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Histochemistry defines a proteoglycan-rich layer in bovine flexor tendon subjected to bending

K.G. Vogel and J.A. Peters

Department of Biology, The University of New Mexico, Albuquerque, NM, USA

Abstract

Mid-substance fibrocartilage develops in bovine deep flexor tendon at the point where the tendon wraps under sesamoid bones of the foot and receives transverse compressive loading during locomotion. Fibrocartilage extends several millimeters into the tendon at this location and the proteoglycan-rich tissue stains intensely with Alcian blue. Using histochemical techniques we demonstrate the presence of aggrecan, type VI collagen, and hyaluronic acid in the extracellular matrix of this region of tendon. Biglycan staining was localized to the cells, however. Adjacent to the fibrocartilage, at the outer curvature of the tendon as it bends, the tissue resembles typical tensile tendon with dense bundles of linearly arranged collagen. Longitudinal sections revealed discrete layers of Alcian blue-stained material between the collagen bundles. We demonstrate that these layers of loose matrix also contain aggrecan, type VI collagen, and hyaluronic acid. However, the dense collagen bundles of this region are negative for these components. Transverse sections of tendon in the area adjacent to fibrocartilage show a distinct Alcian blue-stained structure surrounding vascular elements at the point where several fiber bundles come together. This is concluded to be the same structure as the Alcian blue-stained layers seen in longitudinal sections. These observations suggest that proteoglycan-rich matrices in tendon subjected to mechanical loading other than pure tension may serve multiple roles. Such matrices can not only provide compressive stiffness and separate and lubricate collagen bundles that move relative to each other, but may also protect the integrity of vasculature in tendon subjected to bending and shear.

Keywords: Flexor Tendon, Fibrocartilage, Proteoglycans, Histochemistry, Blood Vessels

Introduction

The adult bovine deep flexor tendon consists of two regions that are distinguished by histology, proteoglycan content and extracellular matrix gene expression[1]. These distinct properties are correlated with the different mechanical stresses experienced in each region[2]. The proximal/tensile portion of this tendon is subjected to longitudinal tensile stress during locomotion. The tissue in this region has the typical tendon appearance with dense, longitudinal collagen bundles and elongated fibroblasts lying between the collagen fibers. Distal to this tensile region the tendon bifurcates and each portion wraps under sesamoid bones of the joint before insertion into the phalangeal bones. During locomotion this mid-substance region of tendon is subjected to transverse compressive loading and shear stresses as well as tension. The tissue at the contact "surface" of this compressed region is characterized by collagen fibers running in many directions and a greatly increased amount of extrafibrillar aggrecan - the same large proteoglycan as accumulates in cartilage[3]. The cells of this region have a rounded shape and express mRNA for type II as well as type I collagen[4]. This is tendon fibrocartilage[5] and in the bovine deep flexor tendon it makes up about one-third of the tendon thickness at this location.

Adjacent to the fibrocartilage, toward the outer curvature of the tendon as it wraps under bone, the tissue appears much like tensile tendon, showing dense longitudinally-oriented collagen fiber bundles. However, there is often a layer of looser matrix between the fiber bundles[6]. Such layers are never observed in the tensile region. The current study reports immunohistochemical localization of tendon matrix components with particular focus on the deeper regions adjacent to fibrocartilage in tendon that wraps under bone.

The authors have no conflict of interest.

Corresponding author: Kathryn G. Vogel, Ph.D., Department of Biology, The University of New Mexico, Albuquerque, NM 87131, USA
E-mail: kgvogel@unm.edu

