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Perspective Article

Hylonome

Tendon proteoglycans: biochemistry and function

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Abstract

Tendon remodeling occurs in response to changes in loading and mobilization. Though the normal mechanical function depends on precise alignment of collagen fibrils, it is proteoglycans that regulate collagen fibrillogenesis and thus, indirectly, tendon function. In this paper we discuss the basic biochemical structure of several members of two proteoglycans families. Decorin, biglycan, fibromodulin and lumican, all members of the small leucine-rich proteoglycans family, bind to collagen fibrils and are active participants in fibrillogenesis. Aggrecan and versican, two members of large modular proteoglycans or lecticans, and their partner hyaluronan likely provide tendon tissues with a high capacity to resist high compressive and tensile forces associated with loading and mobilization. We present data from our laboratory showing that proteoglycans and glycosaminoglycan content increases not only with growth but also with loading of young avian gastrocnemius tendons. Specifically, an increase in the content of keratan sulfate, chondroitin sulfate and hyaluronan was observed. Moderate exercise for several weeks led not only to a further increase in total proteoglycans content but also to qualitative changes in proteoglycan make up.

Keywords: Tendons, Proteoglycans, Glycosaminoglycans, Effect of Age and Loading

Introduction

Tendon function, i.e., the transmission of tensile forces from muscle to bone, depends on the proper structure of the tendon[1]. In response to changes in loading and mobilization (i.e., exercise), as well as in the normal course of development, a tendon will be remodeled[2-4]. Its biochemistry and structure is then adjusted to facilitate its function under altered conditions[5,6]. Remodeling is not to be confused with repair, the replacement of damaged tissue with newly formed connective tissue. Current knowledge of remodeling mechanisms is incomplete and inconsistent but we know that components of the extracellular matrix (ECM) play crucial roles in this process[5]. The majority of macromolecules present in the extracellular matrix of tendons can be classified into three groups: (1) collagen, (2) proteoglycans, (3) glycoproteins. Recent studies have shown that the assembly (i.e., fibrillogenesis) of the chief structural component of the tendon, type I collagen, is regulated by proteoglycans (PGs) present in the ECM[7-12].

Structural integrity and normal mechanical function of the tendon depends on precise alignment of type I collagen fibrils. Those fibrils, identifiable by electron microscopy, are organized into fibers, bundles and fascicles at the light microscopic level. During chicken embryonal development, collagen fibrils are deposited first as discrete segments 10-30 μm long in extracytoplasmic spaces between tendon fibroblasts. The segments are assembled into fibers, and fibers are incorporated into the developing ECM[13]. PGs, most notably decorin, in the ECM modulate the formation and final sizes of the fibrils[3,10].

With exercise the turnover of mature collagen and collagen crosslinks increases[14,15], large diameter fibrils are formed with increased packing density of fibrils[16] and increased tendon stiffness[5]. Exercise also leads to changes in PG content[1]. Strenuous exercise in mature rodents or chickens leads to thickening of collagen fibrils and to an increase of the galactosamine-containing GAGs[17]. In contrast, immature tendons appear to respond to exercise with higher collagen turnover, reduced maturation of collagen[14], and alterations in hyaluronan concentrations[18].

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Designation	Class	Core protein size (kDa)	GAG chains(n)	Properties and role in tendon
Decorin	SLRP	36	CS/DS (1), or CS/DS (1) + KS (1)	Binds to fibrillar collagen, inhibits collagen fibrillogenesis, binds TGFβ and EGF
Biglycan	SLRP	38	CS/DS (1-2)	Binds to fibrillar collagen, absent in avian species
Fibromodulin	SLRP	42	KS (4)	Binds to type I collagen, facilitates formation of mature large collagen fibrils, modulation of tendon strength
Lumican	SLRP	38	KS (2-3)	Binds to type I collagen, inhibits size of collagen fibrils, modulation of tendon strength
Aggrecan	Modular (lectican)	220	CS (~100)KS (~60)	Linked to hyaluronan, provides resiliency, low levels in tensional parts of tendon, high levels in compressed regions, particularly in fibrocartilage
Versican	Modular (lectican)	265-370	CS/DS (10-30)	Linked to hyaluronan, low levels in tensional parts of tendon, somewhat higher levels in compressed regions, increases viscoelasticity, maintains cell shape

Table 1. Properties of most abundant tendon proteoglycans. Modified from Iozzo and Murdoch[23].

PGs play a crucial role in collagen fibrillogenesis, and therefore in tendon function. Complex carbohydrate structures, particularly the glycosaminoglycans (GAGs) endow proteoglycans with unique properties. Mechanical and pressure tensions have differential impact on PG expression in tendons and other load-bearing tissues[19-22]. For example, mechanical tension (or tensional load) induces the synthesis of decorin whereas the production of the large PG aggrecan is stimulated in tendon by compression[20].

Proteoglycans

PGs are composed of a core protein to which one or more GAG chains are co-valently attached. Two groups of PGs have been demonstrated in the tendon. The small PGs are members of small leucine-rich proteoglycans or SLRPs (Figure 1, Table 1)[23]. Their most characteristic feature is the presence of leucine-rich repeats (LRRs), which are 20-30 amino acid long with leucine in conserved positions[24]. These PGs have a small core protein (~ 40 kDa) to which one to two chondroitin or dermatan sulfate (CS or DS) or several keratin sulfate (KS) chains are attached. They can be divided into four classes[25]. Class I PGs, including decorin, contain 10 LRRs. The members of the class II also have 10 LRRs, and, in addition, carry KS chains attached to the LRR. In contrast, the PGs in the class III are smaller, have only 6 LRRs and contain sulfated tyrosine residues. Class IV contains 10 LRRs, but lacks both amino- and COOH- terminal extensions outside the cysteine motifs. Though so far 11 SLRP members have been identified we will discuss only decorin, biglycan, fibromodulin and lumican, all PGs known to bind to collagen fibrils, and thus to play a role in collagen fibrillogenesis in a variety of tissue and organs, including tendons[24]. SLRPs interact with other ECM proteins, e.g., collagens and fibronectin, but also growth factors, e.g., transforming growth factor β (TGFβ)[26] and epidermal growth factors (EGF)27.

