Degenerative suspensory ligament desmitis (DSLD) is a debilitating disorder thought to be limited to suspensory ligaments of Peruvian Pasos, Peruvian Paso crosses, and other horse breeds. It frequently leads to persistent, incurable lameness. The pathogenesis remains unclear, though the disease appears to run in families. Presently, there are no reliable methods of diagnosing DSLD in asymptomatic horses. The goal of this study was to characterize and define the disorder in terms of tissue involvement at the macroscopic and microscopic levels and to identify mechanism of this disease with special attention given to small leucine rich proteoglycans (SLRPs), decorin and fibromodulin.

We examined tissues and organs from DSLD affected and control horses. Histopathological examination revealed excessive accumulation of proteoglycans in the following tissues: suspensory ligaments, superficial and deep digital flexor tendons, patellar and nuchal ligaments, cardiovascular system, and sclerae. Electron microscopy demonstrated changes in diameters of collagen fibrils in tendons, and in smooth muscle cells of the media of the aorta. In biochemical analysis, the proteoglycan overexpressed in affected tendon extracts was identified as decorin. Upon purification from affected tendons, we have demonstrated
presence of a higher molecular weight (~140kDa) decorin. The content of chondroitin-6-sulfate and the glucuronic acid of dermatan sulfate of this abnormal decorin were increased. ELISA showed that this decorin exhibited lower affinity to TGFβ1 than normal decorin. An increase in higher molecular fibromodulin in affected tendons was also observed.

This study demonstrates for the first time that DSLD is a systemic disorder characterized by accumulation of modified proteoglycans in tissues and organs with a significant connective tissue component. We, therefore, propose that equine systemic proteoglycan accumulation or ESPA rather than DSLD is a more appropriate name for this condition. Our biochemical results indicate that decorin and fibromodulin from ESPA affected tendons undergo changes in glycosylation of their glycosaminoglycans chains. In addition decorin purified from ESPA-affected tendons had low affinity to TGFβ1, which might lead to overexpression of TGFβ1 in tissues. These changes in expression of TGFβ1 by abnormal proteoglycan might contribute to the development of clinical picture observed in horses suffering from ESPA.

INDEX WORDS: Degenerative suspensory ligament desmitis, proteoglycan accumulation, Decorin, Fibromodulin, Dermatan sulfate, Keratan sulfate, Transforming growth factor beta 1
BIOCHEMICAL CHANGES IN EXTRA-CELLULAR MATRIX
IN EQUINE DEGENERATIVE SUSPENDED LIGAMENT DESMITIS

by

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Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008
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Dedicated to God, the Creator.
ACKNOWLEDGEMENTS

I’d like to give thanks to everyone who has supported me to stand here;

My academic mentors:
   Dr. Jaroslava Halper
   Dr. Mun-han Lee

My Committee for this dissertation:
   Dr. Jaroslava Halper
   Dr. Cathy Brown
   Dr. Steven C. Budsberg
   Dr. Thomas Krunkosky
   Dr. Eric Mueller
   and Dr. Jagannatha Mysore

Colleagues in my lab:
   Dr. John Bryan
   Jian Zhang
   Youngjoo Choi
   and Dr. Renato Sousa

Technical advisors:
   Mary Ard
   Dr. Jung Hae Yoon

Special thanks to my family:
   My parents
   My sons, Woojin and Hyunjin,
   My adorable and faithful wife, Seungyeon.
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CHAPTER 1

INTRODUCTION

Purpose of the study

Tendons and ligaments suffer many injuries usually as an inappropriate response to exercise and aging. Tendons and ligaments are more easily damaged if the exercise level is too high. Sometimes exercise accelerates a degenerative change that occurs inevitably with aging. There may be a strong relation between age and exercise (Smith, 2003). Degeneration of tendons and ligaments can be caused by mechanical disruption, exercise-induced hyperthermia, hypoxia, or imbalance between the matrix synthesis and degradation of various extracellular matrix proteins (Davis et.al. 2006). These tissues heal extremely slowly and the repaired tissue is inferior in elasticity and strength as compared to the original tissue, predisposing to recurrence and repeated injury in up to 80% of affected horses (Bramlage et.al. 1996, Madison, 1995, Sawdon et.al. 1996, Webbon, 1973). In older horses degenerative changes can be seen more often than in younger animals.

Although the wound healing process in tendon or ligament is similar to repair in other connective tissues, the rate of healing is slower. This is likely the result of the dense and hypocellular composition of tendons and ligaments and hypovascularity (Tozer et. al. 2005). Once the tendon suffers clinical injury which is accompanied by disrupted tendon matrix, damaged blood vessels release platelets. Degranulation of platelets leads to the formation of a fibrin clot. Cells and platelets in the clot quickly release a variety of important cytokines, including platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ)
causing acute inflammatory reaction. This inflammatory phase is a sequence of events: an increase in blood flow, the development of edema, the infiltration of neutrophils, macrophages, and monocytes, and the release of proteolytic enzymes. This is the earliest stage of repair and is designed to remove damaged tendon tissue. This inflammatory period lasts only 1 or 2 weeks and overlaps with the reparative phase, which begins within a few days and lasts several months. The reparative phase is characterized by angiogenic response and fibroblastic cellular infiltration which is responsible for synthesizing the new extracellular matrix (ECM), consisting largely of collagens and proteoglycans, and for synthesizing scar tissue, characterized by randomly arranged collagen. This scar tissue has a higher ratio of collagen types III: type I, a higher hydration, and higher levels of GAGs. The reparative phase of tendon healing merges with the remodeling phase, which begins several months after the injury. During this chronic remodeling phase, there is a decrease in the cellular and vascular content, an increase in the ratio of type I: type III collagen, and an increase in intermolecular collagen cross-linking and collagen fibril diameter. After the healing process is completed, this newly repaired tissue never becomes normal tendon tissue although cellularity, vascularity and collagen composition will return to approximately that of the normal tissues. The healed tendon becomes strong but it is functionally inferior to normal tendon and is thus predisposed to reinjury, often at sites adjacent to the original injury (Evans 1999, Hyman et. al. 2000, Molloy et. al. 2003, Smith 2003, Theoret 2006, and Davis et.al. 2006).

Change in collagen type affects the condition of healing in the tendon and ligament. After tendon injury, expression of type III collagen is upregulated (Williams et. al. 1980). Type III collagen has an important role in the healing process while type I collagen is thought to be responsible for the mechanical strength of the tendon tissue (Liu et.al. 1995). In the cross-
sectioned equine superficial digital flexor tendon (SDFT), the percent of type III collagen in the
degenerated center was elevated compared to the peripheral region (Birch et.al. 1998). Therefore
the increase in type III collagen of healing tendon likely accounts for the decreased resistance of
tendon to tensile forces through the formation of small diameter fibrils which are predominantly
distributed in healing tendon, and may predispose the tendon to reinjury.

Although the molecular mechanism involved in the synthesis or degradation of collagen
is not completely understood, it is clear that different proteoglycans (PGs) regulate fibril
assembly. Results from studies on knockout mice deficient in one or two small leucine rich
proteoglycans (SLRPs) showed abnormal collagen fibrillogenesis in several tissues including
tendons during embryonic development suggesting that they could play specific roles in
regulating different steps of fibril assembly and tendon maturation (Ameye et. al. 2002,
2004). Binding between proteoglycans and collagen fibrils contributes to the biomechanical
strength of tendons and ligaments.

Generally, proteoglycans are classified into two groups, which play an important role in
linking together the fibrous elements of the ECM in tendons or ligaments (Scott 1990). First are
large proteoglycans which are sometimes called lecticans or modular proteoglycans. This group
consists of aggrecan, versican, neurocan, brevican and some others. These proteoglycans provide
tissues with resilience against compressive forces. A second group is small proteoglycans
(SLRPs) which have leucine-rich repeat structure and are considered organizers of collagenous
networks. This group contains decorin, biglycan, fibromodulin, lumican, and keratocan among
others. SLRPs play an important role in stabilizing collagen fibrils by protecting them from
access to collagenases (Geng et.al. 2006). Furthermore SLRPs bind to growth factor or growth
factor receptors. They can modify TGFβ bio-availability and modulate epidermal growth factor receptor (EGFR) or insulin-like growth factor receptor (IGFR) signaling (Droguett et.al. 2006). However, the precise role of these small proteoglycans in modulating the biomechanical properties of tendons and ligaments remains poorly defined.

The basic structure of all proteoglycans includes a protein core and at least one, but frequently more (up to tens or hundreds) carbohydrate chains, so called glycosaminoglycans (GAGs). Glycosaminoglycan chains of proteoglycan consist of repeated disaccharide which can be sulfated at different position. Dermatan sulfate chain attached to decorin is composed of repeated disaccharides of hexuronic acid and N-acetylgalactosamine (GalNAc), mostly sulfated at 4-position or 6-position of GalNAc, or at 2-position of iduronic acid (IdoA), an epimer of glucuronic acid (GlcA) (Trowbridge et.al. 2002) while keratan sulfate of fibromodulin contains repeated disaccharide of galactose (Gal) and N-acetylglucosamine (GlcNAc), sulfated at 6-position of Gal and/or GlcNAc (Funderburgh et.al. 2000). In dermatan sulfate the presence of IdoA instead of GlcA is responsible for inhibition of proliferation of normal fibroblasts (Westergren-Thorsson et.al. 1991), and it also affects the interaction between SLRPs and TGFβs (Hildebrand et.al 1994).

In the ECM of ligaments and tendons, many cytokines are involved in synthesis of collagens and other ECM components through fibroblast activation. For example, members of TGFβ superfamily stimulate the production of proteoglycans in human lung fibroblast culture (Tiedemann et.al. 1997) and regulate glycosylation of proteoglycans. The glycosaminoglycan (GAG) structures of decorin, biglycan, and versican, a member of large PGs, are regulated in part by TGFβ1 (Tiedemann et.al. 2005). On the other hand, decorin, one of the SLRPs, inhibits the overproduction of TGFβ1 in glomerulonephritis model (Border et. al. 1992) and reduces the
binding of TGFβ1 to its receptors in skeletal muscle cell culture (Droquett et.al. 2006). In the hypertrophic scar fibroblasts culture, recombinant human decorin inhibits cell proliferation and reduces TGFβ1 production (Zhang et.al. 2007).

In degenerated equine superficial digital flexor tendon, total amount of sulfated GAGs was found to be significantly increased in central region relative to peripheral region and normal tendon (Birch et.al. 1998). In human patellar tendinopathy, monosulfated disaccharides including ∆Di-4S and ∆Di-6S were increased (Fu et.al. 2007). Besides tissue repair, similar proteoglycan or GAG changes can be identified in genetic diseases. In some patients with Ehlers-Danlos syndrome which is characterized as a defect in collagen synthesis, a substitution of one amino acid in galactosyltransferase I results in longer GAG chain, less iduronic acid, and more core protein of decorin (Seidler et.al. 2006). Although the longer GAG chain of decorin is common in wound healing and genetic diseases, the shortening of dermatan sulfate of decorin in later healing stages does not appear to have a counterpart in genetic diseases (Kuwaba et.al. 2002).

Degenerative suspensory ligament desmitis (DSLD) is a heritable, debilitating syndrome recognized in Peruvian Pasos, Peruvian Paso crosses, Arabians, American Saddlebreds, American Quarter Horses, Thoroughbreds, and some European breeds (Young, 1993). Affected Peruvian Paso horses demonstrate clinical signs at an earlier age than horses of other breeds (Mero et.al. 2002). Horses with DSLD typically develop an insidious onset of bilateral or quadrilateral lameness without a history of trauma or performance related injury (Mero et.al. 2002). Ultrasonography of affected ligaments is characterized by a diffuse loss of echogenicity and an irregular fiber pattern (Dyson, 1996, Dyson et.al. 1995, Gibson et.al. 2002). Unique to DSLD, however, is diffuse enlargement of the affected ligaments despite exercise restrictions (Young, 1993, Mero et.al. 2002). The pathogenesis of DSLD is incompletely understood.
DSLD has been observed to follow familial lines; however, a definitive heritable mechanism has not been established. The presumptive diagnosis of DSLD is obtained from patient signalment and history, clinical examination, and ultrasonographic examination of clinically affected horses, and it is confirmed only at post mortem examination. Recently, four steps for the diagnosis of DSLD in Peruvian Paso horses were suggested: palpation of the suspensory ligaments, observance of baseline lameness, fetlock flexion tests, and sonographic examinations of the suspensory ligament at mid-cannon and both branches (Mero et.al. 2005). Although these four steps are useful, they are not a guarantee for future health until a reliable gene marker test become available. Presently, there are no reliable methods of diagnosing DSLD in asymptomatic horses.

Objectives

Though DSLD has been believed to be a disorder confined to the suspensory ligaments (SLs) of the distal limbs of horses, the mechanism of this disease remains largely unknown. The objectives of this study were to 1) identify whether tissues other than SLs are affected by DSLD, 2) characterize the pathology present in such tissues, 3) identify biochemical changes in DSLD, and 4) present the mechanism of this disease. This research would provide pathological and biochemical insight into pathogenesis of DSLD. It will be very valuable in developing diagnostic method or treatment target for DSLD.

REFERENCES


CHAPTER 2

PREVIOUS STUDIES

LITERATURE REVIEW

Degenerative Suspensory Ligament Desmitis

Tendons and ligaments of the distal limbs of the horse have an anatomic, functional, and clinical importance. Some of the main tendons are superficial digital flexor tendon (SDFT) and its accessory ligament, deep digital flexor tendon (DDFT) and its accessory ligament, and suspensory apparatus including suspensory ligament (SL). Their structures are suitable to sustain very high loads and strains. The functions of these elastic and complex apparatus are to provide support to the fetlock for preventing hyperextension of the carpus, and to restore the energy of impact and full weight bearing during propulsion and lift off (Denoix, 1994).

Generally tendons transfer force produced by muscle to bony attachments on the opposite side of a joint to provide movement while ligaments resist distraction of its two bony attachments. In the equine distal limb superficial digital flexor tendons exhibit considerable elasticity that is used to store energy for energy-efficient locomotion while suspensory ligament supports the fetlock joint with digital flexor tendons when the leg is supporting the horse's weight (Smith, 2003).

The most frequently injured organ in performance horses is the superficial digital flexor tendon. Superficial digital flexor tendon (SDFT) in the forelimb originates from the humeral head of the superficial digital flexor muscle. Its musculotendinous junction is located within the
carpal canal. In the hindlimb, SDFT originates at the musculotendinous junction in the proximal portion of the crus and extends distally along the plantar aspect of the metatarsus. The superficial digital flexor tendon extends along the most palmar aspect of the metacarpal region, forming a ring around the deep digital flexor tendon in the proximal pastern region. It bifurcates into a medial and lateral branch that inserts onto the distal part of the first phalanx and the proximal part of the second phalanx (Reef 1998). Suspensory ligament is the second most frequently injured soft tissue structure in performance horses. The suspensory ligament is a strong tendinous band containing muscle tissue, and originates from the palmar carpal ligament and the proximal palmar surface of the third metacarpal bone. It descends between the second and fourth metacarpal bones in the forelimb while it originates from the plantar proximal of the third metatarsal bone in hind limb (Dyson et. al., 1995). The suspensory ligament continues distally and bifurcates just proximally to the fetlock into medial and lateral branches. Each branch inserts onto the apex of the corresponding proximal sesamoid bone of the fetlock joint. Together, the suspensory ligament and proximal sesamoid bones form a major portion of the suspensory apparatus of the equine distal limb. From the gross ligament down to the collagen molecules, there is a complex structure comprising a series of progressively smaller subunits, (Goodship et. al., 1994). The average minimum force applied on the limb to obtain failure of the suspensory apparatus was 1220 kg and was significantly higher in trained horses than in resting horses. The main function of the suspensory ligament is to support the fetlock when weight is put on the limb during the standing position or stance phase (Denoix, 1994).

