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Glycan profiling of a defect in decorin glycosylation in equine systemic proteoglycan accumulation, a potential model of progeroid form of Ehlers-Danlos syndrome

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ABSTRACT

Defects in glycosylation of decorin can result in systemic hereditary disease. A mutation in the galactosyl transferase I gene is the underlying defect of a progeroid form of Ehlers-Danlos syndrome. We have previously described pathological changes in equine systemic proteoglycan accumulation (ESPA, formerly degenerative suspensory ligament desmitis) as consisting of excessive presence of decorin and other proteoglycans in organs and structures with a high content of connective tissue. Using liquid chromatography/mass spectrometry, and one- and two-dimensional immunoblotting we have determined that decorin from ESPA-tendons had a higher molecular weight than decorin from non-affected control tendons. Glycosaminoglycan structure and monosaccharide composition were determined with HPLC analysis of chondroitinase ABC-digested glycosaminoglycans and gas chromatography/mass spectrometry. This analysis revealed an increase in the total content of sulfated disaccharides, particularly due to enhanced sulfation at 6-position of *N*-acetyl galactosamine (GalNAc) with a subsequent decrease in the ratio of 4-sulfation to 6-sulfation disaccharides in the ESPA decorin. The ESPA-affected decorin also exhibited altered biological activity resulting in (1) diminished binding of TGFβ1 (and of anti-decorin antibody) to ESPA decorin, and (2) increased expression of TGFβ1 in ESPA tissues.

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Introduction

Decorin, a member of the class I small leucine-rich proteoglycans (SLRPs), regulates collagen fibrillogenesis by limiting the growth of diameter of collagen fibrils. It is also the main proteoglycan of tendons contributing to their structural and biomechanical integrity. The small (~40 kDa) core protein of decorin consists of three domains: N-terminus, a central region and C-terminus [1]. The N-terminal domain has an attachment site for a single dermatan/chondroitin sulfate chain. A cluster of Cys residues (CX_3CXCX_6C) is located within this domain as well. The central region includes 10 leucine-rich repeats forming parallel beta sheets which contain a site for several collagen types (type I, II, III, and VI). It also has binding sites for several other proteins such as for transforming growth factor β (TGFβ) [2], and epidermal growth receptor [3]. In addition, the attached glycosaminoglycan (GAG) chain also has binding sites for TGFβ and tumor necrosis factor α [4,5]. A C-terminal region contains, just like the N-terminus, several cysteine residues [1]. Decorin has many other functions besides its regulation of fibrillogenesis. It inhibits TGFβ activity, presumably by sequestering the growth factor [4]. It also participates in regulation of cell proliferation and immune response [6].

Biglycan, another member of class I SLRPs, binds to the same site on collagen as decorin. Fibromodulin and lumican, members of class II, bind to a different site on type I collagen molecule [7–10]. These SLRPs are thought to play similar but so far less defined roles in collagen fibrillogenesis [11]. Studies on mice deficient in one or more SLRPs have contributed to our understanding of their somewhat overlapping roles. The most dramatic effect of decorin deficiency is the emergence of irregular fibrils with large diameter and a decrease in skin and tendon tensile strength as a result of uncontrolled lateral fusion of thin and thick fibrils [12]. Abnormal fibril formation is also seen in biglycan-deficient mice [13], and such abnormalities are accentuated in double knockout mice for both decorin and biglycan [14]. In fibromodulin-deficient mice, lumican substitutes functionally for fibromodulin. In contrast, large diameter collagen fibrils forming disorganized matrix in cornea and skin were found in lumican-deficient mice. It is interesting to note that this did not result in a loss of tendon biomechanical function, and fibromodulin did not substitute for the absence of lumican function [15].

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Defects in glycosylation of proteoglycans can result in both hereditary and metabolic systemic disease. For example, a mutation in the galactosyl transferase I gene is the underlying defect of a progeroid form of Ehlers-Danlos syndrome. On the other hand, decorunt, an alternative form of decorin occurring in aging skin, is likely the product of catabolic changes rather than glycosylation defect [16]. Mutations or variations in xylosyltransferases contribute to development of chronic diseases such as diabetic nephropathy, osteoarthritis or pseudoxanthoma elasticum [17]. Similarly, mutations or variations in sulfatases can lead to mucopolysaccharidoses [18].

We have shown that equine systemic proteoglycan accumulation (ESPA), also known as degenerative suspensory ligament desmitis (DSLD), is characterized by systemic proteoglycan accumulation in tendons, ligaments, cardiovascular system, and sclerae of horses [19]. Affected horses demonstrate lameness [20], and occasionally develop loose skin and aortic aneurysm [10]. In this paper, we describe an abnormal form of decorin present in ESPA tissues. This abnormal decorin exhibits altered biological activity and likely is a result of defect in glycosylation of its GAG chain.

Experimental procedures

Tissue collection

Animal use was approved by the Animal Care and Use Committee, University of Georgia. All horses used in the study were donated to the College of Veterinary Medicine, The University of Georgia, Athens, GA. The diagnosis of ESPA in five horses (four Peruvian Paso and one Appaloosa horse) was based on history, physical and ultrasonographic examination of the distal limbs [20]. Three horses (one Percheron, one quarter horse and one Tennessee walking) donated for reasons other than lameness constituted the control group (Table 1). Immediately after euthanasia with an overdose of sodium pentobarbital, portions of superficial and deep digital flexor tendons, and suspensory ligament were removed from an area located 8–10 cm distal to the accessory carpal bone and the 4–8 cm proximal to the metacarpophalangeal joint. Tissues were either processed for histopathological studies, or frozen at −20 °C until used for biochemical analysis.