The large (M_r ~ 106 Da) PGs are sometimes called modular PGs or lecticans and are, in turn, rich in CS and KS chains. The core proteins of these PGs have multiple domains (one of them C-type lectin-like) where the N-terminal globular domain interacts with hyaluronan, and the C-terminal domain has selectin-like amino acid sequence (Figure 2, Table 2)[23,27,29]. They are negatively charged hydrophilic molecules that can retain water 50 times their weight. They are mostly entrapped within and between collagen fibrils and fibers. By virtue of their high fixed charge density and charge-to-charge repulsion forces, these PGs are stiffly extended to provide the collagen fibrils with a high capacity to resist high compressive and tensile forces. The mechanism is accentuated by the fact that these molecules are compressed by about 20% of their natural solution domain during stress. These PGs also enable rapid diffusion of water-soluble molecules and migration of cells. In addition, the presence of the numerous negatively charged groups attracts many positive counterions in the aqueous milieu, and thus creates Donnan's osmotic pressure[30].

Within a tendon, the tension region proteoglycans are

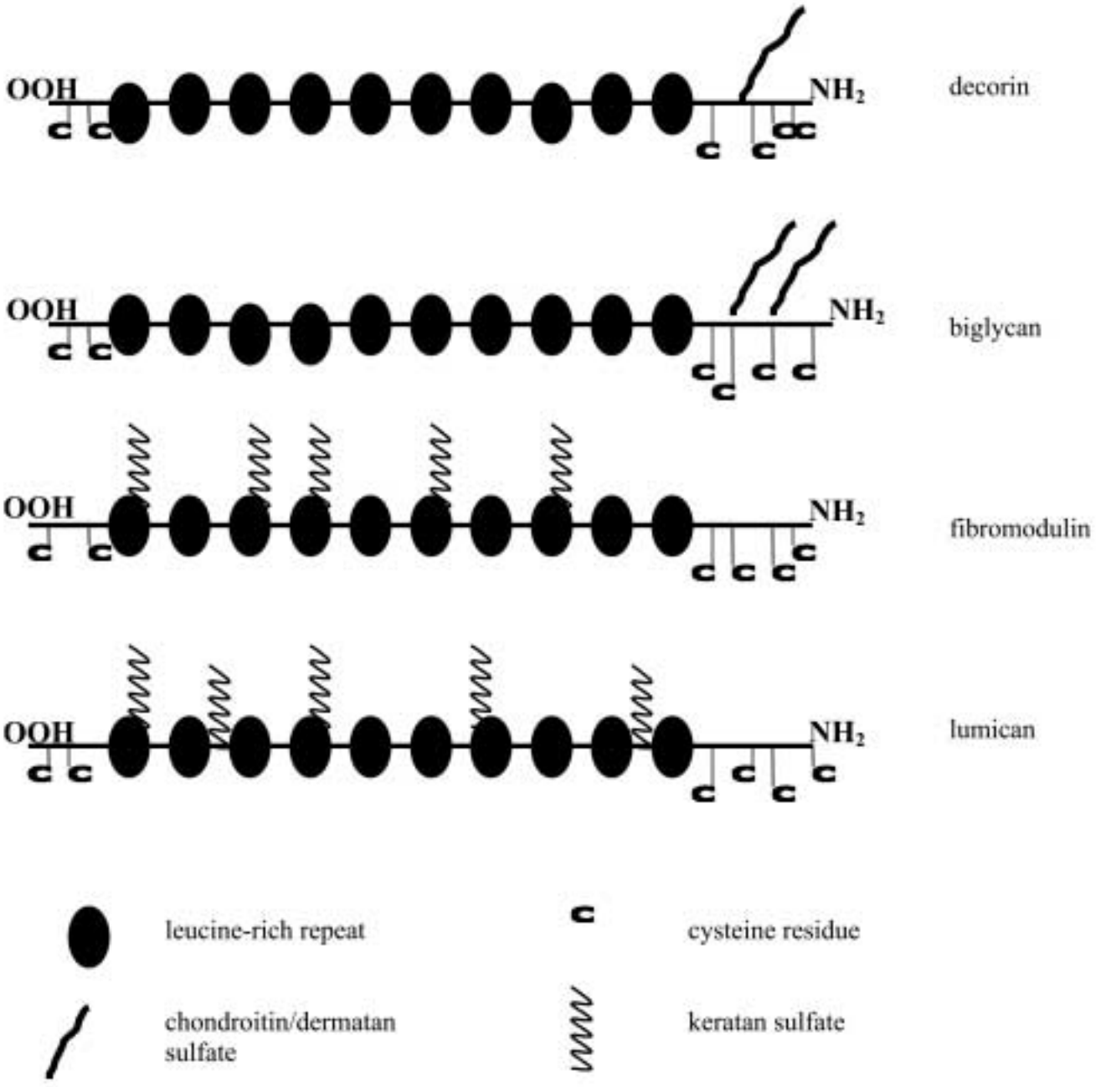

Figure 1. Schematic structures of members of the small-leucine rich proteoglycans present in tendons. Modified from Iozzo and Murdoch[23] and Hardingham[54].

90% small PGs and 10% large PGs. The PG content of the compressed zone is much higher and changed, with 50% small and 50% large PGs by weight[31,32].

PGs function as tissue organizers capable of modulating cell growth and maturation of specialized tissues. They also act as biological filters, modulate growth factor activities, regulate collagen fibrillogenesis and skin tensile strength, affect tumor cell growth and invasion, influence corneal transparency, influence neurite outgrowth[33].

Physical properties of PGs

PGs have (1) low isoelectric points, (2) large hydrodynamic domains resulting from the relatively extended GAG chains and, often, from the multiplicity of chains, and (3) high buoyant densities if the ratio of GAG mass to protein mass is relatively high. The chemical composition of GAGs varies in different types of tissues. The individual chains in a GAG preparation have different molecular weight and com-

Fraction number	ΔDi-HA (μg)	ΔDi-0S (μg)	ΔDi-6S (μg)	ΔDi-4S (μg)
D2	0.23	0.12	trace	trace
D7	0.24	0.10	trace	trace
D10	0.22	0.09	trace	trace
W2	1.2	1.6	trace	2.1
W6	1.3	3.5	trace	3.3
W10	1.4	2.5	trace	1.6

Table 2. HPLC quantification of GAG disaccharides from enzyme treated Sepharose fractions of tendons from 1-day (D2, D7 and D10) and 1-week-old chickens (W2, W6 and W10). Samples digested with Chase ABC were separated by HPLC and quantified based on disaccharide standards[1].

positions. GAG chains from the same tissue, which are linked to different core proteins, may be different[34].

