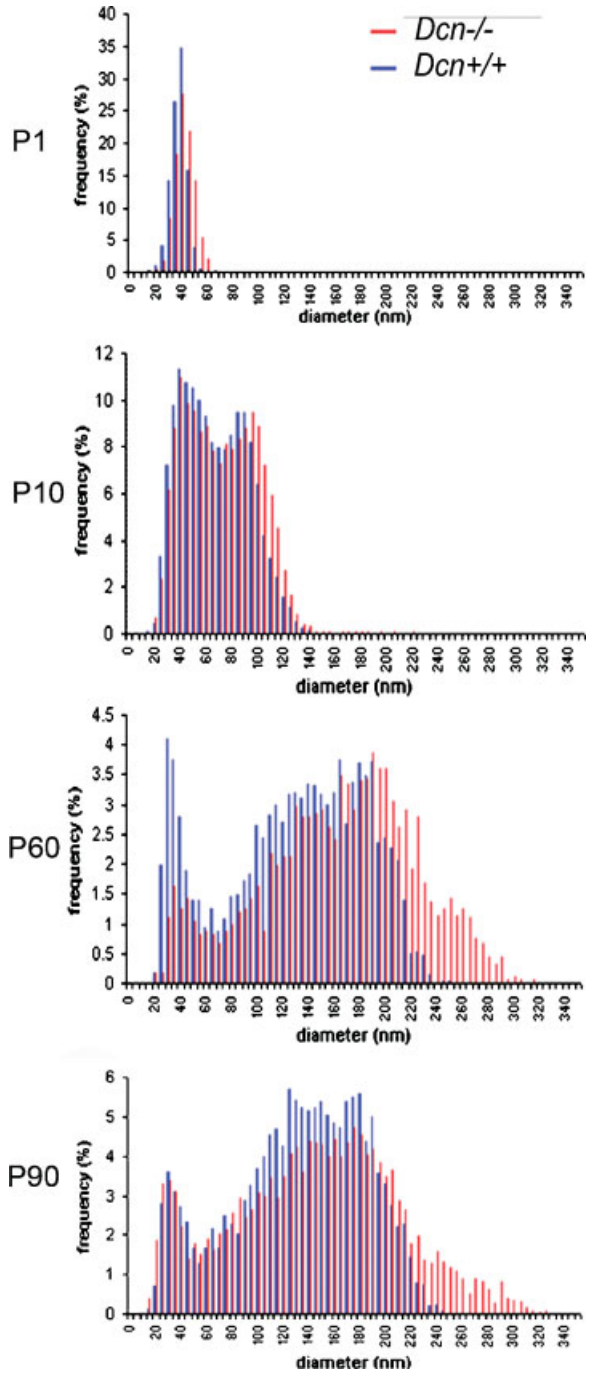

Fig. 2. Collagen fibril diameter distributions are altered in decorin-deficient tendons. At every stage studied, the diameter distributions indicate that, the fibril diameters in mutant tendons were larger than in wild-type tendons. P1 represents a period of fibril intermediate assembly; P10 and P60 represent the beginning and end of the lateral fibril growth stage, respectively; while P90 is representative of a mature tendon. The distributions at P10 to P90 were composed of multiple subpopulations. The data indicate more rapid and premature fibril growth in decorin-deficient tendons compared to the wild-type controls, consistent with a loss in regulation of these steps.

Based on the qualitative fibril diameter distributions in Figure 2, it was assumed that the mature tendon (P90) was composed of a heterogeneous mixture of a few normal distributions or components, so that each component represents a normally distributed subpopulation of fibrils. Individual microscopic fields from P90 tendons were analyzed (Fig. 3). These diameter distributions were best modeled as a three component (subpopulation) system. Subpopulation I represents the immature fibril intermediates characteristic of P1 tendons, subpopulation II the growing fibrils, and subpopulation III the mature fibrils [Ezura et al., 2000].