Usually tendons and ligaments are injured while responding to exercise, and due to aging. In older horses degenerative changes can be seen more often than in younger ones. Tendons and ligaments are more easily damaged if the exercise level is too high. Sometimes exercise
accelerates a degenerative change that occurs inevitably with aging. There may be a strong relation between age and exercise (Smith, 2003). Degeneration of tendons and ligaments can be caused by mechanical disruption, exercise-induced hyperthermia, hypoxia, or imbalance between the matrix synthesis and degradation of various extracellular matrix proteins (Davis, 2006).

Recently, variety intralesional treatments have been developed for the management of injuries of tendons and ligaments to avoid surgical therapies and to reduce reinjury rates. These treatments include polysulfated glycosaminoglycans (PSGAGs), hyaluronan (HA), beta-aminopropionitrile fumarate, growth hormone and growth factors (Dowling et al. 2000 and Dowling et al. 2002).

The term degenerative suspensory ligament desmitis (DSLD) is applied to a heritable, debilitating syndrome first described by Jan Young in 1993. He reported this disease in various breeds of horses, e.g., Peruvian Pasos, Peruvian Paso crosses, Arabians, American Saddlebreds, American Quarter Horses, Thoroughbreds, and some European breeds. Inflammatory changes caused by strains and sprains of a ligament are known as desmitis. Inflammations or injuries of suspensory ligament commonly occur in several breeds of horses, and its development in some breeds is associated with athletic work (Mero et al., 2002). Injuries of the suspensory ligament that occur in athletic horses are primarily confined to the proximal one-third of the suspensory ligament, i.e., to the body of the ligament, or to one or both branches (Dyson et al. 1995).

However, DSLD in Peruvian Pasos primarily affects the branches of the suspensory ligaments (Mero et al., 2002). Degenerative disease of the suspensory ligament was described in several breeds of horses that are used for intense workloads or have suffered from a prior suspensory desmitis. Recently, however, a report described the development of DSLD in 13 of 20 Peruvian Pasos without a history of athletic work (Mero et al., 2002).
Clinically, DSLD causes mild to severe lameness either bilateral or quadrilateral, has no sex or age predilection and can occur in the absence of athletic activity. This disorder is characterized by pain, lameness, thickening of the suspensory ligaments and, in some cases, a dropping of the fetlocks towards the ground. Histologically, affected enlarged fibroblasts may undergo chondroid metaplasia. Fasicles containing affected collagen fiber bundles are increased in diameter in the early stage of the disease. In later stages, giant, pale-staining, paucicellular fascicles are formed in the centers of the branches of the suspensory ligaments and periligamentous and interfascicular fibrosis is prominent (Mero et al., 2002).

Ultrasonographically, enlarged suspensory ligament branches, poor fiber pattern, and discrete hypoechoic lesions are detected and are characteristic for DSLD. Ultrasound examination of the affected limbs represents a useful method to diagnose DSLD. Due to the progressive nature of this syndrome, there is no cure or treatment that is effective (Mero et al., 2002). The abnormalities of affected suspensory ligament were thought to be caused by a defective repair process, which is caused by qualitative or quantitative changes in proteoglycans, growth factors and cytokines (Tozer et al. 2005). The abortive attempts at collagen repair by this defective repair system may be responsible for the weakness and rupture of the affected suspensory ligament.

Because of distinct clinical changes of suspensory ligament, DSLD was thought to be limited to suspensory ligament. However, latest systemic investigation showed this disease is not limited to suspensory ligament but is systemic disease related to proteoglycan accumulation which may affect collagen fibrillogenesis (Halper et al 2006). Accordingly, collagen morphology was also changed indicating proteoglycan accumulation might be a factor that causes DSLD. For
this reason, we proposed that DSLD be renamed as Equine Systemic Proteoglycan Accumulation (ESPA).

Recently, four steps for the diagnosis of DSLD in Peruvian Paso horses were suggested: palpation of the suspensory ligaments, observance of baseline lameness, fetlock flexion tests, and sonographic examinations of the suspensory ligament at mid-cannon and both branches (Mero J.L. and Scarlett J.M 2005). Although these four steps are useful, they are not specific enough to be diagnostic for DSLD. Only a reliable biochemical or genetic marker test would provide diagnostic certainty.

**Wound healing of tendons and ligaments**

Wound in tendons and ligaments can occur in one of two ways, by overstraining or through percutaneous injury (e.g., puncture). Although a sudden overloading of the soft tissue structures exceeding its resisting strength can be an important cause of overstrain-induced injury and wounding, a phase of molecular degeneration is believed to be the most common strain-induced wound in the horse, involving the palmar soft tissue structures of the metacarpal region (Davis et.al. 2006, Tsuzaki et.al. 2003). This degeneration induces neither a clinically evident inflammatory reaction nor any reparative response, but instead progressively weakens the tendons and ligaments (Smith et. al. 2002). These asymptomatic lesions, also described as microtrauma, are the result of healing of low-grade clinical wounds rather than true degeneration and are induced by increasing age and amount of exercise (Birch et.al. 1998). After skeletal maturity is achieved, the synergistic effect of aging and exercise causes an inevitable accumulation of microdamage, which is called degeneration (Smith, 2003 and Davis et.al. 2006). Degeneration is usually the first phase of tendinopathy. Clinical injury of already degenerated tendons and ligaments occurs when the highest stresses is encountered by the structures, and
results in irreversible damage. When there is the load, SDFT and SL bears the load in early part of the weight-bearing phase of gait while DDFT share the load in the later phase (Goodship et. al. 1994). This result can explain why the SDFT and the SL are the most easily traumatized soft tissue structures.

Wound healing is an integrated and complex process that includes the participation of large numbers of regulatory molecules, i.e., proinflammatory cytokines and growth factors, and combined tissue response of numerous cell types. Healing begins very early in the process of acute inflammation after tissue injury and involves two processes. The first phase is inflammatory where infection is combated and injured and/or dead tissue removed by inflammatory cells, such as white blood cells and macrophages. In the second phase the damaged connective tissue is replaced by first young fibrous (granulation) tissue, and then by scarring. Both processes include cell growth, differentiation and cell matrix interaction. Cytokines and soluble growth factors regulate migration, proliferation, and differentiation of cells as well as the synthesis and degradation of extracellular matrix (ECM) components. In the healing process of adult skin tissue, granulation of wound connective tissue represents a transition to mature scar tissue, characterized by continued collagen synthesis and collagen catabolism, i.e., remodeling (Gharaee-Kermani et. al. 2001, Werner S. et. al. 2003). In contrast the healing process in embryo results in essentially perfect repair without scar formation (Nodder et. al. 1997).

Although the wound healing process in tendon or ligament is similar to repair in other connective tissues, the rate of healing is slower. This is likely the result of the dense and hypocellular composition of tendons and ligaments and hypovascularity (Tozer et. al. 2005). Once the tendon suffers clinical injury with disrupted tendon matrix, damaged blood vessels
release platelets. Degranulation of platelets leads to the formation of a fibrin clot. Cells and platelets in the clot quickly release a variety of important cytokines, including platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ) causing acute inflammatory reaction. This inflammatory phase is a sequence of events: an increase in blood flow, the development of edema, the infiltration of neutrophils, macrophages, and monocytes, and the release of proteolytic enzymes. This is the earliest stage of repair and is designed to remove damaged tendon tissue. This inflammatory period lasts only 1 or 2 weeks and overlaps the reparative phase, which begins within a few days and lasts several months. The reparative phase is characterized by angiogenic response and fibroblastic cellular infiltration. The formation of new capillary blood vessels is necessary to sustain the granulation tissue newly formed within the wound. The infiltration of fibroblasts is responsible for synthesizing the new ECM, consisting largely of collagens and proteoglycans, and for synthesizing scar tissue, characterized by randomly arranged collagen. This scar tissue has a composition that is different from that of tendon, with a higher ratio of collagen type III: typeI, a higher hydration, and higher levels of GAGs. The reparative phase of tendon healing merges with the remodeling phase, which begins several months after the injury. During this chronic remodeling phase, there is a decrease in the cellular and vascular content, an increase in the ratio of type I: type III collagen, and an increase in intermolecular collagen cross-linking and collagen fibril diameter. After the healing process is completed, this newly repaired tissue never becomes normal tendon tissue although cellularitiy, vascularity and collagen composition will return to approximately that of the normal tissues. The healed tendon becomes strong but it is functionally inferior to normal tendon and is thus predisposed to reinjury, often at sites adjacent to the original injury (Evans 1999, Hyman et. al. 2000, Molloy et. al. 2003, Smith 2003, Theoret 2006, and Davis et.al. 2006).
Tendons consist of endotendon, epitendon, paratendon, and tendon sheath. The endotendon lies between the tendon bundles and carries vessels, nerves, and lymphatics. Epitenon extends to the endotenon and envelops tendon surface. The outer surface of the epitendon is enclosed by paratendon or tendon sheath. The paratendon is elastic and pliable, and allows the tendon to move back and forth. The tendon sheath reduces frictional forces between the tendon and the surrounding soft tissues where a change of direction or increased friction occurs. This sheath is divided into outer fibrous sheath and inner synovial membrane (McIlwraith 2002, Dyson et. al. 1995). In tendons, there can be two types of healing processes depending on the anatomic location of the injury. One is intrinsic healing in which cellular proliferation by the epitendon and endotendon result in an initial inflammatory phase. The other is extrinsic healing which is related to the supply of new blood vessels and cellular elements made available for the repair by outgrowth and migration from the tendon sheath. This occurs when tendons are injured within the confines of a synovial sheath and there is significant injury to the sheath itself. The restrictive adhesions between the tendon sheath and injured tendon are a result of tendon healing occurring in this synovial region although they have deleterious effects on the function of tendons. Therefore, the lack of the sheath and any surface defect within a tendon sheath will explain the relative poor response in healing (Smith 2003, Jann et.al 2005, and McIlwraith 2002). While tendons have endotenon, epitenon, paratenon, and tendon sheath, ligaments have the endoligament and epiligament. Endoligament refers to the septum of tissue which divides collagen fibers of the ligament into discrete bundles or fascicles, and epiligament refers to the surface layer which is a more vascular overlying layer and which covers the outer surface of ligaments. The endoligament itself is connected to the epiligament which is often indistinguishable from the actual ligament and merges into the periosteum of the bone around the
attachment sites of the ligament. (Frank 2004, Lo et.al. 2002) In medial collateral ligament (MCL) wound healing, the epiligament promote cell proliferation and matrix synthesis. It was suggested that the epiligamnet is suggested source of cells participating in the MCL scar formation (Matthews et.al. 2004).

**The role of collagens in repair of tendon and ligament**

Collagens, mostly fibrillar type I collagen, are the major component of the ECM in tendon and ligament. Type I collagen is composed of two \( \alpha_1 \) chains and one \( \alpha_2 \) chain coiled together in a heterotrimer triple helix and supports structurally extracellular matrices (Vuorio et.al 1990). A number of other collagens are involved in the composition of tendons and ligaments. Type III and V collagens are located in tendons (Niederreither et. al. 1994, Birk et. al. 1997). Fibril-associated collagens with interrupted triple helices (FACITs, for example, types XII and XIV) are localized at the surface of the fibrils (Walchli C. et. al. 1994). Among them, type III collagen is expressed relatively early in wound healing, only to be replaced by type I collagen during later stages of healing process. Type III collagen is a homotrimer composed of three \( \alpha_1 \) (III) chain and is usually abundant in fetal tissues relative to adult tissues (Epstein et.al. 1975). The collagen molecules are assembled into collagen fibrils through collagen fibrillogenesis. This very complex process consists of multiple steps and has been extensively studied during embryonic development (Zhang et.al. 2005, Edon-Vovard et.al. 2004). The individual chains of each collagen are translocated into the endoplasmic reticulum (ER), in which they form the collagen triple helix (Doege et.al. 1986). In ER, the collagen triple helix are hydroxylated and subsequently processed by lysyl oxidase to be secreted extracellularly through Golgi apparatus (Canty et.al. 2002). In ECM, collagen is formed from procollagen by cleavage of the N- and C-propeptides by procollagen metalloproteinases. The members of family of ADAMTS molecules
(ADAMTS-2, -3, and -14) are N-proteinases (whereas bone morphogenetic protein (BMP)-1 has the procollagen C-proteinase activity) (Colige et. al. 2002, and Li et.al. 1996). Once secreted, short immature intermediate fibrils are formed, and collagen fibrils are stabilized inter- and intra-molecularly through crosslinking (Zhang et al 2005). In tendons and ligaments, crosslinking is extremely important because it contributes to the tensile strength of these structures. Collagen fibrils assemble end to end to form longer fibrils (linear growth). At some point tendon fibroblasts synthesize processed collagen molecules as well as surface bound proteoglycans and other molecules for lateral growth (Canty et.al. 2002). Finally, lateral growth occurs in which fibrils associate laterally to generate large diameter fibrils. Collagen fibers branch to form a fiber network within and among fascicles, which provide a higher order of structure stabilization (Canty et.al. 2002 and Zhang et. al. 2005).

Fibrillogenesis of tendon and ligament during healing process is supposed to be related to fibrillogenesis of embryonic development. After tendon injury, expression of type III collagen is upregulated (Williams et. al. 1980). Type III collagen has an important role in the healing process while type I collagen is thought to be responsible for the mechanical strength of the tendon tissue (Liu et.al. 1995). Type III collagen is often codistributed with type I collagen and is concentrated in the endotenon of normal tendon forming small diameter fibril (Stein et.al. 1985). Therefore the increase in type III collagen of healing tendon likely accounts for the decreased resistance of tendon to tensile forces through the formation of small diameter fibrils which are predominantly distributed in healing tendon, and may predispose the tendon to reinjury. During the remodeling period, the increased type III collagen is gradually replaced by type I collagen. In \textit{in vivo} experiments, type III collagen expression is increased initially in healing horse tendon but type III collagen synthesis decreases from 4 weeks after injury and remains a minor collagen
throughout the healing process (Dahlgren et.al. 2005). In the cross-sectioned equine superficial digital flexor tendon (SDFT), the percent of type III collagen in the degenerated center was elevated compared to the peripheral region (Birch et.al. 1998). Type III collagen content is significantly increased at the rupture site of human Achilles tendon when compared to control sites but no changes in the amounts of newly synthesized type I and III collagens were observed suggesting that there was a slow accumulation of type III collagen before rupture due to microwounding of the tendon (Eriksen et. al. 2002). In in vitro healing model, ruptured Achilles tendons contain a significantly greater proportion of type III collagen and large amount of this collagen may exist after repair, which is indicative of an abnormal healing response or incomplete healing process (Maffulli et. al. 2000).