Glycosaminoglycans (GAGs)

GAGs, complex carbohydrate structures, endow proteoglycans with unique properties. GAGs consist of linear polymers with repeating disaccharides of defined structures that contain one hexosamine and one hexuronic acid that may be sulfated (KS is a special case to be dealt with later). GAGs are *O*-linked to serine through a linkage sequence at its reducing end: [GAGs]-GlcUA-β1,3-Gal-β1,4-Gal-β1,4-Xyl-β1,0-serine[35].

Acidic polysaccharides (= GAGs) present in the ECM play a central role in the modulation of cell signals. Modification of functional groups along GAG polymers provides a framework for modulating interactions with cytokines that regulate growth and differentiation. GAGs can bind signal transducers such as fibroblast growth factor and vascular endothelial growth factor and potentially serve as a reservoir, scavenger, or co-factor for cell signaling[36].

At the tissue level, GAGs and PGs participate in regulation of angiogenesis, glomerular permeability, neuron development, joint function and Alzheimer's disease. GAGs also have numerous pharmaceutical applications as anti-coagulant, anti-thrombotic, and anti-lipemic agents, as well as in osteoarthritis[37]. GAGs are usually classified into four classes: (1) hyaluronan, (2) chondroitin sulfate and dermatan sulfate, (3) keratan sulfate, and (4) heparan sulfate (HS) and heparin.

The concentration of GAGs is considerably less in tendons than in cartilage or in other types of connective tissue. The tensional zone of a tendon includes approximately 0.2% GAG (dry mass) of which 60% is DS, while the pressure zone and especially the bone insertion area include 3.5% to 5.0% GAG of which 65% is CS[38]. Hyaluronan constitutes about 6% of the total GAG. HS can be found mostly at the myotendinous junction[39]. In contrast, human nasal septal cartilage contains 2.9% GAG (wet mass)[40] and cultured bovine articular cartilage up to 11% GAG (dry mass)[41].

Chondroitin and dermatan sulfate

Those two rather ubiquitous GAGs arise from the same homogeneous precursor, chondroitin, with the repeated disaccharide unit: -Gal_{NAC} –(β1,4)-GlcUA (β1,3)-. DS (2-8 attached GAG chains, 15-55kDa), but not CS, contains iduronic acid (result of epimerization of GlcUA). CS (20-100 attached GAG chains, 15-70 kDa) has glucuronic acid linked to *N*-acetyl galactosamine. The terms CS A and C refer to GAGs bearing 4- or 6-sulfation, respectively. As with chain elongation and sulfation, there is no template for epimerization, and so the proportion and position of epimerization may vary between DS chains for cartilage from different locations or types of GAGs[42].

Keratan sulfate

Glc_{NAC} –(β1,3) - Gal (β1,4) constitutes the basic repeating unit of KS. It is the only GAG without uronic acid residues. The hexosamine residue is commonly sulfated at its 6-position, but sulfation may also occur at the 6-position of the galactose residues. Two types of KS differ in the linkage region. The molecular weight is typically around 2 to 20 kDa. Porcine type I KS ranges from 10 to 26 kDa, and type II KS is around 5 kDa. Specific enzymes required for polymerization and sulfation of KS may be the key regulators of KS biosynthesis *in vitro* and possibly *in vivo* as well[43].

Type I KS (corneal):

-[Gal-GlcNAc]$_n$-Man Fuc
                ¦
}-Man-GlcNAc-GlcNAc-*N*-asn-protein
NeuNAc-Gal-GlcNAc-Man

Type II KS (skeletal):