The fibril subpopulation composition was analyzed using finite mixture modeling of data from tendons at P1, P10, and P90 (Fig. 4). The

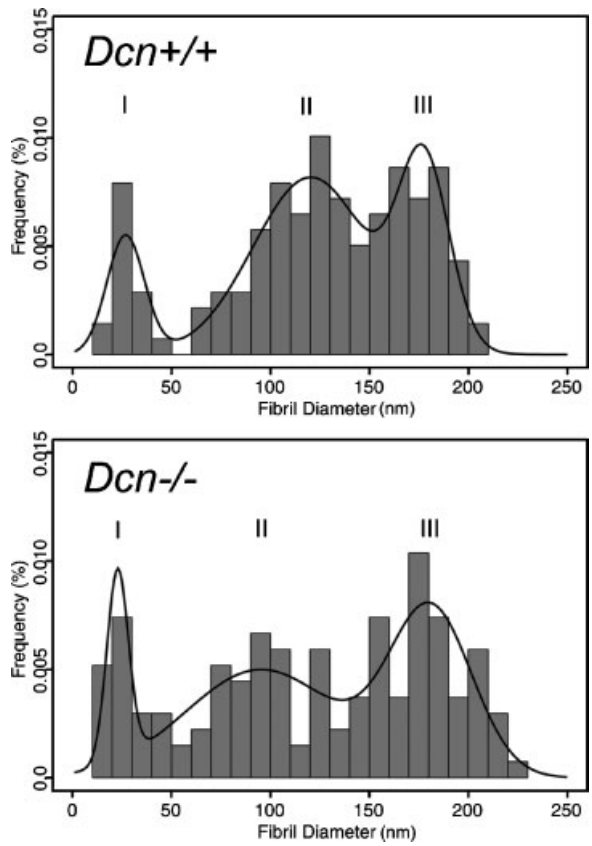

Fig. 3. Mature tendons contain three fibril subpopulations. Representative microscopic fields from wild-type (*Dcn*+/+) and decorin-deficient (*Dcn*−/−) tendons. The fibril diameter distributions from each field were analyzed using finite element modeling. The diameter distributions were best modeled as three fibril subpopulations. The histograms and fitted mixtures of three normal components for a sample microscopic field from *Dcn*+/+ and *Dcn*−/− tendons is presented.

normal, wild-type tendons had a single population of fibrils at P1, representative of a stage where, primarily, fibril intermediates were being assembled. This small diameter, fibril subpopulation (I) was present at all developmental stages studied. At P10, a second larger fibril subpopulation (II) was added, representing the beginning of fibril growth. In the mature tendon (P90), the second subpopulation (II) had broadened and a third larger fibril subpopulation (III) was present, representative of mature fibrils. The average means, standard deviations, and mixing parameters of each subpopulation, estimated from the corresponding linear mixed effects models, are given in Table I.

These subpopulations were analyzed to determine whether there were significant differences between wild-type and decorin-deficient tendons. The means of the subpopulations were analyzed in the model combining all ages. There was no effect of body weight on the means of the mixture distributions. Overall (across developmental stages and subpopulations) decorin-deficiency had a significant effect on the subpopulation means ($P = 0.030$). In P1 and P10 decorin-deficient tendons, there was a consistent, small positive shift (4.2–5.3 nm) in each mean of the corresponding subpopulation relative to the wild-type controls. At P90, there was a significant difference between wild-type and decorin-deficient tendons ($P = 0.011$) jointly in all three subpopulations, but not in each subpopulation considered separately. Only for the second subpopulation (II), the negative shift