Although the molecular mechanism involved in the synthesis or degradation of collagen is not completely understood, it is clear that different proteoglycans (PGs) regulate fibril assembly. Results from studies on knockout mice deficient in one or two small leucine rich proteoglycans (SLRPs) showed abnormal collagen fibrillogenesis in several tissues including tendons during embryonic development suggesting that they could play specific roles in regulating different steps of fibril assembly and tendon maturation (Ameye et. al. 2002, Danielson et. al. 1997, Svensson et. al. 1999, Chakravarti et. al. 1998, and Edom-Vovard et. al. 2004). They regulate collagen fibril assembly and degradation by binding directly to collagen (Zhang et. al. 2006 and Geng et. al. 2006). Like SLRPs, type XII and XIV collagens are localized at the surface of fibrils and play a role in linear fibril growth (Young et.al. 2000). In in vitro experiments using proteoglycans from fetal bovine tendon, type XIII collagen interacted with decorin (or biglycan) through glycosaminoglycan chain of decorin (or biglycan) while fibromodulin (lumican) binds with this collagen through its core protein (Font et.al. 1996, Zhang
et al 2006). In addition to these fibril-associated proteins, some growth factors also play a role in fibrillogenesis. Transforming growth factor β (TGFβ) is a potent regulator of synthesis of collagen. TGFβ stimulates collagen formation and reduces collagen degradation via stimulating the tissue inhibitors of matrix metalloproteinases (TIMPs) together with a suppression of matrix metalloproteinases (MMPs), thus favoring accumulation of ECM and especially of collagen (Edwards et. al. 1987, Overall et. al. 1991). Connective tissue growth factor (CTGF) plays a major role in collagen type I synthesis with close relation to TGFβ (Schild et. al. 2002). Insulin-like growth factor-I (IGF-1) upregulates collagen synthesis in equine flexor tendons in a dose-dependent manner, and enhanced immunoreactivity for type-I collagen, compared with type-III collagen, was evident in treated equine tendon explants (Murphy et. al. 1997).

**Proteoglycans in wound healing**

Proteoglycans are macromolecules that are distributed almost everywhere in the body. As major components of tendon ECM, proteoglycans play crucial role in collagen fibrillogenesis and in tendon function. The basic structure of all proteoglycans includes a core protein and at least one, but frequently more (up to tens or hundreds) carbohydrate chains, so called glycosaminoglycans (GAGs) which are repeats of two monosaccharides. Proteoglycans can be found intracellularly, on the surface of the cells and in the extracellular matrix. Generally, proteoglycans are classified into two groups, which play an important role in linking together the fibrous elements of the ECM in tendons or ligaments (Scott 1990). First are large proteoglycans which are sometimes called lecticans or modular proteoglycans. This group consists of aggrecan, versican, neurocan, brevican and some others. These proteoglycans provide tissues with resilience against compressive forces. They are capable of resisting high compressive and tensile forces because of their high charge density. A second group is small proteoglycans (SLRPs)
which have leucine-rich repeat structure and are considered organizers of collagenous networks. This group contains decorin, biglycan, fibromodulin, lumican, and keratocan among others. They can be divided into 4 different subgroups depending on their number of leucine rich repeat and/or the type of glycosaminoglycan chain (Kresse et. al. 2001, Lorenzo et.al. 2001, Yoon et. al. 2005). Decorin and biglycan which are the members of the first group (class I) have 10 LRRs and one or two chondroitin or dermatan sulfate chains, respectively. Chondroitin sulfate consists of repeated glucuronic acid and N-acetyl galactosamine, and some of glucuronic acid is converted to its epimer, iduronic acid in dermatan sulfate (Peplow 2005). The second group (class II) consists of fibromodulin, lumican, keratocan and so on. They also have 10 LRRs and several keratan sulfate chains. Keratan sulfate has repeated galactose and N-acetyl glucosamine. Epiphycan is the member of the third group (class III) and contains only 6 LRRs. Chondroadherin and nyctalopin comprise the fourth group (class IV). These proteins have 10 LRRs, but lack both amino- and COOH-terminal extensions outside the cysteine motifs.

SLRPs, especially class I and II, play a role in regulation of collagen fibrillogenesis through binding to collagen fibrils. In the mouse with a knockout decorin gene, uncontrolled lateral fusion of collagen fibrils was demonstrated in tail tendon and the tensile strength was reduced in skin (Danielson et. al. 1997). In biglycan/fibromodulin double knockout mouse, fibril collagen in the tendons was altered structurally and stiffness of the tendon was diminished (Ameye et. al. 2002). Decorin and fibromodulin which are major SLRPs in the tendons and ligaments bind to type I collagen via separate binding site because they belong to different subgroup (Keene et.al. 2000, Kalamajski et.al 2007, and Hedbom et.al 1993). The involvement of decorin in the formation of collagen fibrils controls to some extent the diameter of the fibril and prevents lateral fusion of collagen fibrils through binding to collagen triple helix within the
gap zone (Danielson et. al. 1997, Weber et. al. 1996). SLRPs play an important role in stabilizing of collagen fibrils by protecting them from access to collagenases (Geng et.al 2006). Furthermore SLRPs bind to growth factors or growth factor receptors (Hildebrand et.al. 1994, Iozzo et. al. 1999, and Schonherr et.al. 2005). They can modify TGFβ bio-availability and modulate EGFR or IGFR signaling (Droguett et.al. 2006 and Feugaing et.al. 2007).

Normally, there is a significant difference of proteoglycan expression between compressed and tensile regions of tendon. The production of decorin is stimulated in distal/tensional region of biceps tendon whereas compression induces the synthesis of aggreccan (Berenson et.al. 1996, Robbins et. al. 1997). In normal rabbit medial collateral ligament, decorin is the most abundant proteoglycan (accounting for ~80% of the total), and the remainder is biglycan and a large proteoglycan (Plaas et. al. 2000). Like ligaments, tensional region of bovine tendons are comprised of leucine-rich proteoglycans about 90% whereas compressed region consists of up to 50% of large proteoglycans (Vogel et.al. 1985 and 1987). Even in the same equine superficial digital flexor tendon in horses, the amount of decorin decreased in the distal metacarpal region relative to the proximal region whereas the length of GAG chain increased (Watanabe et.al. 2005). In other tissues such as skin or cartilage, GAG structures of SLRPs can be changed according to their age. The length of decorin GAG chain decreases with aging in human skin tissue while the quantity of decorin increases in old skin (Carrino et. al. 2000). Also, the size of the human cartilage fibromodulin decreases with age. In the mature adult the fibromodulin does not possess keratan sulfate, although it appears to possess the same number of N-linked oligosaccharides as the younger tissues (Roughley et. al., 1996).

During wound healing after injury, there are quantitative or qualitative changes in proteoglycans. During healing process of guinea pig skin tissue, CS/DS proteoglycans are
increased and their GAG chains are longer than proteoglycans in quiescent normal tissue (Yeo et al. 1991). After hapten-induced inflammation in mouse skin, molecular size of decorin dermatan sulfate increased whereas there was no change in the size of decorin core protein (Kuwaba et al. 2001). In rabbit ligament undergoing repair, decorin is barely detected, but instead a large proteoglycan and abundant amounts of biglycan accumulate (Plaas et al. 2000). In chronic painful human Achilles tendon (tendinopathy), mRNA expression of biglycan and aggrecan was increased while decorin and versican was not, which was different from mRNA pattern of ruptured Achilles tendon that showed only markedly decreased decorin (Corps et al. 2006). In degenerated equine superficial digital flexor tendon, total amount of sulfated GAGs was significantly increased in central region relative to peripheral region and normal tendon (Birch et al. 1998). In human patellar tendinopathy, monosulfated disaccharides including ∆Di-4S and ∆Di-6S increased (Fu et al. 2007). Most GAGs are sulfated on their core proteins, which make proteoglycans anionic. This acidic property of GAG containing proteoglycans is very important in interaction with biological proteins (Peplow 2005). Besides wound healing, there are similar proteoglycan or GAG changes in certain genetic diseases. Genetic defects in lysosomal enzymes which are related to GAG degradation result in several mucopolysaccharide disorders that lead to accumulation of GAGs within cells (Williams 1967). Alteration of proteoglycan content in several muscular dystrophies is similar to that of normal wound healing. Decorin mRNA was downregulated in Duchenne muscular dystrophy (DMD) and LAMA2-mutated congenital muscular dystrophy (MDCIA) whereas biglycan mRNA level was upregulated in Becher muscular dystrophy (Zanotti et al. 2005). In Ehlers-Danlos syndrome which is characterized as defect in collagen synthesis in most patients, a substitution of one amino acid in galactosyltransferase I results in longer GAG chain, less iduronic acid, and more
core protein of decorin in patients with the progeric form of Ehlers-Danlos syndrome (Seidler et.al. 2006). Although the longer GAG chain of decorin is common in wound healing and genetic diseases, the shortening of decorin dermatan sulfate in later healing stages was not shown to be the case in genetic diseases (Kuwabe et.al. 2002, and Quentin et.al. 1990).

Role of TGFβ1 in wound healing of tendon and ligament

Cytokines and growth factors are small proteins that have the ability to regulate the biological activities of responsive cells. The type of response classifies cytokines as chemokines, growth factors, morphogens and so forth. For example, growth factors stimulate cell proliferation, and also modulate a variety of differentiated functions. The main role of cytokines proper is their participation in immunological processes. In spite of this categorization most cytokines have multiple and in many instances overlapping properties. The interactions among cytokines are complex, sometime antagonistic, and at other times synergistic. Some cytokines activate the synthesis of other cytokines while some inhibit their synthesis. Moreover, various binding proteins, soluble receptors, matrix components, and others regulate activities of many cytokines (Werner et. al. 2003).

Many cytokines are either dormant or activated in the ECM of the ligaments and tendons. Among them are TGFβ, interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), IGF-I, basic fibroblast growth factors (bFGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) and some other, smaller molecules, such as nitric oxide (NO) and prostagladins. In general they regulate collagen synthesis through fibroblast activation (Kjaer 2004). Using the sensitive reverse transcription-polymerase chain reaction technique, transcripts for a number of growth factors and receptors were detected probing RNA isolated from normal and healing rabbit medial collateral ligament tissues. These include TGFβ1, IGF I and II, bFGF,
endothelin-1, and the receptors for insulin and IGF II. The levels of several cytokines were elevated in injured ligament (Sciore et. al. 1998). The function and effect of these cytokines in healing process has been studied using numerous in vitro and in vivo models. In a rabbit flexor tendon wound healing model, repaired tissue showed increased expression of bFGF mRNA by in situ hybridization (Chang et. al. 1998). Increased number of proliferating cells and increased level of expression of type III collagen were noted in rats upon administration of bFG (Chan et. al. 2000). Recombinant human IGFs stimulated the synthesis proteoglycan and collagen in culture of rabbit deep flexor tendons (Abrahamsson et. al. 1996 and Abrahamsson 1997) and accelerated recovery after Achilles tendon operation in the rat (Kurtz et. al. 1999). Connective tissue growth factor (CTGF), usually considered a mediator of TGFβ activity induced collagen synthesis in rat fibroblast cell culture (Duncan et. al 1999).

Among these growth factors, TGFβ is the most potent regulator of extracellular matrix component expression and as such it is very important during wound healing and tissue repair. TGFβ superfamily comprises many related factors involved in cell proliferation, cell differentiation, and embryonic development. This group also includes three known isoforms of TGFβ (TGFβ1-3). TGFβ is produced by most cells involved in the healing process and has been active in almost all stages of tendon healing (Chang et. al. 2000). TGFβ upregulates mRNA levels of collagen in cultured equine tenocytes (Arai et. al. 2002), and elevates the tendinous type I and type III collagen production (Klein et. al. 2002 and Heinmeier et. al. 2003). TGFβ also increases the expression and synthesis of other ECM proteins, e.g., proteoglycans (PGs) (Robbins et. al 1997). Moreover, TGFβ1 inhibits expression of matrix metalloproteinases and augments expression of tissue inhibitors of metalloproteinases, which stimulate the accumulation of ECM components (Edwards et.al. 1996). TGFβs regulate the growth and differentiation of
various cells through Smad proteins (Kawabata et.al. 1999). The effects of TGFβs on ECM components are also controlled by Smad pathway (Verrecchia et.al. 2002). Binding of TGFβ to TGFβ type II receptor (TβRII) recruit TGFβ type I receptor (TβRI) and activate it by phosphorylation. After ligand activation, signaling from TβRI to the nucleus occurs predominantly by phosphorylation several members of the Smad family (Piek et.al. 1999). Recently, however, TGFβ inducible early gene (TIEG) knockout mouse model showed the possibility of tendon healing without the Smad pathway (Tsubone et.al. 2006) suggesting there might be a another pathway of TGFβ expression.

Ligament healing in the rat is accompanied by significant elevation in the expression of TGFβ and this state remains for at least 8 weeks post-injury (Natsu-um et. al. 1997). In the tendon wound environment of the rabbit, TGFβ is activated in both "intrinsic" tenocytes and "extrinsic" tendon sheath fibroblasts, as evidenced by significant mRNA upregulation in these cells (Chang et. al. 1997). This upregulation stimulates the production of proteoglycans in human lung fibroblast culture (Tiedemann et.al. 1997) and regulates the ratio of iduronic acid and glucuronic acid of dermatan sulfate (Tiedemann et.al. 2005). On the other hand, decorin, a small PG and a member of the SLRP family (see above), inhibits the overproduction of TGFβ in glomerulonephritis model (Border et. al. 1992) and reduces the binding of TGFβ to its receptors in skeletal muscle cell culture (Droquett et.al. 2006). In the hypertrophic scar fibroblasts culture, recombinant human decorin inhibits cell proliferation and reduces TGFβ1 production indicating that application of decorin may be a therapeutic method in fibrotic diseases (Zhang et.al. 2007).
References


**PRELIMINARY DATA**


**ABSTRACT**

**Background**

Degenerative suspensory ligament desmitis (DSLD) is a debilitating disorder thought to be limited to suspensory ligaments of Peruvian Pasos, Peruvian Paso crosses, Arabians, American Saddlebreds, American Quarter Horses, Thoroughbreds, and some European breeds. It frequently leads to persistent, incurable lameness and need to euthanize affected horses. The pathogenesis remains unclear, though the disease appears to run in families. Treatment and prevention are empirical and supportive, and not effective in halting the progression of the disease. Presently, the presumptive diagnosis of DSLD is obtained from patient signalment and history, clinical examination, and ultrasonographic examination of clinically affected horses, and is confirmed at post mortem examination. Presently, there are no reliable methods of diagnosing...
DSLD in asymptomatic horses. The goal of this study was to characterize and define the disorder in terms of tissue involvement at the macroscopic and microscopic levels.