Gal-GlcNAc
        }- GalNAc-*O*-ser/thr-protein
NeuNAc-Gal

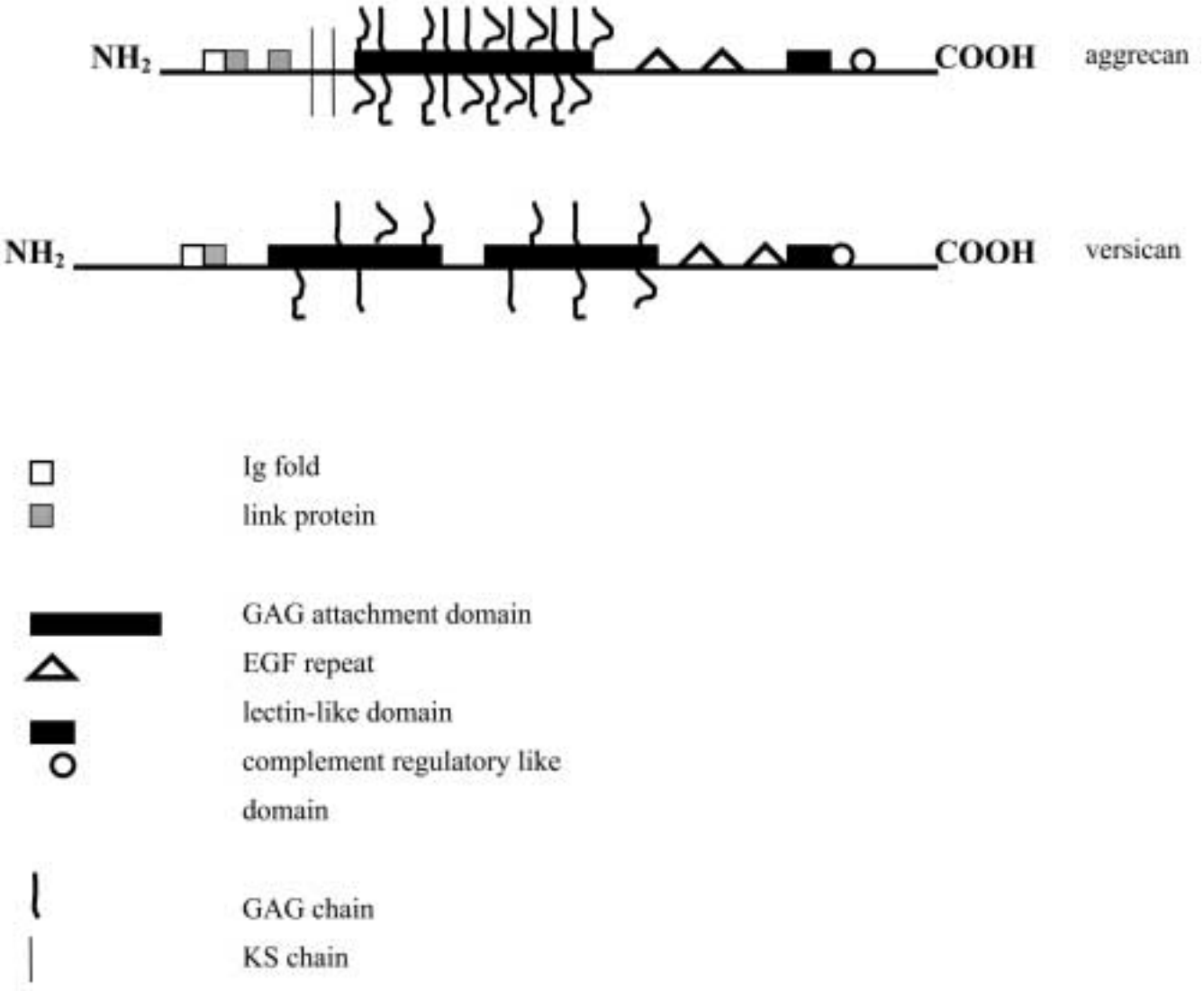

Figure 2. Schematic representation of large modular proteoglycans present in tendons. Modified from Iozzo and Murdoch[23] and Hardingham[54].

After cornea and skeletal tissues, brain appears to exhibit the most KS and is one of the tissues richest in enzymes of KS biosynthesis[43].

HS and heparin

Heparan sulfate proteoglycans (HSPGs) are present on the cell surface of all human (and animal) cells. On fibroblast and hepatocyte cell surfaces core proteins have 4 to 6 chains of 14 to 20 kDa each, while in basement membranes as many as 12 HS chains varying from 25 to 70 are attached to each protein anchor. HS shares the same linkage tetrasaccharide as CS, and the consensus sequence for attachment to serine residues appears to be analogous. The last sulfated GAG, heparin, may be considered a modified form of HS[44].

Decorin

Decorin is the most abundant tendon PG. As a member of class I of the SLRP family decorin contains a leucine rich core protein of M_r ~ 36-40 kD with 10 LRRs and carries one or two CS or DS chains at least in mammals (Figure 1, Table 1)[23,25]. Fibroblasts, chondrocytes, endothelial cells and smooth muscle cells are decorin-producing cells. Decorin binds to almost all types of collagen either through its core protein or GAG chains. The binding site of decorin for collagen is located in the cysteine-free central domain of the core protein[12]. It is ubiquitously distributed in animal and human tissues, and is one of the major components of fibrous tissues or other extracellular matrix. The main functions of decorin[10] could be summarized as follows:

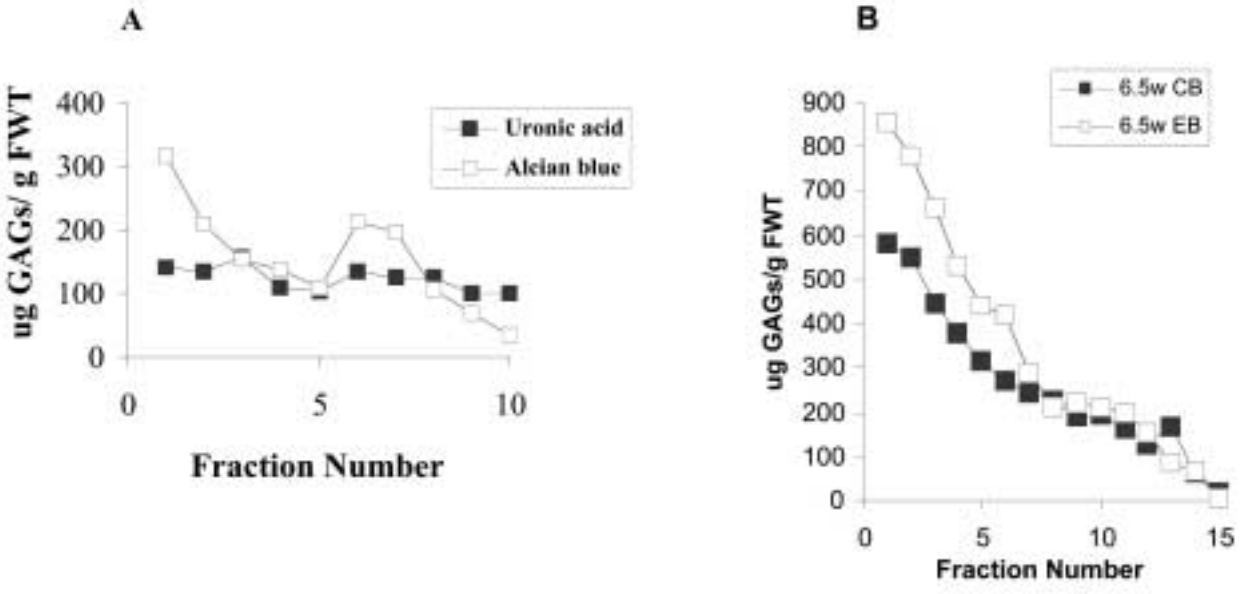

Figure 3. GAG quantification in CsCl fractions. A. The GAG content in CsCl-fractionated gastrocnemius tendons from 1-day-old chickens was determined using both the alcian blue dye binding assay and the uronic acid assay. The average total GAG content per g fresh weight of tendon is shown. Each data point was obtained from the extraction of two tendons. Similar results were obtained in three independent experiments. B. The GAG content of the CsCl fractions from the bottom halves of gastrocnemius tendons from 6.5-week-old control (CB) and exercised (CE) chickens was determined using the alcian blue dye binding assay. The data are averages from 3 replicates from a single experiment. Similar results were obtained in two independent experiments. Increasing number of fraction indicates decreasing density.

1. Maintenance and regulation of collagen fibril structure;
2. Regulation of cell proliferation mainly through inhibition of cell proliferation and spreading;
3. Stimulation of immune responses.