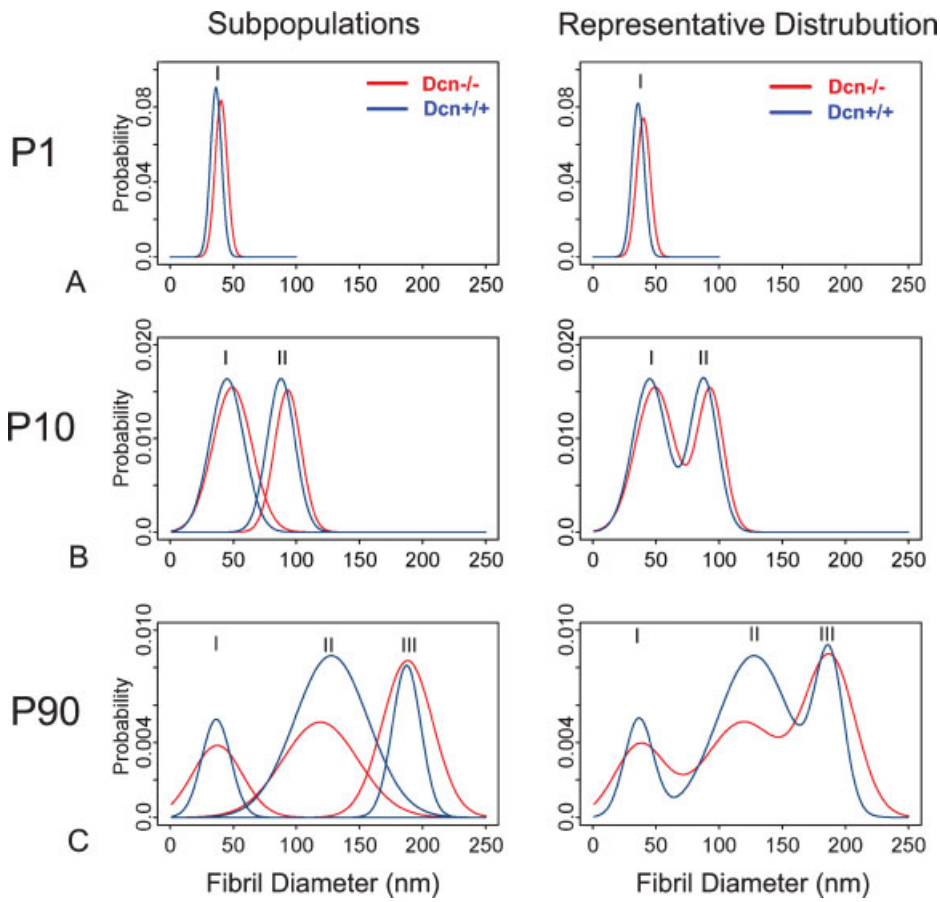

Fig. 4. Alteration of fibril subpopulations in decorin-deficient tendons. The diameter distributions were best modeled as a system composed of three fibril subpopulations. At P1 there is a single subpopulation (I). At P10, a second subpopulation (II) was present. The mature (P90) tendon was composed of three distinct fibril subpopulations. Subpopulation (I) represents the immature fibril intermediates, (II) the growing fibrils, and (III) the mature fibrils. All three subpopulations were altered in the P90 decorin-deficient tendons (*Dcn−/−*, red line) compared to the wild-type (*Dcn+/+*, blue line) tendon, showing increased mean diameters, more variation, and changes in the proportions comprising each subpopulation. The different fibril subpopulations are presented (**left column**). These plots are fitted averages for diameter distributions from microscopic fields from wild-type (blue) and decorin-deficient (red) tendons. A representative fibril diameter distribution is plotted for P1, P10, and P90 tendons (**right column**). The wild-type tendons are plotted in blue; the decorin-deficient tendons are in red.

TABLE I. Fibril Subpopulations in Wild-Type and Decorin-Deficient Tendons During Development

		Dcn+/+		Dcn−/−	
Stage	Subpopulation	Mean (nm)(95% CI[b]) ± SD (95% CI[b])	MixP[a] (%) (95% CI[b])	Mean (nm) (95% CI[b]) ± SD (95% CI[b])	MixP[a] (%) (95% CI[b])
P1	I	35.8 (31.1–40.5) ± 4.9 (4.5–5.2)	—	40.1 (34.6–45.5) ± 5.4 (5.0–5.8)	—
P10	I	45.8 (40.2–49.8) ± 13.4 (10.6–16.2)	55% (48–63)	49.2 (43.7–54.7) ± 15.5 (12.7–18.2)	60% (53–67)
	II	87.8 (81.0–94.7) ± 11 (8.9–13.0)	45% (37–52)	93.1 (87.5–98.8) ± 10.5 (8.6–12.5)	40% (33–47)
P90	I	36.5 (31.8–41.3) ± 11.4 (6.2–16.6)	15% (12–17)	37.3 (31.6–43.0) ± 19.7 (5.4–34.1)	19% (15–23)
	II	127.7 (120.9–134.4) ± 29.1 (18.0–40.2)	63% (55–70)	119.0 (112.8–125.2) ± 29.8 (22.6–36.9)	38% (31–46)
	III	187.3 (179.6–195.0) ± 11.3 (8.2–14.3)	23% (19–27)	188.1 (183.6–192.7) ± 20 (14.7–25.2)	42% (35–50)

[a]Mixing parameter (MixP) is the average percentage of fibrils found in each subpopulation.
[b]Ninety five percent confidence interval.

of −8.7 nm from wild-type to mutant approached significance ($P = 0.062$). No significant difference was found comparing the means of subpopulation I between genotypes at all ages.

The standard deviations and mixing proportions of the subpopulations were analyzed separately for different ages. At P1, body weight had a significant effect on the standard deviations ($P = 0.007$), but the greater spread observed in the decorin-deficient tendons only approached significance ($P = 0.063$). In P10 tendons, the means were consistently larger in the decorin-deficient mice, but there were no statistically significant differences.