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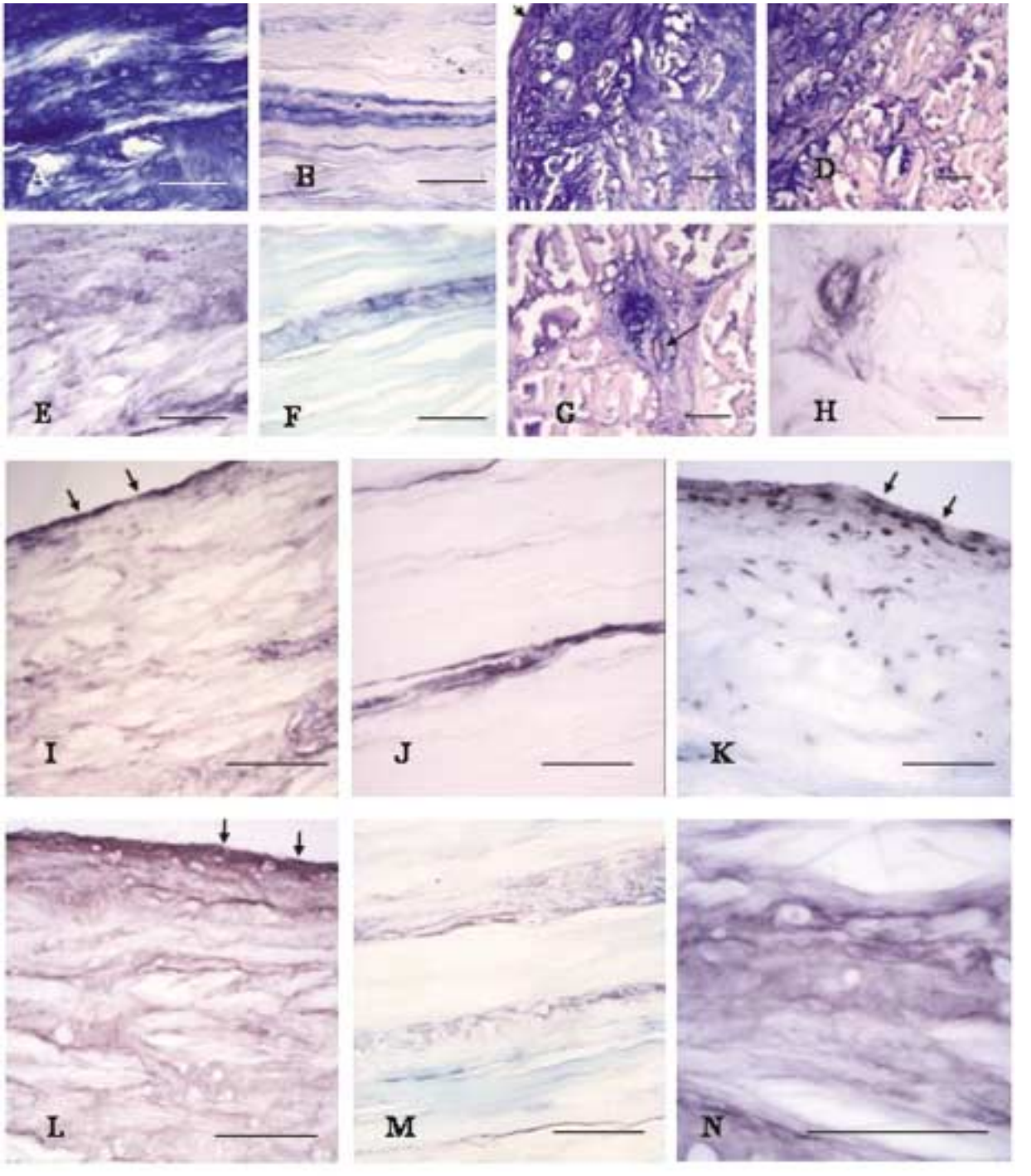

Figure 1. Histochemistry of adult bovine deep flexor tendon in the region of compression. Intense Alcian blue staining was seen throughout fibrocartilage near the contact surface (A) but only in layers between collagen bundles in longitudinal sections of the adjacent deeper region (B). Alcian blue staining was strong in transverse sections of the surface (C), but highly localized in the adjacent deeper region (D). Anti-aggrecan staining was present in longitudinal sections of surface (E) and adjacent deeper region (F). Transverse section of deeper region shows Alcian blue staining and vascular structure (arrow) at the point where several collagen bundles come together (G). This structure is also stained for hyaluronic acid (H). Note that the endotenon surrounding large collagen bundles can be seen in transverse sections of the deeper region even though the collagen fibers themselves do not stick well to the slide. Staining for hyaluronic acid is seen in longitudinal sections of the surface region (I) and in layers between collagen bundles in adjacent deeper tendon (J). Anti-biglycan staining is restricted to cells at surface of tendon fibrocartilage (K). Anti-type VI collagen staining is present in longitudinal sections of the tendon fibrocartilage surface (L) and in layers of looser matrix between collagen bundles in the adjacent deeper regions of tendon (M). At higher magnification, fibrillar material containing Type VI collagen appears to surround the round fibrocartilage cells (N). Bar = 100 μm; arrows in C, I, K and L indicate the contact surface of the tendon.