Decorin is considered a key regulator of matrix assembly because it limits collagen fibril formation and thus directs tendon remodeling due to tensile forces[3,7]. Evidence that decorin plays a role in collagen fibrillogenesis in animals was provided by the observation that decorin-deficient mice have fragile skin that is not able to withstand sudden tensile strain[7]. Electron microscopic examination of the skin in decorin knockout mice shows that, in the absence of decorin, collagen fibrils are coarse, irregular and haphazardly arranged. These changes are accompanied by a decrease in collagen-bound PGs in the skin and tendon. Because neither biglycan nor lumican (two other small leucine-rich PGs) were upregulated at the mRNA level, it was proposed that these PGs did not compensate for the lack of decorin[7]. Decorin is also involved in regulation of cell proliferation through inhibition of TGFβ[26,45] and through direct binding to the EGF receptor[27].

Because chickens and other avian species lack biglycan (see below), a differentially glycosylated form of decorin plays the role of biglycan in avian tissues[46]. The common form of decorin has a single CS/DS chain while the second form, presumably one equivalent to biglycan, has either two DS chains[47] or a CS/DS and a KS side chain attached[1,48].

Biglycan

Biglycan is another member of the class I in the SLRP family (Figure 1, Table 1)[23,25]. Like decorin it contains 10 LRRs and has two DS chains attached to its core protein (M_r ~ 38 – 42 kDa). It is expressed mainly in tendon, cartilage and bone as well as in dermis and blood vessels[11,49,50]. Biglycan also interacts with type I collagen though the nature of this interaction is not well understood. The affinity of biglycan binding to collagen is comparable to decorin and the interaction appears to be independent of the presence of *N*-linked oligosaccharides of the biglycan core protein[11]. Similarly to decorin biglycan also binds to TGFβ, and thus has the potential to participate in modulation of cell proliferation[26,45]. Collagen fibrils in the quadriceps tendons of biglycan-deficient mice had smaller diameter and abnormal morphology. Such knockout mice also developed ectopic tendon and joint ossification accompanied by osteoarthritis after 3 months of age. These changes were more pronounced in biglycan/fibromodulin double null mice. The absence of biglycan and/or fibromodulin (see also below) prevented formation of mature collagen fibrils and thus resulted in weaker tendons with decrease in stiffness. It also appears that fibrocartilage in the double null mice underwent ossification, and thus helped compensate for the decreased stiffness[49].

Fibromodulin

Fibromodulin, another member of the SLRP family, this time of class II, has core protein of M_r ~42 containing 10 internal repeats of 25 amino acid residues rich in leucine[25]. These repeats are located in the center of the protein and constitute some 80% of all amino acid residues (Figure 1, Table 1)[23,25,26]. It has been suggested that fibromodulin functions as a modulator of collagen fibrilllogenesis. Similar to decorin, fibromodulin binds to type I and II collagen but at a different site on each collagen molecule. Unlike decorin it promotes formation of mature large collagen fibrils *in vitro* and *in vivo*[51]. The primary structures of decorin, and fibromodulin deduced from cloned cDNAs of human and bovine sources show extensive homologies to each other and to biglycan and lumican[52]. The N-terminal amino acid sequences bear less similarity to each other. This region contains one CS/DS side chain binding site in decorin and two side chain binding sites in biglycan. Fibromodulin does not have CS/DS side chains but instead it contains four KS chains attached to the core protein via an *N*-glycosidic linkage to asparagines. The different mobilities of fibromodulin from various sources may be taken to indicate that either variable portions of the five potential sites are substituted or the sizes of the side chains vary. Fibromodulin isolated from cartilage and bone is modified with *N*-linked KS chains[53].

Fibromodulin is expressed at high levels in the tendon. It binds to the same region on type I collagen as lumican, another member of the same family. However, this site is distinct from the decorin-binding site[51]. Knockout mice for fibromodulin experienced reduced tendon stiffness (increased laxity), and a compensatory increase in lumican. Tendons from these mice show marked increase in small diameter immature collagen fibrils without progression to mature large diameter fibrils[51]. Fibromodulin deficiency led to impaired tendon function[51]. In other words, fibromodulin contributes greatly to tendon strength[9].

Lumican

The last member of SLRPs to be discussed here is lumican. This PG belongs to class II and is closely related to fibromodulin, with which it shares the collagen binding site[51]. It also contains 10 LRRs (Figure 1, Table 1)[23-25]. It is slightly smaller than fibromodulin with M_r ~ 38 kDa and it has fewer KS chains attached[23]. Lumican was found in skin, cornea, tendon, sclera and cartilage. Studies on knockout mice indicate that lumican is able to substitute functionally for fibromodulin in fibromodulin-deficient mice. In contrast, lumican-deficient mice produced large diameter collagen fibrils forming disorganized matrix in cornea and skin. It is interesting to note that this did not lead to loss in tendon biomechanical function or a compensatory increase in fibromodulin levels[51]. It appears that fibromodulin is more vital to tendon biomechanics than lumican.

Aggrecan

Aggrecan is highly glycosylated with numerous CS and KS chains attached to the large core protein (M_r ~220 kDa). Each aggrecan molecule contains ~100 CS chains, which are typically ~20 kDA each, and the chains are either 4-sulfated, 6-sulfated, or usually both. There are fewer KS chains (up to 60), and they are usually of shorter length (5-15 kDa). The CS chains are all attached to the long, extended domain between globular domains 2 and 3, but the KS chains are more widely distributed. In addition, aggrecan contains a variable number of *O*- and *N*-linked oligosaccharides (Figure 1, Table 1)[23]. The *O*-linked oligosaccharides have a linkage to protein similar to that for KS, and it appears that during biosynthesis some *O*-linked oligosaccharides are extended and sulfated to form KS chains, whereas others are not[54].

The primary site of aggrecan synthesis is in the cartilage, a highly specialized tissue that in higher vertebrates forms the template of long bones during development and is retained in the adult in selected sites, particularly on the weight-bearing surfaces of articular joints. Aggrecan binds to hyaluronan via a link protein or a proteoglycan tandem repeat attached to aggrecan G1 domain[55]. The result is stabilized ECM containing many negatively charged GAG chains. The negative charge leads to increased osmotic pressure, and thus to enhanced tissue hydration. This event in turn exerts a swelling pressure on the collagen network. It is the retention of aggrecan in compressed form within the inextensible collagen network that causes the swelling pressure and makes the tissue ideal for resisting compressive load with minimal deformation, thereby supporting its function as a tough and resilient load-bearing surface. The KS content of aggrecan in cartilage undergoes an age-related increase in KS chain length and sulfation[55].