In the mature (P90) tendons, body weight had a significant effect on the standard deviations and mixing proportions. Considering all three subpopulations together, the decorin-deficient tendons were significantly different from the wild-type mice in terms of standard deviations ($P = 0.043$) and mixing proportions ($P < 0.001$). On average, decorin-deficient mice had more spread in subpopulation I (by 8.3 nm, $P = 0.192$) and subpopulation III (by 8.7 nm, $P = 0.007$). In terms of mixing proportions, the difference between decorin-deficient and wild-type was not significant for subpopulation I, the smaller fibrils (4.7%, $P = 0.059$), and significant for subpopulation II (−24.2%, $P = 0.001$) and for subpopulation III, the largest fibrils (19.5%, $P < 0.001$).

Decreased Biomechanical Properties of Mature Decorin-Deficient Tendons

The effect of the dysfunctional regulation of tendon fibril assembly/structure, due to the absence of decorin, on tendon function was examined. Biomechanical testing was performed on immature, end of fibril growth phase (P60), and mature (P150) tendons (Fig. 5). The mature decorin-deficient tendon demonstrated a significant decrease in mechanical strength and stiffness relative to the wild-type controls ($P < 0.05$). This was the case for all mechanical properties analyzed, that is, maximum load, maximum stress, stiffness, and modulus. These data indicate that decorin-regulated linear and lateral fibril growth is a requisite for appropriate function in the mature tendon. However, this is not the case at the end of the growth stage (P60), when there are no significant differences in these mechanical properties between decorin-deficient and wild-type FDL tendons.

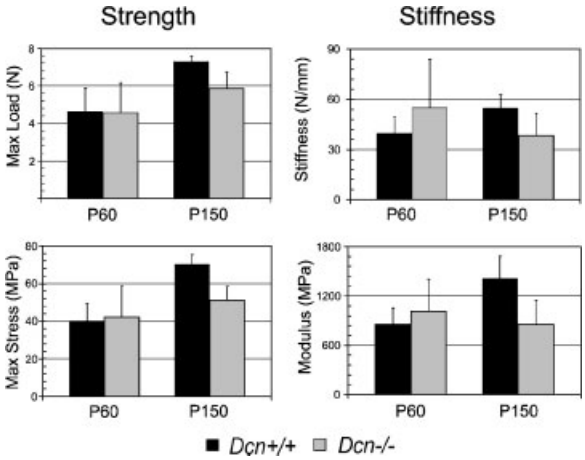

Fig. 5. Reduced strength and stiffness in mature decorin-deficient tendons. Biomechanical testing demonstrated that the mechanical properties of mature 5 month tendons (P150) were significantly reduced in the absence of decorin. However, there was no change at 2 months (P60). FDL tendons from decorin-deficient (*Dcn−/−*) and wild-type (*Dcn+/+*) from 2 month (P60) and 5 month (P150) mice.

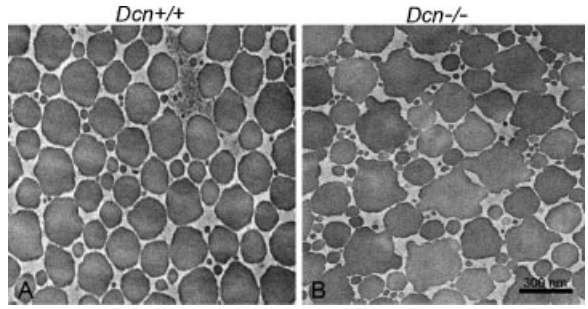

Fig. 6. Tissue-specific fibril structure associated with decorin-deficiency. Transmission electron microscopy of fibril structure in decorin-deficient (*Dcn−/−*) tail tendons. In 7.5-month *Dcn−/−* tail tendons, a high percentage of the fibrils are non-cylindrical and structurally aberrant compared to the wild-type (*Dcn+/+*) controls. The severity of the fibril phenotype is considerably greater than seen in the flexor digitorum longus tendon (see Fig. 1).