**Results**

We examined tissues and organs from 28 affected horses (22 Peruvian Pasos, 6 horses of other breeds) and from 8 control horses. Histopathological examination revealed the presence of excessive amounts of proteoglycans in the following tissues removed from DSLD-affected horses: suspensory ligaments, superficial and deep digital flexor tendons, patellar and nuchal ligaments, cardiovascular system, and sclerae. Electron microscopy demonstrated changes in diameters of collagen fibrils in the tendon, and in smooth muscle cells of the media of the aorta compatible with increased cell permeability in DSLD-affected cells. Separation of tendon extracts by gel chromatography revealed the presence of additional proteoglycan(s) in extracts from affected, but not control extracts.

**Conclusion**

This study demonstrates for the first time that DSLD, a disease process previously thought to be limited to the suspensory ligaments of the distal limbs of affected horses, is in fact a systemic disorder involving tissues and organs with significant connective tissue component. Abnormal accumulation of proteoglycans between collagen and elastic fibers rather than specific collagen fibril abnormalities is the most prominent histological feature of DSLD. Because of this observation and because of the involvement of many other tendons and ligaments beside the suspensory ligament, and of non-ligamentous tissue we, therefore, propose that equine systemic proteoglycan accumulation or ESPA rather than DSLD is a more appropriate name for this condition.
BACKGROUND

Injuries to tendons and ligaments are significant causes of lameness and financial losses in the equine industry. They account for nearly a third of all equine injuries that occur during racing, with a reported incidence of 8–43% [1-4]. These tissues heal extremely slowly and the repaired tissue is inferior in elasticity and strength as compared to the original tissue, predisposing to recurrence and repeated injury in up to 80% of affected horses [5-8]. Tendon and ligament injuries may occur as result of acute overloading of the tendon or ligament or secondary to idiopathic degenerative changes [9-11].

Degenerative suspensory ligament desmitis (DSLD) is a heritable, debilitating syndrome recognized in Peruvian Pasos, Peruvian Paso crosses, Arabians, American Saddlebreds, American Quarter Horses, Thoroughbreds, and some European breeds [12]. Affected Peruvian Paso horses demonstrate clinical signs at an earlier age than horses of other breeds [13]. Horses with DSLD typically develop an insidious onset of bilateral or quadrilateral lameness without a history of trauma or performance related injury [13]. Ultrasonography of affected ligaments is characterized by a diffuse loss of echogenicity and an irregular fiber pattern [14-16]. Unique to DSLD, however, is diffuse enlargement of the affected ligaments despite exercise restrictions [12,13]. The pathogenesis of DSLD is incompletely understood. DSLD has been observed to follow familial lines; however, a definitive heritable mechanism has not been established. The presumptive diagnosis of DSLD is obtained from patient signalment and history, clinical examination, and ultrasonographic examination of clinically affected horses, and it is confirmed only at post mortem examination. Presently, there are no reliable methods of diagnosing DSLD in asymptomatic horses.
Though DSLD has been believed to be a disorder confined to the suspensory ligaments (SLs) of the distal limbs of horses, the mechanism of this disease remains largely unknown. The objectives of this study were to 1) identify whether tissues other than SLs are affected by DSLD and 2) characterize the pathology present in such tissues. This study was initiated because pilot findings from our laboratory suggested that the abnormalities in the collagenous tissue of affected horses are not confined to the SLs distal limbs, but may be manifested systemically, in virtually all collagen containing tissues. In this preliminary work abnormal accumulations of yet to be identified proteoglycans appeared to be present not only in the SL, but also in the superficial and deep digital flexor tendons (SDFT and DDFT, respectively), patellar and nuchal ligaments, aorta, coronary arteries and sclerae of DSLD-affected horses.

RESULTS

Harvested tissues

Twenty eight horses were referred because of clinical diagnosis of DSLD supported by known bilateral (n = 9) or quadrilateral (n = 17) lameness (the lameness status was not noted in two horses). Lameness was accompanied by an excessively dropped fetlock appearance, and/or palpable enlargement of the branches or body of the suspensory ligament. Ultrasound performed on 19 horses showed evidence of suspensory ligament enlargement with changes in echogenicity, e.g., a hypoechoic, irregular fiber ligament disruption, in at least 14 horses (full ultrasound descriptions were not available in all cases) accompanied by intra-ligamentous calcification in 3 cases. At least 16 horses had known family history of DSLD (Tables 2-1 and 2-2). The 28 horses included 22 Peruvian Paso horses (Table 2-1) and 6 horses of other breeds (2 thoroughbreds, 1 Arab, 1 Hanovarian, 1 Appaloosa and 1 quarter horse, Table 2-2). In 25 affected horses SDFTs, DDFTs and SLs from all 4 extremities were examined grossly and microscopically at their
proximal and midmetacarpal regions; SDFT, DDFT and SL from only one extremity were examined in the remaining three animals. One section from the proximal end and another section from the midmetacarpal end of a tendon or ligament were taken for histological examination. The following tissues were collected from most horses: eyes, portions of nuchal ligament, one of the patellar ligaments, portions of the cardiovascular system, lung, skin, muscle and kidney.

In addition, tissues from 8 control horses of both sexes, different breeds (3 quarter horses, 1 Percheron, 1 Arab, 1 Tennessee walking, 1 pony and 1 Peruvian Paso) and ages and with no known medical problems (Table 2-3) were examined as well.

**Pathology of tendons and ligaments**

Normal tendons and ligaments have a uniform, grayish pink color and are pliable on palpation. In normal tendons and ligaments fascicles and bundles of collagen fibers are separated by thin endotenon septa that contain only a few small blood vessels in SDFTs and DDFTs, and in patellar and nuchal ligaments (Figure 2-1A). In addition, small islands of striatus muscle are interspersed with bundles of collagen fibers of the suspensory ligaments; a reminder of the evolutionary past when SL was more a muscle rather than a ligament (or tendon strictly speaking).

All SDFTs, DDFTs and SLs were found to be affected in all four extremities in 25 DSLD horses and the one extremity that was examined in the 3 remaining DSLD horses (no. 10, 14 and 18, Table 2-1). Usually but not always both proximal and midmetacarpal portions of the tendons and ligaments were affected. Grossly the affected tendons and suspensory ligaments felt firm and inflexible, and contained foci of white tissue. The histopathological findings consisted of deposits of acellular amorphous material staining blue with hematoxylin & eosin stain between collagen fibers, in septa and around blood vessels (Figure 2-1B). In some cases the septa were
also infiltrated with small blood vessels. In many tendons and ligaments the diffusely distributed proteoglycans gave the collagen a diffuse blue tinge. The extracellular matrix of affected septa and blood vessels stained for decorin, biglycan and aggrecan. Small foci of cartilage or calcifications were occasionally dispersed among collagen fibers. The cytoplasm of chondrocytes forming these foci stained strongly for biglycan and aggrecan, considerably less for decorin (Figure 2-2). No immunostaining was noted in the extracellular matrix of the cartilage.

In addition, rear suspensory ligaments in one DSLD horse (case no. 6, Table 2-2) revealed the presence of exuberant cellular areas consisting of fibroblasts in young connective tissue, with very little collagen present but with incipient PG accumulation (Figure 2-1D). Hypercellular septa due to proliferation of fibroblasts and increased number of blood vessels together with mild deposits of PGs were observed in a young Peruvian Paso (1.5 yr old male, case no. 5, Table 2-1). In one horse (no. 5, Table 2-2) actively proliferating synovium containing numerous blood vessels was observed in the midmetacarpal portion of the left rear suspensory ligament (data not shown). By light microscopy there was complete absence of inflammatory cells in all types of lesions, including the exuberant proliferating foci.

The histopathological changes in leg tendons and SLs were divided into mild, moderate and severe as indicated in Tables 2-1, 2-2. Because so many tendons were evaluated the grading represents an overall degree of severity of disease for each animal. It was based on the worst lesions present in several tendons and SLs in extremities from the same animal. Nine cases were quantified as mild (based on up to 10% of tissue affected by lesions), 8 as moderate (i.e., up to 30% of tissue affected by lesions) and 5 as severe (i.e., more than 30% of tissue affected by lesions). It is interesting that there was little correlation between the severity of the lesions and signalment or clinical history, such as pain or lameness (data not shown).
Similar histopathological changes, i.e., either focal deposits of PGs or areas of diffuse blue tinge, were present in all examined patellar (6 horses) and majority of nuchal ligaments (17/19 examined samples, Figure 2-3B) from DSLD horses. Figure 2-3D demonstrates that this material gave a strong reaction with alcian blue, indicating that it consists of proteoglycans (PGs).

Interestingly, histological signs of old tendon injuries were found in all examined control horses in at least one tendon (or suspensory ligament), and in all four extremities in 5 horses (Table 2-3). Unlike the DSLD tendons and suspensory ligaments, these tendons and SLs containing lesions, however, were pliable on palpation and no gross lesions were apparent. Histologically, the injury was characterized by the presence of fibrosis accompanied by focal accumulation of proteoglycan material most commonly in the midmetacarpal portion of the tendon or suspensory ligament (Figure 2-1C). The lesions were less extensive than the lesions in the DSLD tendons and were either mild or just consisted of small foci (Table 2-3). One horse, a 9 year old Peruvian Paso, exhibited histopathological changes in tendons of all 4 extremities, the nuchal ligament and other tissues consistent with DSLD (horse no. 7, Table 2-3).

Nuchal ligaments from four control horses revealed the presence of small foci of PGs. In general, the foci were much smaller than the PG deposits found in DSLD ligaments and were limited to only a small area of the nuchal ligament. Another control horse (no. 7, Table 2-3) showed more extensive changes in the nuchal ligament and other tissues consistent with diagnosis of DSLD (see above).

**Electron microscopic changes in DSLD-affected tendons**

Cross-sections from midmetacarpal portions of SDFTs were examined. The majority of collagen fibrils in normal tendon had a bimodal distribution, most fibrils having a fairly large diameter. Only a few fibrils with small diameter were observed (Figure 2-4A). Sections from
DSLD-affected tendon showed a marked increase in the number of mostly small fibrils dispersed among larger fibrils (Figure 2-4B).

**Sepharose CL-2B chromatography of tendon proteoglycans**

We performed an initial analysis of proteoglycan composition in the midmetacarpal portion of the SDFT. Because this analysis is time and labor intensive, it was done only on a limited number of samples. All Sepharose CL-2B chromatographs from one control (no. 1, Table 2-3) and two affected horses (no. 4 and 8, Table 2-1) showed separation into one main peak (Figure 2-5). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on fractions pooled from the main peak from the extract from DSLD tendons showed a distinct band at ~180 kDa (Figure 2-6, arrowhead). The presence of the band seen only in abnormal tendons was independent of treatment with chondroitinase ABC. Treatment with chondroitinase ABC led to the appearance of an 80 kDa band in the DSLD sample (Figure 2-6, arrow). Interestingly, the control sample revealed a band in the same location. Again the presence of the band was independent of treatment with chondroitinase ABC.

**Changes in other tissues and organs**

Focal deposits of proteoglycans were found in several other collagen containing tissues in DSLD affected horses, such as the aorta, pulmonary and coronary arteries and sclera. No tissues besides tendons and ligaments (see above) contained exuberant cellular connective tissue.

The walls of affected blood vessels showed irregular thickening of the intima and media due to the presence of proteoglycans (Figure 2-7B and 2-7F). This was accompanied by collagen and elastic fiber bundle disorganization that was due to separation of fibers by small pools of proteoglycans staining strongly with alcian blue (Figure 2-7D and 2-7H). Three examined heart valves from DSLD-affected horses were vascularized. One examined control heart valve was
Transmission electron microscopy revealed the presence of numerous small vacuoles in the cytoplasm of smooth muscle cells in media from DSLD aorta (Figure 2-8B). The cell membrane of these cells was thinner and discontinuous (Figure 2-8D). However, the collagen fibrils and elastic fibers exhibited similar features and appeared to be intact in normal and DSLD aortas Figure 2-8E and 2-8F).

Unilateral or bilateral scleral involvement was observed in 12/18 of DSLD horses where eyes were examined. The changes consisted of blue discoloration of collagen fibers (data not shown).

In 10 DSLD horses (out of 19 examined) varying in age from 1 to 21 years, and in 3 out of the 7 examined control horses minor lung lesions were present consisting of mild peribronchial, perivascular and septal fibrosis (data not shown). A small collection of mostly chronic inflammatory cells were observed in the peribronchial fibrous tissue of the older, but not of the younger horses (1, 1.5 and 3 years of age). In addition, the fibrosis was more prominent in these 3 young horses, particularly around small arteries. In one young horse (no. 2) small deposits of PGs were discernible in alveolar walls.

Kidney, skeletal muscle and skin appeared normal in all horses. Only one DSLD horse (no. 5, Table 2-2) was diagnosed as having chronic idiopathic granulomatous dermatitis, panniculitis and rhabdomyositis in addition to DSLD.

None of the control horses revealed systemic involvement except for a 9 year old Peruvian Pasofino mare (no.7, Table 2-3). The pathology in this horse was consistent with DSLD (see above).
DISCUSSION

Historically, the pathology associated with the clinical syndrome DSLD has been thought to be limited to the suspensory ligaments of the distal limb of horses. The findings of this study suggest that DSLD is in fact a systemic disorder involving many tissues and organs with a significant connective tissue component. Tissues with histological lesions in addition to the suspensory ligament documented in this study include deep and superficial digital flexor tendons, patellar ligaments, aorta, coronary arteries, nuchal ligaments, and ocular sclerae. In light of these observations, a more appropriate term for this disease process may be equine systemic proteoglycan accumulation (ESPA).

While specific collagen fibril abnormalities have been reported to be a consistent feature of DSLD [13], our observations suggest that abnormal accumulations of proteoglycan between collagen fibers, within the tendon matrix, and between elastic fibers in the blood vessels are the most consistent and prominent histological feature associated with DSLD affected horses rather than primary collagen fibril abnormalities.

The staining properties of material accumulated in connective tissues, the presence of cartilage metaplasia and the appearance of an 80 kDa band after chondroitinase ABC treatment in gel chromatography-separated tendon extracts indicate that DSLD is indeed due to deposits of a yet to be identified PG(s). The difference in immunostaining for PGs between septa and blood vessels on one hand and newly formed cartilage on the other (i.e., extracellular vs cytoplasmic pattern of staining) can be attributed to the fact that the cartilage foci were small and cellular, with only relatively small amounts of extracellular matrix. In other words the cartilage foci were young and thus in the process of formation of extracellular matrix. It is likely that the extracellular presence of PGs in the septa within the tendon indicates long standing lesions.
Because of technical difficulties we did not attempt to "uncover antigens" during the immunostaining procedure (that process leads to the tendon sections slipping off the slide), and it is possible that the immunostaining was incomplete.

We interpret the appearance of the 80 kDa band after chondroitinase ABC treatment as a result of digestion of GAGs attached to a PG(s) of higher Mr (likely around 100 kDa). It is likely that the carbohydrate component is chondroitin or dermatan sulfate [17]. Further studies are in progress to identify this and the 180 kDa band which appears only in the DSLD samples. The marked increase in small collagen fibrils in DSLD tendons is most likely secondary to qualitative changes in the synthesis of PG(s). PGs are involved in regulation of collagen fibrillogenesis, including the rate of formation and final sizes of fibrils [18]. For example, decorin, the most common PG in the tendon, limits collagen fibril growth, and, thereby, directs tendon remodeling in response to tensile forces. It has been shown that knock-out mice deficient in decorin have fragile connective tissues [19]. Similarly, the absence of biglycan and/or fibromodulin, two proteoglycans related to decorin, in knock-out mice prevented formation of mature collagen fibrils and led to ossification of tendon fibrocartilage [20]. Whether the accumulated proteoglycan(s) in DSLD is identical to any of these PGs remains to be determined.