Materials and methods

Tissue preparation. Fresh deep flexor tendons from the hindlimb of adult cows (age 2-3 years) were obtained from a local slaughterhouse. The mid-substance fibrocartilagenous region of each tendon was cut transversely into slices 1-2 cm in thickness, fixed for 24 hours in 4% paraformaldehyde at 4°C and then transferred to 0.5 M sucrose. To make frozen sections the tissue was frozen in O.C.T. Compound (Tissue Tek, Miles, Inc.). Longitudinal or transverse sections (10-12 micrometers thick) were cut and adhered to slides (Superfrost/Plus Microscope Slides, Fisher Scientific). The slides were stored at –70°C.

Alcian blue staining. Slides were rinsed in distilled water and then incubated in 3% acetic acid for 3 minutes. The tissue sections were then incubated with Alcian blue stain for 1 hour at room temperature (0.1% Alcian blue 8GX (Sigma Chem. Co.) in 3% acetic acid, pH 2.5). After rinsing in water for 10 minutes the sections were briefly counterstained in Eosin and again rinsed in tap water. The tissue was dehydrated with two changes of 95% ethyl alcohol, absolute alcohol, cleared with two changes of xylene, and mounted with Permount.

Immunohistochemistry. Frozen sections were removed from the freezer, fixed in acetone for 10 minutes and air dried. All subsequent steps were performed at room temperature. Normal serum, biotinylated secondary antibody and ABC reagent were from the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Sections were rinsed in 10 mM phoshate buffered saline (PBS), pH 7.4, for 5 minutes Sections to be stained with anti-aggrecan were digested with chondroitinase ABC (0.4 units/ml in PBS, 30 minutes, Seikagaku Corp.). Possible interference by endogenous peroxidase was blocked by incubation for 10 minutes in 0.6% H_2O_2 in absolute methanol followed by rinsing in PBS. To block non-specific binding sections were also incubated for 15 minutes in 4% normal goat serum in PBS containing 0.4% Triton X-100 and 1% bovine serum albumin (BSA). Primary polyclonal antiserum recognizing the core protein of bovine aggrecan was provided by Dick Heinegard (Lund, Sweden). Anti-decorin (LF-91) and anti-biglycan (LF-96) antisera were provided by Larry Fisher (Bethesda, MD). Anti-collagen type VI antiserum (279, recognizing the α3 chain) was provided by Eva Engvall (La Jolla, CA). The primary antisera were diluted 1/1000 (anti-aggrecan) or 1/200 (all others) in PBS containing 1% Triton X-100, 1% BSA, 1% normal serum. Individual sections were circled with rubber cement to reduce the volume of antibody used. After incubation for 1 hr with primary antiserum the sections were rinsed with PBS, incubated for 1 hour with biotinylated anti-rabbit secondary antibody (1/200 in 1% Triton X-100, 1% BSA), and rinsed again with PBS for 5 minutes. Sections were then incubated 1 hr with ABC reagent diluted in PBS containing 1% BSA. Finally, sections were rinsed in sodium acetate-imidazole buffer (0.2 M imidazole, 1 M sodium acetate, pH adjusted to 7.2 with glacial acetic acid) and developed by observing color change in the chromagen solution. This solution was made by dissolving 62.5 mg nickel (II) sulfate in 2.5 ml of acetate-imidazole buffer and adding 1 mg 3', 3'-diaminobenzadine (DAB). When development reached an appropriate level the sections were rinsed in acetate-imidazole buffer, followed by subsequent rinses with PBS and distilled water. Primary antiserum was omitted in the preparation of control sections and no chromagen color developed in the controls. Sections were counterstained with methyl green and mounted with Permount.