This provides tissues with a considerable degree of resistance to compressive loading. This resilience is important not only in cartilage but also in regions of weight-bearing tendons experiencing compression[55]. Sulfated GAGs provide compressive stiffness, with CS being more abundant in tensional part, and KS more common in tissue subjected to compression. For example, upper tensional part of a tendon such as gastrocnemius has been reported to contain CS, but no KS[56]. Lower portion of the same tendon, which is exposed to compression and tension, contains a mixture of CS and KS. Cartilage contains the highest level of KS. It is thought that aggrecan expressed in areas of tendons subjected to less compression and more to tensional forces contains both CS and KS chains, and more KS chains in areas of tendons experiencing compression[56].

Parts of tendons subjected to compression reveal the presence of fibrocartilage, rich in aggrecan and type II collagen, the main components of cartilage. In gastrocnemius tendon, fibrocartilage is found adjacent to the bone insertion site[57]. Aggrecan interactions with collagen reduce collagen network stiffness and apparent viscosity. Aggrecan also contributes

significantly to the viscoelasticity and acts as a lubricant, allowing the fibrils to slide more easily over each other. Some of these properties are also exhibited by hyaluronan, a large polymer of disaccharides of glucuronic acid and N-acetylglucosamine. In cartilage, hyaluronan and aggrecan are responsible for the formation of prominent pericellular matrix and it is likely that the same happens in fibrocartilage[57].

Versican

Versican is another member of the hyaluronan-binding PGs (Figure 1, Table 1)[23,58]. It is expressed in many tissues, especially in fast growing cells of soft tissues. Versican is found in the dermis of skin and in the media of the aorta. It appears transiently during mesenchymal condensation in developing chicken limb buds[59]. An increase in versican content leads to expansion of ECM and to increased viscoelasticity of pericellular matrix that supports cell-shape changes necessary for cell proliferation and migration. It also has an effect on tension exerted on the cell itself and the traction forces generated by the cell[60].

Hyaluronan

Hyaluronan differs in several aspects from other GAGs. First of all, it is not bound co-valently to a core protein (i.e., is not part of a PG) and it is not sulfated. It is considerably larger than the other GAGs with chain lengths from 500 to several thousand disaccharides (M_r 100 to 1000 kDa). Because of direct synthesis at the cell membrane, the elongating hyaluronan molecule is secreted directly into the extracellular matrix by the consecutive union of glucuronic acid and N-acetyl glucosamine: Glc_{NAC} –(β1,4)- GlcUA-(β1,3).

The globular N-terminal G1 domain of aggrecan contains a lectin-like binding site with high affinity for hyaluronan and is responsible for the formation of aggregates. Hyaluronan can bind a large number of aggrecans to form aggregates up to several hundred million in molecular weight. The binding of each aggrecan to hyaluronan is further stabilized by a small glycoprotein (45 kDa) that serves as link protein. It is interesting to note that in young, developing cartilage, the amount of hyaluronan is about 1% of the aggrecan. With age the content rises to almost 10%[61].

GAG and PG content changes with age

Carrino et al. described a decrease in large PGs and an increase in modified small PGs in aging human skin[62,63]. Particularly, they observed the presence of a catabolic form of decorin in adult but not fetal tissues. This form, termed "decorunt", had a markedly reduced capacity to bind type I collagen, and thus could be responsible for changes in skin stability (wrinkle formation?) in older subjects[63]. Whether such modifications are limited to aging skin or are distributed in other tissues, including tendons, awaits determination.

Here we report changes in PGs in young growing tissues, specifically, in avian gastrocnemius tendons. Gastrocnemius tendons were removed from 1 day, 1, 2.5, 5 and 6.5-week-old chickens. In addition, starting at 2 weeks of age half of these chickens underwent a 4.5-week long exercise training. This exercise consisted of treadmill pacing at 1 mph for three 10 minute intervals for 5 days a week as described[1]. The tendons were first homogenized, then extracted with 4M guanidine HCl buffer with protease inhibitors at 4°C for 24 hours[1]. The density of the extract was adjusted (final density 1.4 g/ml) by CsCl. A density gradient was formed by ultracentrifugation (108,000 x g, 10°C, 72 hr). PGs were quantified in collected fractions using a modified dye binding method[64] in which alcian blue binds in proportion to the number of negative charges on the GAG chains attached to the core protein[65]. We also measured the content of uronic acid present in GAGs[66].

While the amount of GAGs in 1-week-old tendons (3.9 mg/g fresh weight) was only slightly greater than the amount in 1-day-old tendons (2.2 mg/g), there was a significant increase in 2.5-week-old tendons (8.5 mg/g), and the amount remained stable in older tendons (8.2 mg/g at 6.5 wk)[1]. In general, the dense (i.e., bottom) CsCl fractions from all tendons contained the most GAGs as measured by the uronic acid and alcian blue assays (Figure 3). In addition, the lower half of the tendon always had a higher (by approximately 20%) GAG content than the tendon upper half. The alcian blue assay but not the uronic acid assay identified a distinct GAG peak in the intermediate fractions from 1-day and 1-week-old tendons (Figure 3A). This alcian blue peak was less prominent at 2.5 weeks (data not shown). We hypothesized that the GAGs in this distinct peak belonged to the negatively charged (and therefore binding to alcian blue) KS class. This assumption was subsequently confirmed by using a sensitive dotblotting assay for KS using the mouse anti-KS antibody 5-D-4[64]. This method involves the fixation of polyanionic GAGs to nitrocellulose membrane by precipitation of KS with neodymium, a trivalent metal. The detection of KS by dotblotting was performed on untreated and Chase AC treated aliquots of dense and intermediate CsCl fractions from 1-day-old tendons. Our data showed that the content of immunoblotted KS remained constant at 1 day and 1 week of age[64]. The KS content was greater in the dense fractions recovered following CsCl fractionation of 1-day and 1-week-old chicken gastrocnemius tendons, and was proportionately less in intermediate and top fractions. The content of KS in samples from 1-day-old tendons was as follows. CsCl fraction 1: 144 ng, fraction 5: 78 ng, fraction 7: 87 ng and fraction 9: 75 ng. The content of KS in samples from 1-week-old tendons: CsCl fraction 1: 156 ng, fraction 2: 78 ng, fraction 6: 90 ng and fraction 10: 48 ng. These results correlate well with the amount of total GAGs present in CsCl fractions of tendons as determined with the alcian blue assay (Figure 3A).