Proteoglycan extraction and purification

Portions of superficial digital flexor tendons (SDFTs) from five affected horses and from three control horses were cut up into pieces smaller than 1 mm^3, defatted briefly with a chloroform:methanol solution (1:1.6 ml/1.0 g of dry weight sample), and dried in Speedvac [21]. The dry tissue was extracted twice with 10 volumes of guanidine HCl extraction buffer (4 M guanidine HCl, 0.05 M sodium acetate, pH 6.0, 0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.05 M EDTA, 5 mM benzamidine, 5 mM iodoacetamide, 1 mM PMSF, and 1 mM pepstatin A) for 2 × 12 h on a rotator at 4 °C [22]. After centrifugation the supernatants were combined and dialyzed against deionized water three times overnight at 4 °C.

Dialyzed extracts were lyophilized and dissolved in 2 ml of 4 M guanidine HCl, 0.05 M sodium acetate, pH 6.0. Aliquots of samples (protein content ranged from 7.51 to 12.28 mg/2 ml) were applied to Sepharose CL-2B column (1.3 × 110 cm) preequilibrated with 4 M guanidine buffer [23]. Two milliliter fractions were collected. The main peak was divided into four pools (I–IV). The fractions from each pool were combined, dialyzed against deionized water, and lyophilized. For further purification by Q-Sepharose combined fractions from pool III were used. Lyophilized material was resuspended in smaller volume of deionized water. After protein determination, protein aliquots were concentrated using centrifugal Microcon (Millipore YM-10, cut off 10 K, Millipore, Billerica, MA). Concentrated samples were gently mixed with 2 ml of Q-Sepharose anion exchange gel equilibrated in 7 M urea, 0.05 M sodium acetate buffer, pH 6.0, for 2 h at 4 °C. The suspension was loaded on a column (1.3 × 15 cm) packed with Q-Sepharose anion exchange gel equilibrated in 7 M urea buffer, and washed with 7 M urea buffer (10 ml). Bound proteoglycans were sequentially eluted with 7 M urea buffer in steps of 0.1, 0.3, 0.5, and 0.7 M NaCl [23].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)/western blotting

Aliquots of CL-B or Q-Sepharose fractions were precipitated by ethanol at −80 °C for several hours. After centrifugation (3400 rpm for 10 min) the pellet was dried at 37 °C and (for certain experiments) samples were incubated with chondroitinase ABC (Chase ABC), 0.5 U/ml (Sigma–Aldrich Co., St. Louis, MO) in Tris buffer (100 mM, pH 8.0) at 37 °C overnight [24].

For 1D gel electrophoresis, aliquots (25 μg) of ethanol-precipitated, Chase ABC-treated or -untreated samples were separated on 10% SDS–polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250 or alcian blue/silver nitrate [25].

For 2D gel electrophoresis, aliquots (50 μg) of ESPA and control CL-2B pool III not treated with Chase ABC were resolubilized in 7 M urea, 2 M thiourea, 2% CHAPS, 2% 3-[*N*,*N*-dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate (ASB-14), 0.5% 3–10 immobilized pH gradient (IPG) buffer, and 18.2 mM dithiothreitol (DTT). Proteins were separated on IPG strips (pH 3–10 for 22,000 Vh in the BioRad isoelectric focusing cell using active rehydration at 50 V. After isoelectric focusing, IPG strips were equilibrated in 6 M urea, 2% SDS, 65 mM DTT, 30% glycerol, 50 mM Tris, pH 8.8, and 0.002% bromophenol blue for 15 min at room temperature (RT), and then equilibrated with the above buffer, replacing DTT with 135 mM iodoacetamide for 15 min at RT. Proteins were then separated on Criterion 10% SDS polyacrylamide gels (BioRad

Table 1
The ratio of ΔDi-4S and ΔDi-6S in SDFT GAGs. Although both 4S and 6S are increased in ESPA samples (E1–E5), 6-sulfation at GalNAc is significantly increased, especially in E3 and E4 samples as is disaccharide/protein ratio.

Horse	Age (sex)	Breed	Protein content in HPLC samples (μg)	ΔDi-4S (ng)	ΔDi-6S (ng)	4S/6S ratio	4S + 6S/protein (ng/μg) ratio
C1	5 months, ♂	Percheron	3.26	164.78	65.99	2.50	70.8
C2	8 years, ♀	Quarter horse	3.02	116.50	34.79	3.35	50.1
C3	8 years, ♂	Tennesse walking	HPLC ND				
E1	18 months, ♂	Peruvian Paso	3.19	386.00	241.18	1.60	196.6
E2	3 years, ♂	Peruvian Paso	3.70	320.30	192.49	1.66	138.6
E3	3 years, ♀	Peruvian Paso	5.00	612.33	1375.62	0.45	397.6
E4	5 years, ♀	Appaloosa	4.12	467.91	923.73	0.51	337.8
E5	21 years, ♂	Peruvian Paso	3.60	254.39	215.12	1.18	130.4

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