The proliferative tendon lesions found in three horses with DSLD are significant as they represent in all likelihood early lesions which eventually progress to a less cellular stage characterized by increasing PG accumulation. Characteristically, no inflammatory or fibrotic changes accompanied these deposits or proliferative lesions. We hypothesize that the proliferating fibroblasts secrete PGs which then accumulate in tissues. The stimulus for the proliferation of fibroblasts and the subsequent production of proteoglycans is unknown. The
proliferation of fibroblasts and growth of the exuberant connective tissue may explain the presence of pain in the early stage of the disease.

Though flexor tendon microinjuries and subsequent histological lesions can be found in virtually all horses, the tendon lesions in horses afflicted with DSLD differed significantly from "control" horses. All 28 horses with DSLD had histopathological lesions in all SDFTs, DDFTs and SLs. It is of interest that in our study many control SDFTs and DDFTs had signs of old microinjuries consisting mostly of small foci of fibrosis, i.e., regular scar tissue and small deposits of PGs in the midmetacarpal (and proximal) region. This is different from other studies claiming the sparsity of injuries to DDFT in normal horses [21,22]. The lack of systemic involvement in control horses is also indicative that they did not have DSLD.

It is not clear whether the changes observed in the blood vessels and eyes progress over time and lead to clinical manifestations. Several horse breeders brought to our attention that they have encountered horses with a record of clinically diagnosed DSLD dying suddenly without a precipitating disease. Abnormalites in connective tissues components of the aorta or major vessels may predispose to rupture of aortic aneurysm and sudden death [23,24]. Further epidemiologic studies are necessary to determine the clinical significance of these findings.

DSLD is thought to run in families and bears some similarity to several hereditary diseases affecting connective and musculoskeletal tissues in people. The first is called Marfan's syndrome, which affects joints, the aorta, heart valves and eyes. Affected individuals are typically tall, have heart and eye problems and frequently die of a catastrophic rupture of the aorta. The genetic and biochemical defect of Marfan's syndrome is a mutation in the gene for fibrillin-1, an extracellular matrix protein involved in proper organization of fibrils [23]. Because fibrillin-1 regulated activation of transforming growth factor β is impaired in Marfan's patients
and fibrillin deficient mice, it is quite likely that accumulation of proteoglycans causing the prolapse of mitral valve, a prominent feature in Marfan's syndrome, is one of the consequences [24]. Whether secondary changes in regulation of growth factors and other mediators play a role in DSLD is not known at the present time. Elastic fragmentation and cystic changes can be found in Marfan's syndrome and Erdheims' cystic medionecrosis [25] (and also in DSLD). In addition the EM findings of small vacuoles in the cytoplasm of smooth muscle cells in the media from DSLD aorta and of thinner and discontinuous membrane of these cells appeared to be similar to ultrastructural changes observed in endothelial cells in aortas from patients with Marfan's syndrome and Erdheim's disease and are indicative of increased cell permeability [26].

The second human disorder or group of related disorders is Ehlers-Danlos syndrome. People with this disorder experience increased fragility of the skin and hyperflexibility of the joints. The skin may be very loose, forming folds, and prone to scarring. Because Ehlers-Danlos syndrome comprises a group of clinically similar diseases rather than one defined entity, its pathogenesis is more complicated. It is likely that different mutations lead to different forms of Ehlers-Danlos syndrome [27]. Mutation in the gene encoding type III collagen leads to the most severe form of Ehlers-Danlos syndrome, type IV [28]. Patients with this form often develop and die of rupture of large blood vessels, uterus or intestines [29,30].

The third, called Williams syndrome is characterized by distinctive facial features, mental retardation, and cardiovascular disease. Sudden death often in young people is associated with stenoses and other abnormalities of coronary arteries [31]. Other cardiovascular malformations include aortic stenosis and arch hypoplasia [32]. The underlying defect is a mutation in the gene encoding for elastin (ELN) [33]. The fourth entity, Caffey syndrome or infantile cortical hyperostosis is characterized by subperiosteal bone formation, some Ehlers-Danlos features (e.g.,
joint hyperlaxity and hyperextensible skin), but also by a localized inflammatory response. The symptoms are attributed to a mutation in the gene encoding the α1(I) chain of type I collagen (COL1A1) [34]. It is interesting to note that some DSLD horses or their offspring develop osteopetrosis (David Burrell, personal communication).

As far as we know none of these human hereditary disorders is known to be caused by a defect in proteoglycan biochemistry or genetics, thus making it different from DSLD. However, because all mutations occur in genes encoding for structural components of tendons and blood vessel walls (e.g., collagen, elastin, fibrillin) that contribute to biomechanical integrity of these tissues, the diseases share certain similarities in their presentation.

Current experiments in our laboratory are directed at the characterization and identification of the specific proteoglycans involved in DSLD. Further characterization and understanding of the pathogenesis of DSLD will allow us develop diagnostic tests to identify asymptomatic horses and help prevent the propagation of the syndrome through a selective breeding program.

**CONCLUSION**

The findings of this study demonstrate that so called degenerative suspensory ligament desmitis (DSLD) thought to be limited to suspensory apparatus is actually a systemic disorder affecting tissues with a high content of connective tissue, such as tendons and other ligaments, blood vessels and sclerae. Initial investigations suggest that accumulation of still to be identified proteoglycans between collagen and elastic fibers and around small blood vessels is the underlying pathology. Due to the systemic nature of the disease we feel that the term equine systemic proteoglycan accumulation or ESPA is more appropriate than DSLD.

**METHODS**
Cases and tissue collection

All horses were donated for research to the College of Veterinary Medicine, The University of Georgia, Athens, GA. All procedures were approved by the Animal Care and Use Committee, University of Georgia, IACUC# A2001-10120). Twenty-eight horses clinically diagnosed with DSLD (22 Peruvian Paso horses and 6 horses of other breeds) were compared to 8 horses donated for reasons other than lameness (control group) using light and electron microscopy, and gel chromatography. Postmortem examination was conducted immediately following euthanasia and tissues of interest were either processed for histopathological and electron microscopy studies, or frozen at -20°C until used for biochemical analysis. Superficial and deep digital flexor tendons and suspensory ligaments were removed proximally at the level of the proximal third metacarpus (= proximal portion) to the level of the metacarpophalangeal joint above the fetlock (or above the bifurcation of the SL, labeled by us as the midmetacarpal portion). One section from the proximal end and another section from the midmetacarpal end were taken for histological examination. In many horses the following tissues were examined for histopathology: eyes, proximal portion of the right coronary artery, arch and/or thoracic aorta, nuchal and patellar ligaments, lung, skin, muscle and kidney.

Histopathology and immunohistochemistry

Five µm thick sections from formalin fixed, paraffin embedded tissues were stained with hematoxylin & eosin, and with alcian blue (pH 2.5 and pH 1.0).

The core proteins of decorin and biglycan were identified with rabbit polyclonal antibodies LF-30 or LF-136 to decorin and LF-106 to biglycan (generous gift from Dr. Larry Fisher, NIDCR, NIH [35]. The antibody to the core protein of human aggrecan was a generous gift from Dr. Peter Roughley [36]. We used an immunohistochemistry protocol used by us
previously [37]. Briefly, after deparaffinization and rehydration in descending grades of ethyl alcohol, tissue sections were quenched in 0.3% H$_2$O$_2$ in methanol and non-specific sites were blocked with normal goat serum (Vector laboratories Inc., USA) for 30 minutes. Slides were incubated at 4°C overnight with primary antibodies (1:500 in PBS) to decorin, aggrecan or biglycan. Next day, slides were treated with biotinylated anti-rabbit IgG (H+L) secondary antibody, made in goat (Vector Laboratories Inc.) at dilution 1:1000 in PBS for one hour. Antigen-antibody complexes were detected using an avidin-biotin complex detection system (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA). Slides were stained with DAB Substrate kit (Vector laboratories), rinsed in water and briefly counter stained with hematoxylin and washed in water.

**Electron microscopy**

Samples of midmetacarpal regions of SDFTs from a DLSD-affected (horse no. 8, Table 2-1) and a control (horse no. 1, Table 2-3), and upper thoracic aorta from a control horse (no. 1, Table 2-3) and thoracic aorta of two DSLD-affected horse (case no. 9, 11, Table 2-1) were fixed at 4°C in 2% glutaraldehyde/2% (para)formaldehyde/0.2% picric acid/0.1 M cacodylate buffer, pH 7.2. The fixed tissue was rinsed at 4°C in 0.1 M cacodylate buffer/300 mM sucrose, pH 7.2. Secondary fixation was performed for 1 hr at 4°C in 1% OsO$_4$/0.1 M cacodylate buffer, pH 7.2. After dehydration in solutions of increasing ethanol concentration the fixed aortic tissues were infiltrated with propylene oxide and propylene oxide/Epon 812 mixture, and embedded in Epon 812. Sections were positioned on 400 mesh grid, stained with uranyl acetate and lead citrate, and examined using JEOL 100CXII Transmission Electron Microscope.

The dehydrated fixed tendon tissues were infiltrated with acetone/Spurr resin mixtures (50% Spurr for 1 hr, 50 % Spurr overnight, and 100% Spurr twice for 1 hr. The tendon blocks
were polymerized for 1 day at 60°C and were sectioned with glass knives on a Leica Reichert Ultramicrotome. Sections were positioned on 400 mesh grids, stained with uranyl acetate and lead citrate, and examined using JEOL JEM-1210 Transmission Electron Microscope.

**Proteoglycan extraction**

Midmetacarpal portions of SDFTs from 2 affected horses (no. 4 and 8, Table 2-1) and from one control horse (no. 1, Table 2-3) were dissected, minced, defatted with a chloroform: methanol solution (1:1, 6 ml/1.0 g of dry weight sample), and dried using a Speedvac (SVC 100 H; Thermo Savant, Holbrook, NY). The dry tissue was homogenized and extracted twice with 10 volumes of 4 M guanidine HCl extraction buffer for 24 h on a rotator at 4°C as described by us previously [18]. The combined extracts were dialyzed against deionized water three times for 24 h at 4°C.

**Sepharose CL-2B chromatography**

Dialyzed extracts were lyophilized and dissolved in 3 ml of 4 M guanidine HCl. Two ml of dissolved sample (at protein concentrations 4.4 mg/ml for horse no. 8 and 5.75 mg/ml for the control horse) were separated on a molecular sieve Sepharose CL-2B column (1.25 × 110 cm, equilibrated and eluted in 4 M guanidine HCl) at 0.1 ml/min [18]. The Vt or total volume of the column was 146 ml and the Vo or void volume was 52 ml as determined by Dextran blue elution.

**SDS-PAGE**

Aliquots (30 µg) of Sepharose CL-2B pools were precipitated for 2 hrs at -80°C with 8 volumes of 100% ethanol. The precipitates was dissolved in 15 µl of 100 mM Tris buffer, adjusted to pH 8.0 with concentrated acetic acid and digested with 20 mU of chondroitinase ABC (Proteus vulgaris, Sigma, St. Louis, MO) at 37°C for 24 h. Chondroitinase ABC digested
samples and non-digested replicate aliquots were separated on 10% SDS-polyacrylamide gels. The gels were stained with silver nitrate [38].

Authors' contributions

JH conceived and designed the study, supervised and evaluated all experimental aspects, examined all histological slides and wrote the final version manuscript. BK was involved in post-mortem examination, immunohistochemistry and proteoglycan analysis. AK was involved in post-mortem examination, immunohistochemistry and in writing of the manuscript. JHY participated in proteoglycan analysis. POEM was involved in selection of horses, and in clinical evaluation and diagnosis. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by a grant from The University of Georgia Research Foundation, David and Laura Burrell, Peruvian Paso Horse Registry of North America, and DSLD Foundation, Inc. Dr. Ahrar Khan was supported by the Ministry of Science and Technology, Government of Pakistan. We would like to thank Dr. K.P. Carmichael for helpful discussions, Mary Ard, John Bryan and Dr. Renato Sousa for technical assistance, and Drs. Larry Fisher and Peter Roughley for their generous gifts of antibodies to decorin, biglycan and aggrecan.

REFERENCES


16. Gibson KT, Steel CM: **Conditions of the suspensory ligament causing lameness in horses.** *Equine Vet Ed* 2002, **4**:50-64.


## FIGURES AND TABLES

**Table 2-1.** Peruvian Paso horses affected with DSLD

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<td>sire</td>
<td>4 mod</td>
<td>ND</td>
<td>cor +</td>
<td>ND</td>
<td>+</td>
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Age: age at necropsy; ND: not done; legs: SDFT, DDFT and SL; cor: coronary artery; PA: pulmonary artery; nuchal: nuchal ligament

Mild, mod (moderate), severe: describes the average severity of pathology in examined leg tendons and ligaments; +: accumulation of proteoglycans in other tissues; -: no proteoglycan accumulation

*only limited tissue was available for examination
Table 2-2. DSILD-affected horses of other breeds

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<td>Cor</td>
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<td>+</td>
<td>both+</td>
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Age: age at necropsy; ND: not done; legs: SDFT, DDFT and SL; cor: coronary artery; PA: pulmonary artery; nuchal: nuchal ligament; Han: Hanovarian; Appal: Appaloosa; thoro: thoroughbred

Mild, mod (moderate), severe: describes the average severity of pathology in examined leg tendons and ligaments; +: accumulation of proteoglycans in other tissues; -: no proteoglycan accumulation
### Table 2-3. Control horses

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<th>hear t</th>
<th>PA</th>
<th>aort a</th>
<th>eye s</th>
<th>other tissues</th>
<th>ultrasound</th>
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<tr>
<td>1</td>
<td>young, F, quarter</td>
<td>NA</td>
<td>healthy</td>
<td>no</td>
<td>2 fibr. tendons, mild</td>
<td>ND</td>
<td>-</td>
<td>N</td>
<td>D</td>
<td>-</td>
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<td>2</td>
<td>5mo, M, Percher on</td>
<td>NA</td>
<td>healthy</td>
<td>no</td>
<td>foci in 4 legs</td>
<td>ND</td>
<td>-</td>
<td>N</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td>nuchal +</td>
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<tr>
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<td>6y, F, quarter</td>
<td>NA</td>
<td>Cushing</td>
<td>no</td>
<td>fibrosis in four, mild</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td>lung +</td>
</tr>
<tr>
<td>4</td>
<td>8y, F, quarter</td>
<td>NA</td>
<td>healthy</td>
<td>no</td>
<td>foci in 4 legs</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td>ND</td>
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<td>slight fever</td>
<td>no</td>
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<td>-</td>
<td>-</td>
<td>N</td>
<td>D</td>
<td>-</td>
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<td>no</td>
<td>foci in 4 legs</td>
<td>-</td>
<td>-</td>
<td>N</td>
<td>D</td>
<td>-</td>
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<td>healthy</td>
<td>no</td>
<td>four, mild</td>
<td>+</td>
<td>cor +</td>
<td>N</td>
<td>D</td>
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<td>foci in 4 legs</td>
<td>-</td>
<td>cor +</td>
<td>N</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td>nuchal, lung +</td>
</tr>
</tbody>
</table>