Polyacrylamide gel electrophoresis (PAGE) of tendon GAGs

Further analysis was aimed at determining which other GAGs were present in young tendons. Trypsin and proteinase K treated aliquots of the dense CsCl fractions from 1-day and 1-week-old tendons were analyzed untreated or further digested with Chase ABC or Chase AC, separated on 28% polyacrylamide gels and stained with alcian blue-silver[1]. The positions of alcian blue-silver stained bands of GAGs were compared with the positions of alcian blue-silver stained GAG standards (Figure 4, lanes 1-2; see also Figure 5, lanes 1-7). In general, samples from 1-day and 1-week-old tendons were found to have a low GAG content. Digestion with Chase ABC or Chase AC led to the disappearance of most of the high molecular weight alcian blue-silver stained bands that likely corresponded to CSs or hyaluronan-like material (at the top of the gel, Figure 4, lanes 3 and 6) in extracts from both 1-day and 1-week-old tendons (Figure 4, lanes 3-5 for 1-day-old tendons, lanes 6-8 for 1-week-old tendons).

We also used PAGE to evaluate qualitative differences in GAG content in somewhat older tendons from control and exercised 6.5-week-old chickens. Analysis of fractions after extraction and gradient ultracentrifugation indicated much higher GAG content in exercised tendons (Figure 3B). The four bottom dense CsCl fractions from the control and the exercised 6.5-week-old tendons were pooled and purified over a Sepharose CL-2B column. We divided the PG containing fractions into 5 pools from exercised (E1-E5) material and 5 pools from control (C1-C5) material[1].

PAGE analysis was then performed on E3 and C3 middle pools of the 5 Sepharose peaks of extracted PGs from exercised and control 6.5-week-old tendons (Figure 5). The position of alcian blue-silver stained material from untreated aliquots (Figure 5, lanes 8 and 12) and aliquots digested with Chase ABC (Figure 5, lanes 9 and 13), Chase AC (Figure 5, lanes 10 and 14) or keratanase (Figure 5, lanes 11 and 15) were compared with the position of GAG standards: CS A (Figure 5, lane 1), CS B (Figure 5, lane 2), CS C (Figure 5, lane 3), KS (Figure 5, lane 4), HS (Figure 5, lane 5), HA (Figure 5, lane 6) and oligogalacturonides of increasing degrees of polymerization of 7-23 (Figure 5, lane 7).

In contrast to extracts from very young tendons, the fractions from 6.5-week-old tendons had significantly increased GAG content (Figure 3). As in extracts from very young tendons, digestion with Chase ABC led to the disappearance of a wide high molecular weight band of CS-like material in lanes containing GAGs from control (Figure 5, lane 9) and exercised (Figure 5, lane 13) samples from 6.5-week-old tendons, this time, however, uncovering unidentified GAGs and GAG products. Extensive digestion of control extracts with Chase ABC and Chase AC indicated the presence of CS A+B+C in intact material (Figure 5, lanes 9 and 10). The oligosaccharide ladder present in Figure 5 (lane 14) was likely the result of Chase AC digestion of CS A+C in the sample and suggested the presence of chondroitin oligosaccharide 4-sulfates in extracts from exercised 6.5-week-old tendons. The latter results were confirmed by HPLC analysis in another paper[1]. Hyaluronan migrated as a narrow band at the top of the gel (Figure 5, lane 6). A band seen in the same position was apparent in lanes containing Sepharose pooled material from 6.5-week-old tendons. This band was much stronger in the exercised than the control lanes (Figure 5, lanes and 12-15 and 8-11, respectively) and though it most likely represents hyaluronan, a large PG aggregate cannot be excluded. Treatment with keratanase led to the appearance of additional alcian blue-silver stained bands ranging in size from 1,000-15,000 Da (Figure 5, lanes 11 and 15).

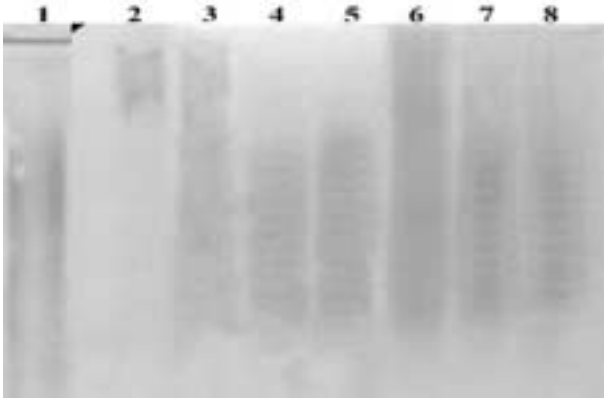

Figure 4. Aliquots of CsCl fractions were treated with trypsin and proteinase K, some were further digested with Chase ABC or Chase AC, and then separated on 28% polyacrylamide gels and stained with alcian blue-silver. Lanes 1-2: standards, HA (1), CS A+B+C (2). Lanes 3-8: undigested fraction 6 from 1-day-old tendon (3), fraction 6 digested with Chase ABC (4) or Chase AC (5), undigested fraction 7 from 1-week-old tendon (6), Fraction 7 digested with Chase ABC (7) or Chase AC (8).

HPLC analysis

For further evaluation of GAG components, aliquots of CsCl fractions from 1-day (fractions 2, 7 and 10) and 1-week-old (fractions 2, 6 and 10) tendons were digested with Chase ABC, and the resulting oligosaccharide mixture was analyzed by HPLC. This analysis revealed a low content of GAG (mostly CS) disaccharides in 1-day and 1-week-old tendons (Table 2). Somewhat higher levels of hyaluronic disaccharide (ΔDi-HA) were detected. Because it is known that Chase ABC can digest hyaluronan[67], it is not surprising that no hyaluronan was detected in Chase ABC digested extracts from 1-day and 1-week-old using PAGE (Figure 4). Though the content of disaccharides was still low in 1-week-old tendons, it was significantly higher than in 1-day-old tendons (Table 2). Small amounts of hyaluronan were present in these tendons as well. The significant increase in GAG content with growth in 6.5-week-old tendons was described by us