Hyaluronan staining. Frozen sections were removed from the freezer, fixed in acetone for 10 minutes and air dried. All subsequent steps were performed at room temperature. Sections were rinsed in 10 mM PBS, pH 7.4 for 5 min. Possible interference by endogenous peroxidase was blocked by incubation for 5 minutes in 10% H_20_2 in absolute methanol followed by rinsing in dH_20 for 2 minutes and equilibration in PBS for 5 minutes. The probe for hyaluronan was biotinylated HA-binding fragments of bovine aggrecan prepared as described[7] and provided by Charles Underhill (Washington, D.C.). This probe was diluted to 4 μg/ml in PBS containing 10% BSA and incubated on the sections for 1 hour. The sections were then rinsed in PBS and incubated in ABC reagent for 1 hour followed by rinsing in PBS. Sections were rinsed in sodium acetate-imidazole buffer and developed by observing color change in the chromagen solution, followed by subsequent rinses with PBS and distilled water.

Results

Alcian blue. Longitudinal sections of tendon fibrocartilage stained intensely with Alcian blue (Figure 1A), indicating accumulated proteoglycan. Characteristic layers of looser extracellular matrix material could be seen between the dense collagen bundles in tissue adjacent to the fibrocartilage. These layers were stained with Alcian blue whereas the collagen bundles on either side of these layers were not stained (Figure 1B). The tensile region of the tendon was not stained with Alcian blue[6] (data not shown).

Transverse sections of tendon fibrocartilage show a jumble of collagen fiber bundles and a great deal of staining with Alcian blue (Figure 1C). Somewhat deeper in this tissue the cross-sections revealed collagen fiber bundles delineated by endotenon and little Alcian blue staining (Figure 1D). However, at the point of junction between several collagen bundles a unique structure was frequently seen. This structure consisted of vasculature surrounded by Alcian blue-stained material (Figure 1G). There was also staining for hyaluronic acid in this structure and the hyaluronan staining appeared to outline vasculature (Figure 1H). Although fiber bundles surrounded by a thin endotenon was also a clear feature in cross-sections of tendon from the tensile region, the vasculature in tensile regions of tendon was never surrounded by Alcian blue-stained material (data not shown).

Immunohistochemistry. Longitudinal sections of fibrocartilage showed positive staining with antibodies to the core protein of aggrecan (Figure 1E). In tissue adjacent to the

	Region of Tendon			
	Tensile	Compressed		
		Surface	Middle	
		Fibrocartilage	Collagen Bundles	Looser Layer
Alcian Blue	--	++	--	+
Aggrecan	+	++	+/-	+
Decorin	++	++	+	+
Biglycan	--	+ (cellular)	--	--
Collagen type VI	++	++	+/-	+
Hyaluronic acid	+/-	++	--	+

--, absent; +/-, minimally present; +, present; ++, strongly present

Table I. Histochemistry of adult bovine tendon extracellular matrix.

fibrocartilage, aggrecan staining was found in the looser layer of material corresponding to Alcian blue-stained layers described above (Figure 1F). Similar sections were stained for hyaluronic acid, biglycan and type VI collagen. The immunohistochemical reactions showed both hyaluronic acid and type VI collagen in a fibrillar pattern in the fibrocartilage (Figures 1I and 1L). Staining for type VI collagen was distinctive in that it appeared to closely surround cells in the fibrocartilaginous tissue (Figure 1N). In contrast, biglycan staining was localized to the cells of the fibrocartilage but was not found associated with the extracellular matrix (Figure 1K). Both hyaluronic acid and type VI collagen reaction product was found in deeper tissue, localized to the layers of loose matrix material located between dense collagen fiber bundles (Figures 1J and 1M). All regions of tendon matrix were positive for decorin (data not shown). These results are summarized in Table 1.

Collagen fibers cut as frozen cross sections did not stick well to the slides, creating the image of ragged bundles seen in Figure 1G. It was not possible to do satisfactory immunohistochemistry on these cross-sections.

Discussion

The biochemical composition of fibrocartilaginous (compressed) regions of tendon is very similar to the tensile regions, except that type II collagen is found only in the compressed region. Many molecules initially discovered as components of cartilage have been sought in tendon, and each of these has been found to be present not only in tendon fibrocartilage but also in the tensile region of tendon[3,8,9]. These include COMP, PRELP, aggrecan, biglycan and type VI collagen. However, some of these components (aggrecan, biglycan and type VI collagen) are clearly present in greater quantity in the fibrocartilaginous tissue which develops at the site where tendon wraps under a bony structure and receives compressive/transverse loading[10,11]. This is particularly true of the proteoglycan aggrecan, which is often present at more than 10-fold greater amounts in the compressed region than in adjacent tensile regions of the same tendon.