Abnormal Fibril Structure Associated With Decorin-Deficiency is Tendon-Specific

The initial demonstration of abnormal collagen fibril structure in decorin-deficient tendons was done in the non-weight bearing tail tendon [Danielson et al., 1997]. The mature tail tendons of the decorin-deficient mice were examined and as expected, the data are comparable and demonstrate a severe fibril phenotype with large numbers of structurally aberrant fibrils with very irregular contours (Fig. 6). However, in the tail tendons, the fibrils become more irregular with age in the wild-type mice (data not shown), but the structural differences between the genotypes remain striking. The differences between the phenotype in FDL versus tail tendon illustrate that there are site/tissue-specific differences in the dominance of decorin regulation in functionally different tendons.

Relationship Between Decorin and Biglycan in FDL Tendons

Decorin and biglycan have differing temporal expression patterns. To determine whether these closely related SLRPs had coordinate roles in tendon development, their developmental expression patterns were analyzed. Decorin and biglycan were differentially expressed during normal tendon development. Expression of decorin and biglycan mRNA in normal mouse tendon development was studied by semi-quantitative RT-PCR at different developmental stages. Decorin mRNA expression was high at P1, peaked at P4–10, then decreased dramatically, and was sustained at a stable level of about 50% of peak expression. In contrast, biglycan mRNA expression was highest at P1 and decreased rapidly to a barely detectable level at P30 (Fig. 7). Decorin and biglycan protein core demonstrated a reciprocal expression pattern. Semi-quantitative immuno-blotting demonstrated that decorin increased gradually with development, 30% and 70% from P4 to P10 and P30, respectively. Presumably the relatively constant decorin mRNA level beginning at P30 allowed decorin to accumulate in the

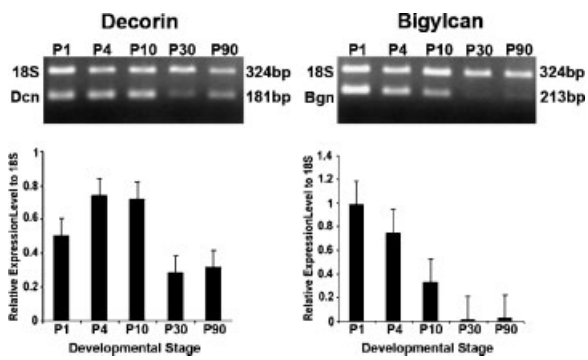

Fig. 7. Differential expression of decorin and biglycan mRNA. Decorin mRNA peaked at P4–10, decreased and sustained stable levels in mature tendons. Biglycan mRNA peaked at P1, decreased quickly and shut off in mature tendons. The gels are representative experiments and the graphs are mean relative expression levels ± SD from three independent PCR. Values are expressed relative to 18S RNA.

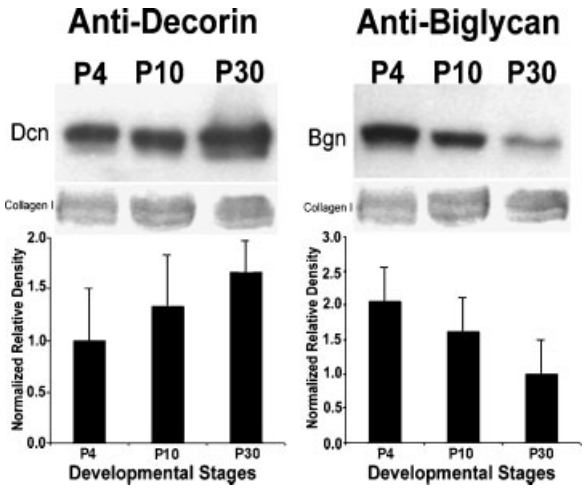

Fig. 8. Decorin and biglycan protein core content in developing tendons. Decorin increased and reached its highest level at P30 while biglycan decreased to very low levels at P30 of development. The blots are representative experiments using 10 μg total protein per well and the graphs are mean relative levels ± SD from three independent immuno-blots. Expression is relative to type I collagen.

matrix during this developmental period. In contrast, biglycan protein core decreased 37% and 50% from P4 to P10 and P30, respectively (Fig. 8). The spatial localization of decorin and biglycan were comparable. In early stages of development, for example P4, the localization of both proteoglycans was homogeneous throughout the tendon matrix. However, by P30, both decorin and biglycan were enriched peri-cellularly with weaker signal in the tendon matrix (Fig. 9).