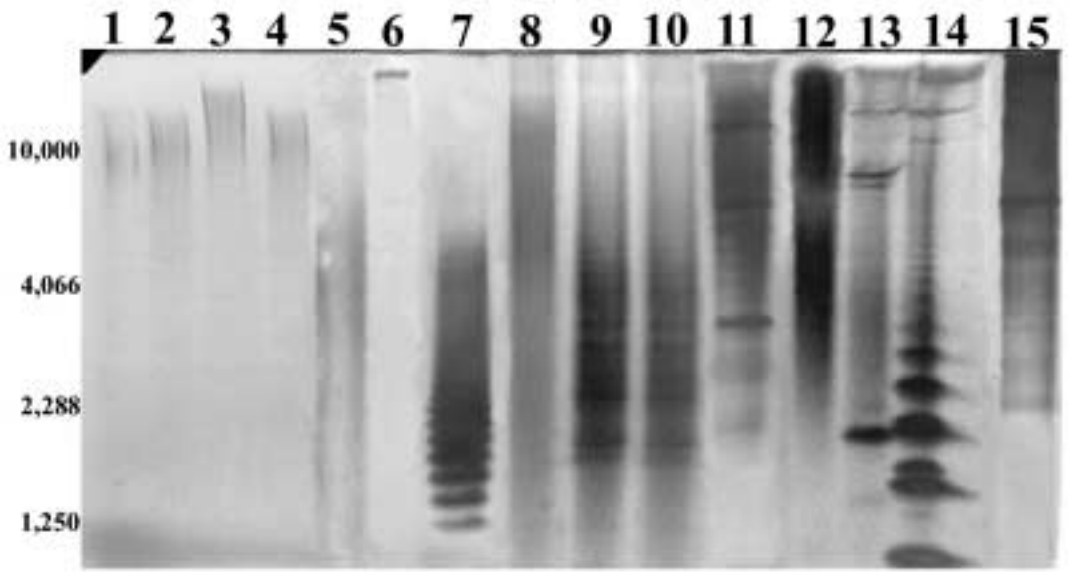

Figure 5. PAGE profile of GAGs present in pools E3 and C3 from 6.5-week-old tendons. Aliquots of the dense CsCl fractions from 6.5-week-old exercised and control tendons were treated with trypsin and proteinase K, and separated on 28% polyacrylamide gels. The gel was stained with alcian blue-silver. Lanes 1-7: standards, CS A (1), CS B (2), CS C (3), KS (4), HS (5), HA (6), oligogalacturonides (DP 7-23) (7). Lanes 8-15 (30 µl aliquots): C3 pool untreated (8), C3 treated with Chase ABC (9), C3 treated with Chase AC (10), C3 treated with keratanase (11), E3 pool untreated (12), E3 treated with Chase ABC (13) and E3 treated with Chase AC (14) and E3 treated with keratanase (15).

previously[1]. This increase was mostly due to marked increases in ΔDi-HA and chondroitin 4-sulfate disaccharide (ΔDi-4S), particularly in exercised tendons. Control tendons had higher content of ΔDi-6S. These findings confirm the results described in PAGE analysis above. We conclude that the growth of avian tendons in the first weeks of life is characterized by an increase in total GAG content, especially in the dense CsCl fractions from the tendon lower parts. CSs and hyaluronan are major GAG components in tendons at this age, followed by KS. The presence of CS indicates the likely occurrence of decorin, which we have shown to be present in relatively high levels in 1-week-old tendons[1]. The increasing concentrations of GAGs and PGs (presumably mostly in the form of decorin) in growing tendons correlate well with a rapid increase in diameter of collagen fibrils at this stage[3,10,13].

Changes in PGs and GAGs with exercise

In normal tendon and other load-bearing tissues, the profile of PGs changes with the type of load the particular tissue is exposed to[19,20,22]. In general, decorin is upregulated with tensional load while the large PG aggrecan is upregulated with compression load[20]. This indicates specific functions for individual PGs in collagen fibrillogenesis and during loading. Some of the changes occurring with loading are described above under individual PGs or GAGs. Whereas fetal bovine tendon contains almost exclusively only small PGs containing DS chains, i.e., most likely decorin, the PG content of adult bovine tendons changes considerably, particularly in the region undergoing compression[31]. Though a small PG (M_r ~48 kDa) with a DS chain predominated in all regions of tendon, a high level of a large PG (M_r ~200 kDa) capable of interacting with hyaluronan (presumably aggrecan) was identified in high levels in the pressure-bearing region of the tendon, and in low levels in the tensile region[32]. In addition, Vogel et al. have found the presence of other PGs, such as decorin, fibromodulin, biglycan and even aggrecan in the tensile region[68]. The aggrecan present in the tensile region lacked the G1 domain, and, consequently, was predominantly bearing CS, rather than KS, chains[69]. Results from our laboratory indicate that exercise leads not only to an increase in the size of the avian gastrocnemius tendons and to an increase in total GAG content but also induces qualitative changes in PG profiles in gastrocnemius tendons of 6.5-week-old chickens[1]. The content of hyaluronan and decorin increased with exercise consisting of treadmill pacing, i.e., of mostly tensional load. High levels of KS were found in the lower halves of gastrocnemius tendons, although, the amount of KS decreased with exercise. This corresponded well with lower content of aggrecan in lower halves of exercised tendons[1]. In contrast, as Vogel and her group demonstrated in many elegant studies, increased expression and synthesis of aggrecan particularly in fibrocartilage is associated primarily with the application of compressional load[20,70].

Such findings suggest that PG composition likely under-

goes changes with growth and development, and that different types of loading have regional and very specific local effects on tendons resulting in changes in the structure and assembly of collagen fibrils, development of fibrocartilage, and thus in adaptation of biomechanical function to new conditions.

Conclusions

The field of proteoglycan biochemistry has been very active, with new members of this multifunctional gene family still being discovered. As a result we now have a better conceptual grasp of the structure of various PGs as well as that of several different types of GAGs, each of which is heterogeneous with respect to molecular size, disaccharide composition and sulfate content. Biological and structural studies have focused on GAGs and/or PGs, and several potent and effective methods have been developed to obtain compositional qualitative and quantitative analysis of GAG chains.

The proteoglycan field has reached maturity and as a consequence it has led to novel, and sometimes unexpected, findings. The biological functions of PGs are quite pleiotropic and their effects on cell function are far reaching. Some proteoglycans regulate cell growth, others promote differentiation and neurite cell growth, and others act as biological barriers and repellents. In the tendon PGs play a major role in structural and biochemical adaptation to changes in loading, and are thus responsible for maintaining proper biomechanical function. The isolation, characterization, and mapping of novel proteoglycan-encoding genes, together with the utilization of mutant animals with targeted disruption of single proteoglycan genes, or a combination of genes, will provide definitive answers about their specific functions.

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