Age: age at necropsy; ND: not done; legs: SDFT, DDFT and SL; cor: coronary artery; PA: pulmonary artery; nuchal: nuchal ligament

Mild, mod (moderate), severe: describes the average severity of pathology in examined leg tendons and ligaments; +: accumulation of proteoglycans in other tissues; -: no proteoglycan accumulation
**Figure 2-1. Comparison of normal and DSLD-affected tendons.** A. Only thin septa (*) separate bundles of collagen and elastic fibers in a normal tendon. Hematoxylin & eosin, magnification × 200. B. A section of DSLD-affected tendon reveals PG deposits (*) between collagen fibers and in septa. Hematoxylin & eosin, magnification × 200. C. A section of tendon from a horse without DSLD shows the presence of fibrosis or scar tissue (*) between collagen fibers and in septa. Hematoxylin & eosin, magnification × 200. D. A proliferative lesion found in one DSLD case consists of swirls of active fibroblasts in young, well vascularized tissue. Hematoxylin & eosin, magnification × 200.
**Figure 2-2. Immunostaining of metaplastic cartilage.** A. Immunostaining reveals very little decorin, magnification × 500. B. The cartilage reveals the presence of aggrecan (▶) in the cytoplasm, magnification × 400. C. The same chondrocytes were also positive for biglycan (▶), magnification × 400. Countestain: hematoxylin.
Figure 2-3. Histopathological changes in nuchal ligament. A. Only thin septa separate bundles of collagen and elastic fibers in a normal nuchal ligament. Hematoxylin & eosin, magnification × 200 (× 200). B. In DSLD – affected tissue streaks of proteoglycans (▶) separate bundles of collagen and elastic fibers. Hematoxylin & eosin, magnification × 200. C. Alcian blue stains very lightly normal nuchal ligament, magnification × 200. D. PGs accumulated among bundles stain intensively with alcian blue (▶) in DSLD-affected nuchal ligament.
**Figure 2-4.** Electron micrographs of normal and DSLD-affected tendon. A. A cross-section of normal tendon reveals that most collagen fibrils have fairly large diameters. B. A marked increase in small collagen fibrils was observed in cross-sections of DSLD-affected tendon.
Figure 2-5. Sepharose CL-2B chromatography of PGs in extracts from normal and DSLD-affected tendons. Guanidium HCl extracts from midmetacarpal portions of SDFTs from 2 affected horses (no. 20 and 21, Table 2-1) and from one control horse (no. 1, Table 2-3) were separated on a molecular sieve Sepharose CL-2B column (1.3 × 110 cm, equilibrated and eluted in 4 M guanidine HCl) at 0.1 ml/min. The majority of PGs eluted in one wide peak (fractions 66–81).
Figure 2-6. SDS-PAGE of Sepharose CL-2B peaks. Aliquots of pooled Sepharose CL-2B fractions were precipitated with 100% ethanol, dissolved in 15 µl of 100 mM Tris buffer, adjusted to pH 8.0 with concentrated acetic acid and digested with 20 mU of chondroitinase ABC at 37°C for 24 h. Chondroitinase ABC digested samples and non-digested replicate aliquots were separated on silver stained 10% SDS-polyacrylamide gels. Lane 1: untreated sample from control SDFT no. 1 (Table 2-3); lane 2: untreated sample from DSLD SDFT no.8 (Table 2-1); lane 3: chondroitinase ABC treated sample from control SDFT no. 1 (Table 2-3); lane 4: chondroitinase ABC treated sample from SDFT no. 8 (Table 2-1).
**Figure 2-7. Histopathological changes in arteries.**

Figure 2-8. Ultrastructural features of normal and DSLD-affected aorta. A. Normal smooth muscle cell from media from a healthy aorta. B. A smooth muscle cell from the media of DSLD-affected aorta reveals the presence of numerous cytoplasmic vacuoles (*). C. normal smooth muscle cell has a well defined cell membrane (▶). D. The cell membrane of a diseased media is disrupted and missing in places (▶). E and F. Organization of collagen fibrils and elastic fibers is similar in normal (E) and DSLD aorta (F). SMC: smooth muscle cell, EL: elastic lamina, C: collagen fibrils.
CHAPTER 3
PURIFICATION AND CHARACTERIZATION OF PROTEOGLYCANS
ALTERED IN EQUINE SYSTEMIC PROTEOGLYCAN ACCUMULATION

ABSTRACT

Equine Systemic Proteoglycan Accumulation (ESPA) was known for a long time as degenerative suspensory ligament desmitis (DSLD), a debilitating disorder thought to be limited to suspensory ligaments in horses. However, we have shown that DSLD is a systemic disorder characterized by accumulation of proteoglycans in many tissues and organs with high content of collagen in affected horses, and renamed it as ESPA. Proteoglycans, and in particular, small leucine rich proteoglycans (SLRP) such as decorin, biglycan and fibromodulin, are involved in regulation of collagen synthesis and cytokine activity by binding directly. Of the latter ones, transforming growth factor β1 (TGFβ1) is a potent inducer of collagen synthesis. Although it plays an important role in modulating SLRPs synthesis, its activity is inhibited by SLRPs. The purpose of this study was to identify mechanism of this disease with special attention given to SLRPs, decorin and fibromodulin.

Superficial digital flexor tendons from control and ESPA-affected horses were used for proteoglycan analysis. After extraction with 4M-guanidine buffer and purification by CL-2B sepharose gel filtration, analysis of overexpressed bands in SDS-polyacrylamide gel was performed by nano-LC tandem mass spectrometry and Western blotting. A high molecular protein band (~140kDa) was found to be overexpressed in affected, but not in control tendons, and was identified as decorin by both nano-LC tandem mass spectrometry and Western blotting. Two-dimensional gel electrophoresis showed this band located in the highest pH. After purification on a Q-sepharose ion exchange column, analysis of purified proteoglycans was performed by western blotting and high pressure liquid chromatography (HPLC). Decorin purified from ESPA-affected tendons was eluted at 0.7M NaCl on Q-sepharose column, and was shown to be of a higher molecular weight than normal decorin as demonstrated by western
blotting. The glycosaminoglycan chain was shown to have more chondroitin 6-sulfate than chondroitin 4-sulfate compared to controls when analyzed by reverse phase HPLC. The content of higher molecular fibromodulin was also increased in ESPA-affected tendons. Its keratan sulfate chain showed 6-sulfated pattern when it was digested by keratanase II but not by keratanase. Gas chromatography mass spectrometry (GC/MS) indicated that glucuronic acid was increased in dermatan sulfate chains of purified decorin from ESPA-affected tendons whereas iduronic acid was decreased. Binding assay with ELISA showed that purified decorin from ESPA-affected tendon had lower affinity to TGFβ1 than control suggesting that this might be a factor to cause the overexpression of TGFβ1 observed in tissue by in situ hybridization.

Our results indicate that decorin and fibromodulin from ESPA affected tendon undergo changes in glycosylation of their glycosaminoglycans chains. In addition decorin purified from ESPA-affected tendon had low affinity to TGFβ1 which we hypothesize likely led to overexpression of this growth factor in tissues. Such changes in expression of TGFβ1 would have direct effect on collagen synthesis and proteoglycan activity, and would thus become an active participant in the pathogenesis of ESPA.

INTRODUCTION

Equine Systemic Proteoglycan Accumulation (ESPA) was known for long time as degenerative suspensory ligament desmitis (DSLD), a debilitating disorder thought to be limited to suspensory ligaments in horses. Many diverse breeds of horses are afflicted with ESPA. ESPA is largely an incurable disease, leading to euthanasia of many afflicted animals (Mero et.al. 2002). This disorder is characterized by pain, lameness, thickening of the suspensory ligaments and, in some cases, a dropping of the fetlocks towards the ground. Recently, we have shown that DSLD
is a systemic disorder characterized by accumulation of proteoglycans in many tissues and organs with high content of collagen in affected horses, and renamed it as ESPA (Halper et al. 2006).

The extracellular matrix (ECM) of tendons and ligaments consist of collagen and noncollagenous matrix (e.g., proteoglycans and glycoproteins). Binding of proteoglycans, in particular, of small leucine rich proteoglycans (SLRP) such as decorin and fibromodulin to collagen fibrils plays a major role in regulation of collagen fibrillogenesis (Kresse et al. 2001). Binding between proteoglycans and collagen fibrils contributes to the biomechanical strength of tendons and ligaments. The basic structure of all proteoglycans includes a protein core and at least one, but frequently more (up to tens or hundreds) carbohydrate chains, so called glycosaminoglycans (GAGs). SLRPs play an important role in stabilizing collagen fibrils by protecting them from access to collagenases (Geng et al. 2006). Furthermore SLRPs bind to growth factor or growth factor receptors. They can modify TGFβ bio-availability and modulate EGFR or IGFR signaling (Droguett et al. 2006). However, the precise role of these small proteoglycans in modulating the biomechanical properties of tendons and ligaments remains poorly defined.

In the ECM of ligaments and tendons, many cytokines are involved in synthesis of collagens and other ECM components through fibroblast activation. Especially, members of TGFβ superfamily stimulated the production of proteoglycans in human lung fibroblast culture (Tiedemann et al. 1997) and regulated glycosylation of proteoglycans. The glycosaminoglycan (GAG) structures of decorin, biglycan, and versican, a member of large PGs, are regulated in part by TGFβ1 (Tiedemann et al. 2005). On the other hand, decorin, one of the SLRPs, inhibits the overproduction of TGFβ1 in glomerulonephritis model (Border et al. 1992) and reduces the
binding of TGFβ1 to its receptors in skeletal muscle cell culture (Droquett et.al. 2006). In the hypertrophic scar fibroblasts culture, recombinant human decorin inhibits cell proliferation and reduces TGFβ1 production (Zhang et.al. 2007).

Glycosaminoglycan chain of proteoglycan consists of repeated disaccharide which can be sulfated at different position. Dermatan sulfate of decorin has repeated disaccharide of hexuronic acid and N-acetylglactosamine (GalNAc), majorly sulfated at 4-position or 6-position of GalNAc or at 2-position of iduronic acid (IdoA), an epimer of glucuronic acid (GlcA) (Trowbridge et.al. 2002) while keratan sulfate of fibromodulin contains repeated disaccharide of galactose (Gal) and N-acetylglucosamine (GlcNAc), sulfated at 6-position of Gal and/or GlcNAc (Funderburgh et.al. 2000). In dermatan sulfate, the presence of IdoA instead of GlcA inhibits the proliferation of normal fibroblast (Westergren-Thorsson et.al. 1991) and affects the interaction between SLRPs and TGFβs (Hildebrand et.al 1994).

In degenerated equine superficial digital flexor tendon, total amount of sulfated GAGs was found to be significantly increased in central region relative to peripheral region and normal tendon (Birch et.al. 1998). In human patellar tendinopathy, monosulfated disaccharides including ∆Di-4S and ∆Di-6S were increased (Fu et.al. 2007). Besides tissue repair, similar proteoglycan or GAG changes can be identified in genetic diseases. In some patients with Ehlers-Danlos syndrome which is characterized as a defect in collagen synthesis, a substitution of one amino acid in galactosyltransferase I results in longer GAG chain, less iduronic acid, and more core protein of decorin (Seidler et.al. 2006). Although the longer GAG chain of decorin is common in wound healing and genetic diseases, the shortening of dermatan sulfate of decorin in later healing stages does not appear to have a counterpart in genetic diseases (Kuwaba et.al. 2002).
Our initial study has shown that degenerative suspensory ligament desmitis (DSLD) is a systemic disorder. The main characteristics is excessive accumulation of proteoglycans in organs with high content of connective tissue, such as suspensory ligaments, superficial and deep digital flexor tendons, patellar and nuchal ligaments, cardiovascular system, and sclera. We have proposed that equine systemic proteoglycan accumulation or ESPA rather than DSLD is a more appropriate name for this condition (Halper et. al. 2006). Our hypothesis was supported by results obtained from preliminary gel chromatography revealing the presence of overexpressed proteoglycan(s) in extracts from ESPA affected-tissues, but not from control extracts. Because the diameter of collagen in ESPA affected tendon was also markedly changed, we hypothesized that these overexpressed proteoglycans consist of small leucine rich proteoglycans (SLRPs) which bind to collagen and regulate fibrillogenesis (Zhang et. al. 2006 and Geng et. al. 2006). Our biochemical analysis of the accumulated proteoglycans showed, indeed, that decorin comprised a large proportion of these proteoglycans. In addition, this decorin underwent faulty glycosylation of its glycosaminoglycans chains.

The purpose of this study was to identify qualitative and quantitative changes in decorin and fibromodulin structure and to present mechanism of this disease.

**EXPERIMENTAL PROCEDURES**

*Tissue Collection* – All horses were donated to the College of Veterinary Medicine, The University of Georgia, Athens, GA. Procedures and care were approved by the Animal Care and Use Committee, University of Georgia, IACUC# A2001-10120). Five horses clinically diagnosed with DSLD (4 Peruvian Paso horses and 1 Appaloosa horse) were compared to 2 horses donated for reasons other than lameness (control group). Postmortem examination was
conducted immediately following euthanasia and superficial digital flexor tendons were removed proximally at the level of the proximal third metacarpus (= proximal portion) to the level of the metacarpophalangeal joint above the fetlock (or above the bifurcation of the SL, labeled by us as the midmetacarpal portion). Tissues were either processed for histopathological studies, or frozen at -20°C until used for biochemical analysis.

**Proteoglycan Extraction** – Midmetacarpal portions of SDFTs from 5 affected horses and from one control horse were dissected, chopped into less 1mm² size., defatted with a chloroform:methanol solution (1:1, 6 ml/1.0 g of dry weight sample), and dried using a Speedvac (SVC 100 H; Thermo Savant, Holbrook, NY). The dry tissue was extracted twice with 10 volumes of 4 M guanidine HCl extraction buffer (4M guanidine HCl, 0.05M sodium acetate, 0.5% CHAPS, 0.05M EDTA, 5mM benzanidine, 5mM iodoacetamide, 1mM PMSF, and 0.1mg/ml pepstatin A) for 24 h on a rotator at 4°C (Yoon et. al. 2003). The combined extracts were dialyzed against deionized water three times for 24 h at 4°C.