The relationship between expression of decorin and biglycan in the decorin-deficient tendon was examined. The stage in normal tendon development (P30) where decorin peaked and biglycan decreased to its lowest level was utilized for these analyses. Immuno-localization demonstrated an increased reactivity for biglycan in the decorin-deficient versus the wild-type tendon (Fig. 10). There were significant differences in biglycan protein core expression in decorin-deficient versus wild-type tendon (Fig. 11). Biglycan protein core was present in minor amounts in the wild-type tendon. However, in the decorin-deficient tendons the

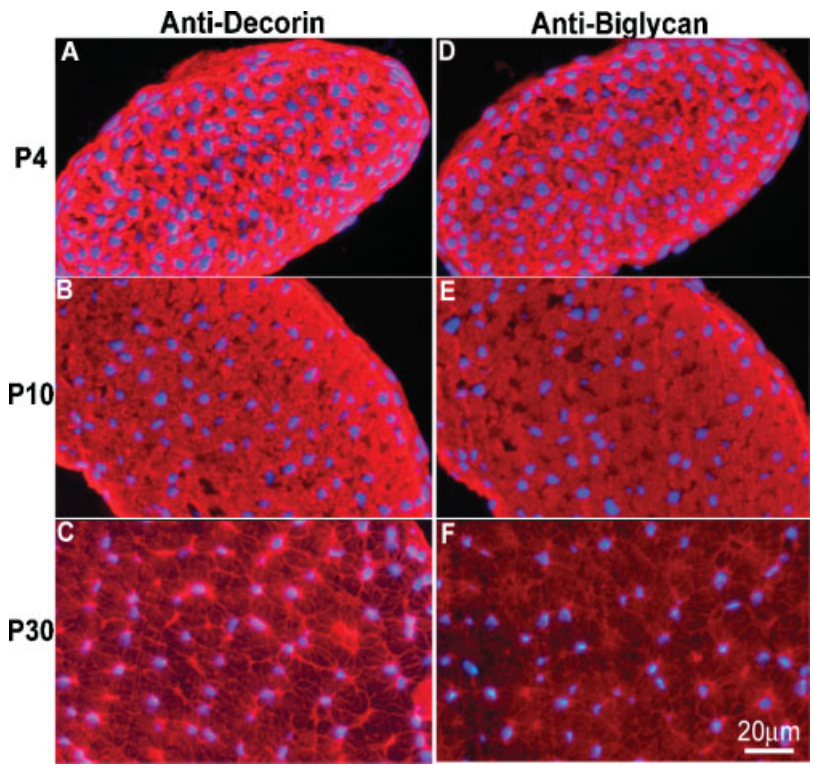

Fig. 9. Spatial localization of decorin and biglycan. The localization of decorin and biglycan was comparable. At early developing stages, P4 and P10, both decorin and biglycan were homogeneously expressed throughout the tendon. At later stages, P30, the signal was enhanced around cells, while the reactivity in the tendon proper became weaker.

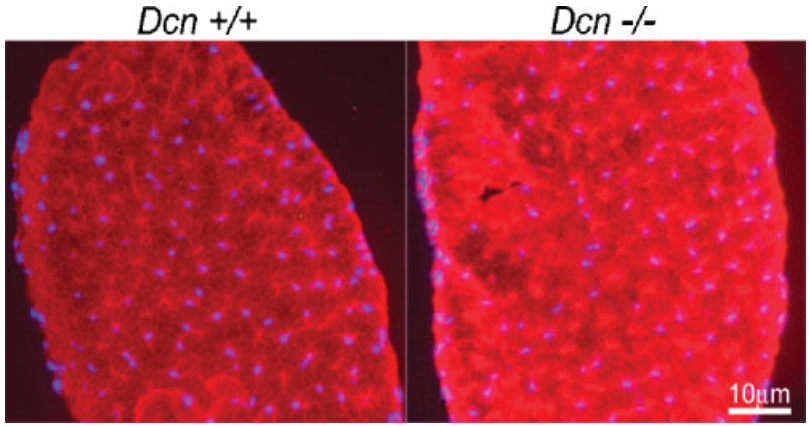

Fig. 10. Upregulation of biglycan in decorin-deficient tendons. Stronger biglycan reactivity was observed in decorin-deficient (*Dcn−/−*) tendons compared with wild-type (*Dcn+/+*) controls by immuno-localization. However, the spatial localization was comparable. This suggests that upregulated biglycan may partially compensate for the lack of decorin.

biglycan protein core was strongly expressed. As expected, immuno-blot analyses demonstrated that decorin protein core was absent in the decorin-deficient mouse tendon while strongly positive in wild-type tendons. The localization of the biglycan protein core was comparable in wild-type and decorin-deficient tendons. These data indicate that biglycan increased in the decorin-deficient mouse tendon in compensation for the lack of decorin, suggesting that biglycan may partially substitute for decorin in the regulation of collagen fibrillogenesis in tendon development.