Several types of experiments indicate that aggrecan accumulation in tendon subjected to compression is a response to the load. For example, *in vitro* cyclic loading leads to increased large proteoglycan synthesis and increased aggrecan gene expression[12,13]. In addition, *in vivo* experiments have shown that removal of transverse load by translocation of the rabbit flexor digitorum profundus tendon leads to a rapid decrease in tendon glycosaminoglycan content[14,15]. The translocated tissue showed a significant increase in equilibrium strain under compressive creep load[15]. The accumulation of aggrecan at the point of compressive loading can provide compressive strength to the tendon right where it is needed. This is presumed to be the primary function of the aggrecan that is accumulated.

One of the first reports of the histology of bovine flexor tendon from the region under transverse load showed that staining with Alcian blue (indicating the accumulation of glycosaminoglycans) was present not only in the cartilage-like tissue near the contact surface, but also in layers of loosely-organized tissue located between longitudinal collagen fiber bundles[6]. This curious organization was found below the contact surface of the tendon, under the fibrocartilage, at the outer curvature of tendon as it bends around bone. The cells in this material are round like cells in the compressed tissue, and unlike the elongated cells in adjacent collagen bundles and in tensile tendon. Furthermore, the glycosaminoglycan content in this region of tissue was higher than tensile regions but lower than the fibrocartilage at the contact surface[10]. We now show by immunohistochemical techniques that this looser material located between longitudinal fiber bundles has a particularly high content of aggrecan and type VI collagen - an observation consistent with the tissue being more like fibrocartilage than tensile tendon.

The function of proteoglycan-rich layers between collagen bundles at the point of tendon bending is not known. We have speculated that they may facilitate the movement of collagen fiber bundles relative to one another, providing mobile planes to relieve shear stress as the tissue bends[16]. In other tendons, where the amount of aggrecan is increased compared to the amount in purely tensile tendon, this proteoglycan may play a similar role. For example, somewhat elevated levels of glycosaminoglycan, and corresponding elevated levels of the proteoglycans aggrecan and biglycan, have been noted in ligament as compared to tensile tendon[17,18]. A detailed investigation of the functional morphology of the human supraspinatus tendon described fascicles that are structurally independent and can slide past one another to accommodate changing joint angles[19]. Proteoglycans appear to be accumulated between the collagen layers where they may serve to separate and lubricate the collagen bundles as they slide relative to each other during normal, non-uniform, loading of the tissue[19,20]. This is a second probable role for proteoglycan in tendon.

It is difficult to cut transverse sections of large adult tendons. For this reason the common image of tendon tissue organization is based on longitudinal sections. We initially believed that the layers of Alcian blue-stained material seen in longitudinal sections of wrap-around tendon were expanded endotenon. We predicted that these layers surrounded the collagen fiber bundles. Indeed, some images from the fibrocartilage region suggest that this may be the case and this leads to the intriguing speculation that fibrocartilage in mid-substance tendon might be derived from expanded endotenon. However, cross-sections of deeper tissue revealed that the Alcian blue-stained material did not entirely surround the fiber bundles. Although this fibrocartilaginous material may still be derived from endotenon, it was located only at the points where several collagen bundles meet. At this location, it appeared to contain vasculature. Thus, the images of the current study suggest a third role for the accumulation of a proteoglycan-rich matrix in wrap-around tendon - a role in protecting the tendon's vascular supply during bending of the tissue.

It is now clear that vasculature in the mid-substance regions of gliding or wrap-around tendons can take two forms. There may be a completely avascular zone through the entire tendon, such as that between the A2 and A4 pulley in the long flexor tendon of the rabbit forepaw[21]. Alternatively, the fibrocartilaginous contact zone may be avascular but there can still be a complete vascular network running along the aspect of the tendon that is opposite to the surface in direct contact with a pulley or bone. This pattern has been described along the posterior part of the human tibialis anterior tendon and the dorsal part of the long flexor tendon of the rabbit hindpaw[21,22]. The bovine deep flexor tendon appears to have this latter pattern, with a rich network of vessels in the peritenon on the tendon's posterior surface as well as longitudinal core vessels in the tendon substance. Without some material to resist compressive loading these interior longitudinal vessels might be pinched or occluded by the bending and stretching that occurs in a wrap-around tendon.

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