**Purification of Proteoglycans** – Dialyzed extracts were lyophilized and dissolved in 2ml of 4 M guanidine buffer (4M guanidine HCl and 0.05M sodium acetate), pH 6.0. Three quarter of dissolved sample was applied on a molecular sieve Sepharose CL-2B column (1.3 x 110 cm) preequilibrated with 4M guanidine buffer (Yanagishita, 2001). Extracts were separated at 0.1 ml/min of flow rate and collected as 2ml/tube by fraction collector (Gilson FC203B). Protein concentration of every fraction was measured by coomassie blue dye with BioRad protein assay kit. The main peak was pooled, dialyzed against deionized water, and lyophilized for the next step of separation. Lyophilized samples were dissolved again in 8ml of 4M guanidine buffer. One mg aliquots of pooled samples were concentrated by centrifugal Microcon (Millipore YM-10, cut off 10k) for buffer changing to 7M urea buffer (7M urea and 0.05M sodium acetate), pH
6.0 and concentrating samples to 200µl. Concentrated samples were mixed with 2ml of Q-
Sepharose anion exchange column preequilibrated with 7M urea buffer and were stirred gently
for 2h at 4°C. Mixtures were loaded on the column (1.3 x 15cm) and washed with 10ml of 7M
urea buffer. Bound proteoglycans were sequentially eluted with 10ml of 7M urea buffer
containing 0.1M NaCl, 0.3M NaCl, 0.5M NaCl, and 0.7M NaCl by gravity (Yanagishita, 2001).

Enzyme Treatment – Five hundred µl aliquots of 0.7M Q-Sepharose fractions were mixed
with 4 ml ethanol (absolute, anhydrous) to precipitate protein. After incubation at -80°C freezer
for several hours, the sample was centrifuged for 10 min at 3,400 rpm. Supernatant was
discarded and pellet was dried at 37°C. The buffered samples were incubated with
chondroitinase ABC (Chase ABC) (Sigma-Aldrich Co., 0.5U/ml; in 100mM Tris buffer, pH 8.0),
keratanase (Seikagaku Co., Japan, 0.1U/ml; 50mM sodium phosphate buffer pH 7.4), or
keratanase II (Seikagaku Co., Japan, 0.1U/ml; 10mM sodium acetate buffer pH 6.0) at 37°C
overnight for each enzyme (Roughley et. al., 1996).

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) / Western
Blotting – For 1D gel electrophoresis, ethanol precipitated undigested samples dissolved in
100mM Tris buffer or enzyme-treated samples (see above) were mixed with 2x sample buffer
and loaded in wells of 10% SDS–polyacrylamide gels with 4% SDS-polyacrylamide stacking gel.
Samples were separated for 40min at 50V and then 1hr 40min at 100V in the buffer system of
Laemmli (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R-250 or silver
nitrate. For 2D gel electrophoresis, samples were resolubilized in 7M urea, 2M thiourea, 2%
CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 2% ASB-14 (3-
[dimethyl-3-(tetradecanoylamino)propyl]azaniumyl]propane-1-sulfonate), 0.5% 3-10
immobilized pH gradient (IPG) buffer, and 18.2mM dithiothreitol (DTT). Proteins were
separated on 7cm pH 3-10 IPG strips for 22,000 Vhr in the IEF (isoelectric focusing) cell (BioRad Laboratories) using active rehydration at 50V. After isoelectric focusing, IPG strips were equilibrated in 6M urea, 2% SDS, 65mM DTT, 30% glycerol, 50mM Tris, pH 8.8, and 0.002% bromphenol blue for 15 min at RT, and then equilibrated with the above buffer, replacing DTT with 135mM iodoacetamide for 15 min at RT. Proteins were then separated on Criterion 10% SDS polyacrylamide gels (BioRad Laboratories) at 150V for 90 min. Gels were stained with Coomassie Blue G-250. Two-D gel electrophoresis was done at Proteomics Resource Facility, UGA. For western blot, 1D and 2D gels were transferred to nitrocellulose membranes for 1 hr in transfer buffer (25mM tris, 192mM glycine, 0.1% SDS and 20% methanol, pH8.3). Non-specific binding sites on the membranes were blocked in 5% non-fat dry milk for 1 hr at RT for blocking, blotted with primary Ab (1:1000) to decorin (polyclonal rabbit LF122 antibody to human recombinant decorin from NIH) and fibromodulin (polyclonal rabbit LF149 antibody to mouse fibromodulin from NIH) at 4°C overnight and secondary Ab (biotinylated anti-rabbit IgG, 1:1000, Vectors Laboratories) for 1 hr at RT. After incubating with horse-radish peroxidase for 30 min at RT, antibody-antigen complexes were detected with a DAB kit (Vector Laboratories) (Yoon et. al. 2003).

**High Performance Liquid Chromatography (HPLC)** – The ethanol precipitated samples of Q-Sepharose 0.7M NaCl fractions were dissolved with 95 µl of 50mM sodium phosphate buffer (pH 7.4) and digested with 5 µl ChaseABC (10 U/mL) for 12 h at 37°C. The ChaseABC-treated samples were transferred onto a centrifugal filter (cut off 3K, Millipore Co.) and centrifuged at 10,000 rpm for 10 min. Thirty µl of clear filtrate were injected onto the Nova-Pak C-18 analytical column (150mm×3.9mm internal diameter, 4µm particle size; Waters Co.) attached to Beckman System Gold® 126 Solvent Module. An isocratic elution program was run.
at a flow rate of 0.7 ml/min with 92% of eluent A (0.4mM tetrabutylammonium hydrogen sulfate and 1mM sodium chloride) and 8% eluent B (acetonitrile) for 60 min. The eluate was monitored by a Beckman System Gold® 166 Detector at 230 nm. The data was acquired and processed with 32 Karat Software, Beckman (Upreti et.al. 2006).

**Nano-LC Tandem Mass Spectrometry (MS)** – After SDS-PAGE, excised gel was chopped and washed with deionized water in eppendorf tube. Coomassie blue was removed by washing with methanol and acetonitrile. Proteins in the dried gel were digested by incubating with trypsin (1:50) for 30min in the refrigerator and at 37°C for 16hrs and eluted by washing with 50mM ABC and 50% ACN/0.1%TFA. Pooled supernatant was dried completely (Rosenfeld et. al. 1992). The Nano-LC tandem MS was carried out by Dr. Jung Hae Yoon at Columbus Children's Research Institute, OH. USA. It was performed on an LC-Packing system connected to a Linear Ion Trap (LTQ) mass spectrometer (Thermo Electron Corporation). Each sample was injected via an autosampler (Agilent 1100 HPLC) onto a C18 reversed-phase column (100µm x 12cm) and separated at a flow rate of 200nl/min for 150 min mobile phase of water with 0.01% of formic acid (A) and 80% acetonitrile (B) using a linear gradient. Protein sequence search was performed with the BioWorks 3.3 software (Thermo Electron Corporation, San Jose, CA). Each file was searched against the human NCBInr and human SwissProt databases using Sequest search engine. The acceptance criteria were DeltaCn (ΔCn) > 0.1, a variable threshold of Xcorr vs charge state (Xcorr = 1.9 for z = 1, Xcorr = 2.5 for z = 2, Xcorr = 3.8 for z = 3) and a peptide probability.

**Gas Chromatography/Mass Spectrometry (GC/MS)** – This spectrometry was performed at Complex Carbohydrate Research Center, University of Georgia, GA. USA. Twenty five µg or 50 µg of 0.7M NaCl fraction from Q-sepharose chromatography was dialyzed with deionized
water for 24hrs and added to separate tubes with 5µg of Inositol as the internal standard. After freeze-drying, the samples were hydrolyzed by 3N methanolic HCl at 100°C for 2 hrs, followed by N-acetylation with pyridine and acetic anhydride in methanol. The hydrolysis and acetylation were repeated two more times. The samples were then per-O-trimethylsilylated by with Tri-Sil (Pierce) at 80°C (0.5 hours). GC/MS analysis of the TMS methyl glycosides was performed on an AT 6890N GC interfaced to a 5975B MSD, using a Supelco EC-1 fused silica capillary column (30m × 0.25 mm ID) (Merkle et.al. 1994).

**Enzyme Linked ImmunoSorbent Assay (ELISA)** – Each well of ELISA plates (CovaLink™ NH modules, Nunc) was coated with 100ng/100µl coating buffer (0.1M sodium bicarbonate buffer, pH9.6) of Q-Sepharose-purified decorin (for the standard binding curve, 0, 0.064, 0.32, 1.6, 8, 40, 200, 1000ng decorin/100ul coating buffer were coated in each experiment). After coating for 24hrs at 4°C, non-specific binding sites on the membranes were blocked in 200µl 1x power block solution (BioGenex) for 1½hr at RT. Polyclonal decorin antibody (1:200, 100ul) was added and incubated with coated decorin at 4°C overnight. In another set of experiments (TGFβ1 binding assay) 10ng of TGFβ1 (recombinant human, R&D Systems) was incubated in decorin coated wells at 4°C overnight, and then TGFβ1 antibody (rabbit polyclonal antibody against C-terminus of TGFβ1 of human origin, Santa Cruz Biotechnology Inc.) was applied for 1½hr at RT. After primary antibody binding, biotinylated secondary antibody (1:200,100µl, Vectors Laboratories) was incubated for 1hr at RT. After incubating with alkaline phosphatase for 30min at RT, antibody-antigen complexes were detected with p-nitrophenyl phosphate solution (Vectors Laboratories). After incubation for 30min at 37°C, color development was read at 405nm in spectrophotometer (Ultraspec 3000, Pharmacia Biotech.) (Engvall et.al. 1971).
**Immunohistochemistry** – Deparafinized histology slides were dried and quenched with 3% H$_2$O$_2$ for 10min at RT. Slides were microwaved in antigen-retrieval unmasking solution (Vector Laboratories, Inc.) for 10min and were blocked with 1x power block solution (BioGenex) for 7 min at RT. After washing with PBS slides were incubated with primary Ab (1:250, 4°C, overnight) and then with biotinylated secondary Ab (1:250, RT, 1hr). After incubating with horse-radish peroxidase for 30min at RT, antibody-antigen complexes were detected with a DAB kit (Vectors Laboratories). Antibodies used were same as those used for western blot (Halper et.al. 2006).

**In Situ Hybridization** – The slides were heated at 70°C for 10min and deparafinized. Tissue sections were allowed to dry and then rehydrated with 5mM MgCl$_2$ in PBS for 10min at RT. Slides were incubated in proteinase k (100ug/ml, 2mM CaCl$_2$ in 10mM Tris pH7.5) for 15min at 37°C and the reaction was stopped with 0.1M glycine in 0.2M Tris, pH 7.5. Prehybridization solution was added to sections for 1h (42°C). This followed by the addition of the probe and kept in a humid chamber overnight (42°C). The next day, after several washes and blocking with 10% non-fat dry milk for 15min at RT, the slides were incubated with anti-dig alkaline phosphatase in 2% normal serum in buffer 1 (150mM NaCl in 100mM Tris pH7.5) for 2hrs at 37°C. After washing, substrate (NBT/BCIP) in buffer 3 (100mM NaCl and 50mM MgCl$_2$ in 100mM Tris-HCl pH 9.5) was used for color detection (Jin et.al. 1997). The riboprobe used for in situ hybridization was based on the sequence of the equine TGFβ1 gene obtained from Genbank (AF175709). A pair of primers was designed to create a 204 bp long probe:

- TGFβ1-Sense: 5’---CAG CAA TAA TTC CTG GCG CTA C---3’
- TGFβ1-Antisense: 5’---CTG GAA CTG AAC CCG TTG ATG C---3’
The mRNA was extracted from snap frozen horse liver and/or skin with Qiagen RNeasy Midi Kit. The first strand cDNA was synthesized using Superscript III kit (Invitrogen) followed by RT-PCR. After purification from agarose gels the PCR products were ligated into the TA-vector (pGEM-Teasy, Promega). Ligation products were introduced into *E.coli* by heat-shock. Positive colonies were confirmed by DNA sequencing performed by The Sequencing and Synthesis Facility at University of Georgia. A confirmed positive colony (it is in a reversed direction in pGEM-Teasy) was cultured and plasmid DNA prepared using a Promega mini-prep kit (Promega). The resulting constructs were cleaved with restriction enzyme (*SacI*). This was followed by *in vitro* transcription with T7 RNA polymerase to generate an anti-sense RNA for used for *in situ* hybridization of formalin-fixed, paraffin-embedded tissue samples to detect the production of TGFβ1 in horse tissues.

**RESULTS**

*Purification of Proteoglycans from ESPA-Affected Tendon* – We have previously shown that proteoglycan accumulation is systematic in ESPA affected horses (Halper et.al. 2006). Abnormal accumulation was revealed not only in suspensory ligaments but also in superficial and deep digital flexor tendons, patellar and nuchal ligaments, cardiovascular system, and sclera. We have now identified some of the proteoglycans accumulated in tissue. Our efforts have concentrated on the purification and partial characterization of two major proteoglycans from tendons from two controls and five ESPA horses. Superficial digital flexor tendons, one from each horse, were extracted by 4M guanidine buffer (Table 3-1). Because quantitative and qualitative changes in proteoglycans occur in tendons with aging, samples were arranged in Table 3-1 according to age. As a first step in separation proteoglycans from major tendon ECM
components, Sepharose CL-2B gel filtration was performed with these extracts (Figure 3-1). The early peak was discarded and the main peak was divided into 4 fractions (I-IV). An aliquot of each fraction was separated on 10% SDS-PAGE. The Coomassie blue-stained gel showed multiple bands with high molecular weight proteins eluting in early fractions while low molecular weight proteins eluting in late fractions (Figure 3-2, B and D). SDS-PAGE analysis of a pool combining protein from fractions I-IV (Figure 3-2, B and D, lane T) revealed the presence of an additional band in the ESPA lane (Figure 3-2D, *) so further analysis was necessary.

Identification of Overexpressed Proteins by Mass Spectrometry – When the combined total protein of every sample from the main CL-2B Sepharose peak was separated on 10% SDS-PAGE, several bands representing overexpressed proteins were detected in some ESPA samples (Figure 3-3A, #1-4). To characterize these proteins four different overexpressed bands were excised, eluted, digested with trypsin and applied to Nano-LC tandem mass spectrometer at the Children’s Hospital at The Ohio State University. Protein sequence search was performed with the BioWorks 3.3 software. Each file was searched against the human NCBInr and human SwissProt databases using Sequest search engine. Two of them (Figure 3-3A, #1 and 2) were identified as decorin (Figure 3-3B) and two of them (Figure 3-3A, #3 and 4) were identified as hyaluronan and proteoglycan link protein 1 precursor (HAPLN1, Figure 3-3C).

Abnormal Immunostaining for Decorin and Fibromodulin in ESPA Tissues – To evaluate whether the distribution of decorin differs in normal and ESPA-tissues we performed immunostaining tissues for decorin. Because of availability of an antibody we also immunostained tissues for fibromodulin, proteoglycan related to decorin. Sections of control SDFTs were immunostained for decorin and fibromodulin more evenly and less intensively than the sections of ESPA affected SDFT (Figure 3-4) suggesting that there might be an abnormality
in structure of both proteoglycans, and binding to collagen. Sections of normal and ESPA aorta showed similar differences in distribution of decorin and fibromodulin (data not shown).

Immunoblotting for Decorin and Fibromodulin. To further study abnormalities in decorin and fibromodulin in ESPA tissue aliquots of total pooled protein from the main Sepharose CL-2B peak were separated by 10% SDS-PAGE and probed by polyclonal antibodies against decorin and fibromodulin. The concentration of higher molecular decorin and fibromodulin was higher in ESPA affected samples than in controls (Figure 3-5A, C). After treatment with Chase ABC, overexpressed decorin (Figure 3-5B, *) with about 140kD of molecular weight was clearly detected while most of glycosaminoglycan containing decorin were cleaved and moved to the protein core level (Figure 3-5B, =). To further characterize higher molecular decorin, aliquot of total protein samples was subjected to 2D electrophoresis. In the 2D gel, overexpressed decorin (Figure 3-6, *) and heterogeneous decorin (Figure 3-6, ↓) were separated by pI. Overexpressed decorin was revealed to have 140kD molecular weight and approximately pl 10.