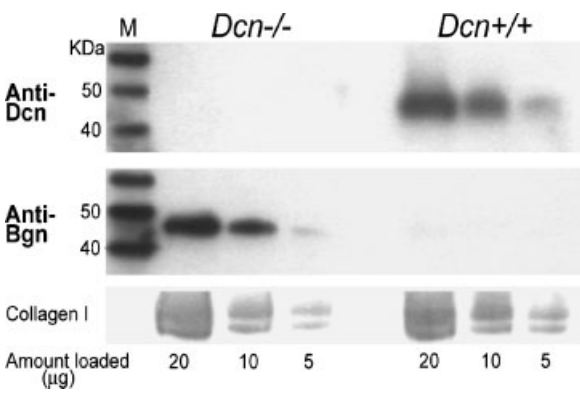

Fig. 11. Biglycan protein core expression is upregulated in decorin-deficient tendons. Semi-quantitative immuno-blots with serially diluted extracts from decorin-deficient (*Dcn−/−*) and wild-type (*Dcn+/+*) tendons showed that biglycan reactivity was strongly positive in *Dcn−/−*, but barely detectable in *Dcn+/+* tendon extracts. While decorin reactivity was normal in *Dcn+/+* and absent in *Dcn−/−* tendons. Collagen type I bands show comparable sample loadings between two groups.

DISCUSSION

In the decorin-deficient mouse tendon, fibril formation is altered at all stages (P1 through P90) of tendon development relative to the normal tendons. The structural changes include increased overall diameters and increased diameter variability with the progression through developmental stages. In addition, the fibrils demonstrate abnormal profiles beginning at P10 with the number increasing during development. These data demonstrate that decorin–fibril interactions are an important regulatory mechanism in fibrillogenesis during tendon development.

Fibril diameter analysis is utilized to follow fibril assembly and growth [Ezura et al., 2000; Zhang et al., 2005]. During tendon development, collagen fibrils are initially assembled as immature fibril intermediates. This is followed by linear and lateral growth of mature fibrils from the preformed intermediates [Birk et al., 1995; Graham et al., 2000; Canty and Kadler, 2002; Zhang et al., 2005]. In the development of the weight bearing mouse flexor digitorum longus (FDL) tendon, P1 represents the stage of immature fibril intermediate formation. The initial stages of linear and lateral growth of longer, larger diameter fibrils via association and fusion of the preformed intermediates are characteristic of P10. At P90, the mature tendon is characterized by long, large diameter mature fibrils [Ezura et al., 2000]. In the present study, altered fibril structure is observed beginning at P10, during early stages of linear and lateral

growth from immature fibril intermediates. Therefore, this indicates that decorin functions in the regulation of growth steps producing mature fibrils characteristic of the functional tendon.

It has previously been shown that the mature non-weight bearing tail tendon has altered fibril structures in decorin-deficient mice [Danielson et al., 1997]. The very aberrant fibril structure seen in decorin-deficient tail tendon fibrils is confirmed in our study. We demonstrate that fibril structure in the load-bearing FDL tendon is less severely affected. This indicates that the regulatory role(s) of decorin is modulated/modified in a tissue/tendon-specific manner. Regulation presumably involves the differential expression of multiple members of the leucine-rich proteoglycan family. We have previously demonstrated that fibromodulin and lumican are important regulators of FDL structure and mechanical function [Ezura et al., 2000; Jepsen et al., 2002]. Presumably, this pair of class II leucine-rich proteoglycans functions in conjunction with decorin in regulation of FDL development, which may not be the case in the tail tendon. Compound biglycan/fibromodulin deficient mice demonstrate synergistic effects of the double mutation on tendon fibrils [Ameye et al., 2002]. This supports the possibility of the coordinate regulation by class I and class II SLRPs in tendon fibrillogenesis.

The different steps in fibrillogenesis in decorin-deficient and wild-type FDL tendons are characterized using finite mixture modeling. Tendon fibrillogenesis is best modeled as a three-subpopulation mixture of fibrils. In early development (P1), where the immature fibril intermediates are formed, the tendon consists of a unimodal population of homogeneously small diameter fibrils. At the beginning of fibril growth (P10), the fibril diameters are heterogeneous containing two subpopulations of fibrils. The first is identical to the fibril intermediates in P1 and a second larger diameter subpopulations representing the growing fibrils. The mature tendon (P90) contains three fibril subpopulation, the fibril intermediates, the growing fibrils, and a population of large mature fibrils. Alteration of any subpopulation and abnormal diameter variation in this model system implies abnormal fibril growth and loss of a regulatory step(s) in fibrillogenesis.

During development, the mean diameter of each subpopulation is consistently larger in mutant mice, although statistical significance was detected only at P90. Also, the diameter distributions and the percentage of fibrils in each subpopulation are altered. These data indicating the absence of decorin is associated with dysfunctional regulation of fibril growth steps resulting in abnormal lateral fibril fusion in fibrillogenesis. Statistical analysis of fibril diameters revealed that all three subpopulations are significantly different in mature, P90 mutant tendons. Larger mean diameters and more variation in all three subpopulations in mutant compared to wild-type tendons indicate a more rapid and premature progression of fibrillogenesis related to decorin deficiency. This is associated with a loss of regulation and control of fibril growth steps that result in very large, abnormally packed fibrils in mature tendons. However, at early developing stages, P1 and P10, the fibril population in mutant tendon is comparable to the wild-type control, except for slightly larger fibril diameters. Thus, alterations in tendon structure due to decorin deficiency are related to mature tendons. This is the structural basis for the significantly reduced biomechanical properties observed in mature (P150), but not in growing (P60) tendons. As tendons mature, the structural defects compromise tendon function.

Mice deficient in decorin, biglycan, fibromodulin, or lumican, the four most prominent and widely expressed SLRPs have been generated [Danielson et al., 1997; Chakravarti et al., 1998; Xu et al., 1998; Svensson et al., 1999]. Each of the four single deficient animals develops distinct phenotypes demonstrating distinct functions that reflect their major physiological sites of SLRP expression. However, high protein conservation, striking structure similarity, and partially overlapping tissue distribution among SLRPs imply coordinate and redundant functions shared among these closely related molecules. The synergistic effects in double-deficient animals and more severe phenotypes in double compared to single mutant animals suggested a rescue and/or compensation mechanism in the single deficient animals and provided evidence for the existence of functional overlap between SLRPs [Ezura et al., 2000; Ameye et al., 2002; Corsi et al., 2002]. The study of fibromodulin and lumican proved the existence of compensation mechanisms between the two class II

members. In fibromodulin-deficient tendons, lumican deposition is increased, which indicates that the fibromodulin molecules are putatively replaced by lumican and lumican can partially substitute and compensate for the function of fibromodulin [Svensson et al., 1999]. Similarly, in compound mutant mice for class I members, decorin and biglycan, the phenotypes are synergistic suggesting possible coordinate regulation and possible compensation mechanisms [Corsi et al., 2002].

In this study, we examine decorin and biglycan expression to determine whether they demonstrate consecutive and reciprocal patterns analogous to fibromodulin and lumican. Our data demonstrate significantly increased biglycan protein levels in the decorin-deficient tendon, suggesting that biglycan compensates for, and potentially provides the regulatory roles normally associated with decorin. In addition, the expression of biglycan in the FDL tendon decreases dramatically and was sustained at a barely detectable level, while decorin expression remains relatively high during development, consistent with previous studies on human cartilage [Roughley et al., 1994]. The localization of biglycan in tendon by immunohistochemistry is comparable with decorin. Both decorin and biglycan compete for collagen binding, suggesting the use of identical or adjacent binding sites on the fibril [Schonherr et al., 1995a,b]. Therefore, in the decorin-deficient tendons, increased binding of biglycan to fibrils with a partial compensation for the lack of decorin would provide partial regulation. This also explains the accumulation of structural and biomechanical defects in decorin-deficient tendons mainly in the stages associated with decorin expression and low/no biglycan expression.

Overall, our data indicate a dysfunctional regulation of tendon fibrillogenesis in the absence of decorin. While consistent differences exist between decorin-deficient and wild-type tendons at all developmental stages studied, the structural abnormalities accumulate after the beginning of tendon fibril growth (P10). The accumulation of structural defects during fibril growth, a period associated with steady decorin expression and lower biglycan expression, may be the cause of compromised mechanical function in the mature, but not in the immature decorin-deficient tendon. The data suggest that the temporal switch from biglycan to decorin is an important event in the coordinate regulation of fibrillogenesis and development in load-bearing tendons such as the FDL.

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