Purification of Glycosylated Decorin and Fibromodulin – To further purify decorin and fibromodulin, aliquots of the main peak from Sepharose CL-2B column were loaded on a Q-Sepharose anion exchange column, and individual proteoglycans were eluted by stepwise NaCl gradient from 0 to 0.7M. Glycosylated decorin was eluted in the 0.7M NaCl fraction (Figure 3-7A), and ESPA decorin had higher molecular weight than control decorin (Figure 3-7A, ↑). Aliquots of this fraction were then treated with Chase ABC to remove attached chondroitin/dermatan sulfate chains. An aliquot of the treated sample showed a doublet of Mr 45kD after separation in silver stained 10% SDS polyacrylamide gel (Figure 3-7C, =). The remaining bands on the gel corresponded to Chase ABC bands in a lane where only Chase ABC
was loaded (Figure 3-7C, <). Unfortunately, decorin stained with silver stain only after treatment with Chase ABC. That this doublet is the protein core of decorin was confirmed in a western blot using decorin antibody binding to the protein core after Chase ABC treatment (Figure 3-7B, =).

As humans (and likely other mammals) grow older, amount of decorin in the skin tissue increases while molecular weight of decorin decreases (Carrino et al. 2000). This may explain why molecular weight of decorin in C1 sample (from a 5 month old horse) was higher than that of decorin in C2 sample (8 year old horse). Our findings of an additional form of high molecular weight forms of decorin in young horses, E3 (3 years) and E4 (5 years), suggest that this is a pathological change. The quantitative difference between glycosylated decorin present in ESPA tissues (Figure 3-7A) and decorin protein core (after Chase ABC treatment) from the same tissues (Figure 3-7B) is due to the low binding affinity of glycosylated decorin purified from ESPA affected tendon to decorin antibody (Figure 3-11). Highly glycosylated fibromodulin was readily eluted in 0.5M NaCl fraction (Figure 3-8B) while low molecular weight fibromodulin was eluted in 0.1M NaCl fraction (Figure 3-8A). The size of the human cartilage fibromodulin also decreases with age, from 70-110 kDa of 6 week-old neonate to 67 kDa of the mature adult. This is mainly due to loss of keratan sulfate chains while N-linked oligosaccharides remain linked to fibromodulin even in aging tissues (Roughley et al., 1996). This effect of aging may explain the absence of highly glycosylated fibromodulin in C2 (8 year old horse) and sustained expression of abnormal, high molecular weight fibromodulin with age in 0.5M NaCl fraction in material purified from ESPA affected tendons (Figure 3-8B).

**Sulfation Pattern of Glycosaminoglycan Chain(s) Attached to Decorin and Fibromodulin** – In both proteoglycans, increase of molecular weight in extracts from ESPA-affected tendon is most likely due to changes in attached glycosaminoglycan chains, chondroitin or dermatan sulfate.
sulfate in decorin and keratan sulfate chains in fibromodulin. To analyze sulfation pattern of repeated disaccharide in decorin chondroitin/dermatan sulfate, aliquots of 0.7M NaCl Q-Sepharose fractions were cleaved with Chase ABC. The cleaved disaccharides were separated from the protein core by centrifugal filter (cut off 3K) and were loaded into reverse phase HPLC column (C18). The elution pattern of sulfated disaccharides was monitored at 230 nm. As Figure 3-9 indicates the total content of sulfated disaccharides was increased in ESPA affected sample, and sulfation at 6-position of N-acetyl galactosamine was more increased than sulfation at 4-position. The ratio of 4-sulfation over 6-sulfation disaccharide was significantly decreased in ESPA samples, especially in E3 and E4 (Table 3-2).

To analyze sulfation pattern in fibromodulin keratan sulfate, aliquots of each fraction eluted with 0.1, 0.3, or 0.5M NaCl were cleaved with keratanase or keratanase II. Because keratanase does not act in the presence of 6-sulfated galactose while keratanase II requires the sulfate at 6-position of the glucosamine, the fact that fibromodulin in 0.5M fraction had no reaction to keratanase (Figure 3-8D) and was cleaved by keratanase II (Figure 3-8E) indicates sulfation at 6-position in both galactose and N-acetyl glucosamine of fibromodulin keratan sulfate in 0.5M NaCl fraction.

Monosaccharide Composition of Glycosaminoglycans of Decorin – Decorin has one dermatan sulfate chain where some of the glucuronic acid of chondroitin sulfate are converted to its epimer, iduronic acid by chondroitin C5 epimerase (Malmström A., 1981). To investigate the ratio of glucuronic acid over iduronic acid, an aliquot of 0.7M NaCl fraction was analyzed by GC/MS. In ESPA affected sample, the content of glucuronic acid and N-acetyl glucosamine were increased while the content of mannose and iduronic acid were decreased (Figure 3-10). The ratio of glucuronic acid over iduronic acid in ESPA affected sample was increased about 4
times compared to the ration in the control sample (Table 3-3) indicating there might be an abnormal form of ESPA decorin. In addition, hexuronic acids and N-acetyl galactosamine, other monosaccharides such as mannose or N-acetyl glucosamine are present in this fraction confirming the fact that decorin has a few N-linked glycans.

**Binding Affinity of Purified Decorin to its Antibody and TGFβ1** – Because dermatan sulfate affects interactions between proteoglycan and other proteins (Trowbridge et.al. 2002), changes in dermatan sulfate of ESPA decorin may alter its binding affinity to TGFβ1. To investigate the binding affinity of purified decorin, wells of ELISA plates were coated with an aliquot of 0.7M NaCl fraction and the bound decorin was detected by decorin antibody or TGFβ1 antibody. Binding affinity of purified decorin to decorin antibody was increased with increasing concentration of decorin bound to the bottom of the well but was saturated at > 0.2µg decorin/well in both control and ESPA samples (Figure 3-11A). Binding affinity of anti-decorin antibody for ESPA decorin is significantly decreased (P<0.05) compared to the affinity of the same antibody to control decorin (Figure 3-11B), suggesting that change in sulfation of decorin dermatan sulfate affects the binding affinity of decorin. The binding of TGFβ1 to decorin was also significantly decreased (P<0.05) in ESPA samples (Figure 3-12) suggesting that ESPA decorin might not be able to inhibit the activity of TGFβ1.

**In situ hybridization for TGFβ1** – To investigate expression of TGFβ1, histological sections of superficial digital flexor tendon were hybridized with TGFβ1 antisense probe produced from horse liver. TGFβ1 mRNA was increased in ESPA tendon, especially in septa (Figure 3-13). TGFβ1 mRNA was also increased in ESPA aorta and coronary artery (data not shown) indicating that aberration in dermatan sulfate of ESPA decorin may be not limited to SDFT.
DISCUSSION

We have identified some of the biochemical changes occurring in ESPA, a chronic equine disease characterized by abnormal accumulation of proteoglycans in tendons, ligaments and other connective tissues. Firstly, the accumulations of proteoglycans in the tendons of ESPA affected horses contained high amounts of an abnormal, high molecular weight isoform of decorin. We attribute the presence of this form of decorin to abnormal sulfation and glycosylation of its glycosaminoglycan. These changes in decorin structure also led to decreased binding affinity of decorin to TGFβ1. Secondly, another proteoglycan, fibromodulin, also underwent changes in sulfation and O-glycosylation. Finally, these biochemical changes were reflected in abnormal distribution of decorin and fibromodulin as documented by immunostaining of SDFT and in overexpression of TGFβ1 revealed by in situ hybridization in affected tissues.

Decorin synthesized in ESPA tissues undergoes biochemical changes affecting its glycosaminoglycan chain but not the protein core. Interestingly, those modifications varied among horses affected with ESPA. Decorin in extracts from E3 and E4 horses (Table 3-1) clearly revealed biochemical changes (higher molecular weight, increase of 6 sulfation, and extremely low binding affinity to antibody) when compared to control (C2) horse of similar age while decorin purified from E1 horse showed the same molecular weight and moderate binding affinity when compared to control (C1) of similar age. Moreover, no change in molecular weight and moderate binding affinity of decorin purified from E2 and E5 horses was observed. This might be due to late onset of symptom or too old age, especially in E5 horse (21 years of age). The change in the expression of decorin was not unexpected because this chondroitin/dermatan sulfate proteoglycan is a well known regulator of collagen fibrillogenesis and inhibitor of TGFβ1.
activity. TGFβ1 in turn is known as a potent stimulator of collagen synthesis and inhibitor of collagen degradation via stimulating the expression of tissue inhibitors of matrix metalloproteinases (TIMPs) together with inhibition of metallomatrix proteinase (MMP) expression (Edwards et. al. 1987, Overall et. al. 1991). The decorin identified in ESPA affected tendons had higher molecular weight, about 140kD, and was resistant to treatment with Chase ABC. Several explanations are possible. One is that this alteration is an experimental artifact as polymerization of decorin protein cores can exist at high molar ratio of cross-linker or after dialysis against water and lyophilization (Goldoni et.al. 2004). Molecular weight (about 140kD) of this overexpressed decorin is similar to a trimer of decorin core protein (45kD). The isoelectric point of decorin core protein (pI 9.6) (Kresse et.al. 1993) is identical to pI of the overexpressed decorin in 2D gel electrophoresis. The binding properties of this presumptive trimer may not be normal because the polymers did not dissemble in reducing condition of SDS-PAGE suggesting that this presumptive trimer could not work properly as the normal monomer. In a recent paper, the monomer is regarded to be the biologically active decorin (Goldoni et.al. 2004) although there is assertion that decorin dimers may represent an active form favorable for folding and stability of SLRPs (Scott et.al. 2006).

Another, biologically more relevant and plausible explanation is that glycosaminoglycan chains attached to decorin synthesized by ESPA tissues are abnormally produced due to a mutated gene(s) encoding for one of the enzymes participating in glycosylation. Glycosaminoglycan chains consist of repeated units of disaccharides which are sulfated at different positions. The chain of dermatan sulfate of decorin consists of repeated disaccharide units of hexuronic acid and N-acetylgalactosamine (GalNAc), which undergo sulfation at 4-position or 6-position of GalNAc or at 2-position of iduronic acid (IdoA), an epimer of
glucuronic acid (GlcA) (Trowbridge et al. 2002). Our HPLC results showed increased sulfation of ESPA decorin, specifically at 6-position of GalNAc. The ratio of 4-sulfation over 6-sulfation in ESPA decorin revealed similar pattern to molecular weight pattern showing that the ratio 4:6 sulfation was extremely low in E3 and E4 samples while it was only moderately low in E1, E2 and E5 samples. Because the E1 sample came from a young animal it is likely that the high molecular weight and only moderately low sulfation ratio might be due to its young age rather than due to a pathological change, and that the animal had a defect in another proteoglycan other than decorin. The presence of IdoA in dermatan sulfate distinguishes it from chondroitin sulfate and high amount of IdoA inhibit the proliferation of normal fibroblast (Westergren-Thorsson et al. 1991). Our GC/MS data showed the ratio of GlcA over IdoA in ESPA affected sample is 4 times higher than in control sample indicating most of GlcA was not converted to IdoA. The decreased IdoA content may be responsible for an increase in 6-O-sulfation because IdoA enhances 4-O-sulfation of GalNAc in the course of the biosynthesis of dermatan sulfate chains (Eklund et al. 2000).

The other proteoglycan which underwent changes in glycosylation was fibromodulin. It is interesting that the biochemical changes affecting glycosaminoglycans attached to fibromodulin were more consistent from one case of ESPA to another. Highly glycosylated fibromodulin eluting in 0.5M NaCl fraction was identified in all extract from ESPA tendons, including the samples from the 21 year old (E5) but not in the 8 year old control (C2). The amount of fibromodulin core protein (i.e., without keratan sulfate chains) eluting in 0.1M NaCl fractions decreased with age in both controls and ESPA affected samples.

Keratan sulfate chains of fibromodulin contain repeated disaccharide units of galactose (Gal) and N-acetylglucosamine (GlcNAc), sulfated at 6-position of Gal and/or GlcNAc
(Funderburgh et.al. 2000). In our study keratanase treatment of high molecular weight fibromodulin in 0.5M NaCl fractions did not lead to changes in molecular weight. However, treatment with keratanase II led to the appearance of a band with faster mobility on a Western blot. This indicated that keratan sulfate chains attached to high molecular weight fibromodulin synthesized in ESPA tissues are sulfated at 6 position of both Gal and GlcNac because keratanase does not act on 6-sulfated galactose while keratanase II requires the sulfate at 6-position of the glucosamine for its activity.

Increase of sulfation in decorin and fibromodulin may affect the binding affinity of both proteoglycans due to the increase of negative charge provided by sulfation. Binding affinity of ESPA decorin to decorin antibody was decreased compared to control decorin, and this result also showed good correlation with the sulfation ratio. Binding affinity of E3 and E4 (both had extremely low ratio 4:6 sulfation) was only about 50% of control whereas that of E1 and E2 was about 70% and E5 was about 60% of control. This result suggests that the binding affinity of decorin to decorin antibody increases in inverse proportion to 6-O-sulfated dermatan sulfate.

Dermatan sulfate proteoglycans bind to specific site on collagen (Iozzo, 1997), growth factors, including TGFβ (Yamaguchi et.al. 1990, Tumova et.al. 2000, Penc et.al. 1998) and other ECM components (Schmidt et.al.1987, Elefteriou et.al. 2001). TGFβs form a large family of multifunctional growth factors that includes three isoforms of TGFβ (1-3). TGFβ1 is produced by most cells, it is involved in the healing process and is active in almost all stages of tendon healing (Chang et. al. 2000). TGFβ1 activates proliferation of fibroblasts, elevates the tendinous type I and type III collagen production (Klein et. al. 2002 and Heinmeier et. al. 2003) and increases the expression and synthesis of other ECM proteins, e.g., proteoglycans (PGs) (Robbins et. al 1997). In addition, TGFβ1 suppresses the activity of proteases that degrade
extracellular matrix by inhibiting MMP expression and by inducing synthesis of protease inhibitors, such as plasminogen activator inhibitor (PAI)-1 and TIMPs (Schiller et.al. 2004). In the hypertrophic scar fibroblasts culture, however, recombinant human decorin inhibits cell proliferation and reduces TGFβ1 production (Zhang et.al. 2007). Binding affinity of decorin in 0.7M NaCl fraction of ESPA affected sample to TGFβ1 was markedly decreased to one third of control activity suggesting that ESPA decorin may not be able to inhibit production and activity of TGFβ1. This low affinity may be due to the presence of aberrant glycosaminoglycans of ESPA decorin because normal dermatan sulfate chains attached to SLRPs can inhibit the interaction between SLRPs and TGFβs (Hildebrand et.al 1994).

Birch et al have noted that in degenerated equine superficial digital flexor tendon, total amount of sulfated GAGs was significantly increased in central region relative to peripheral region and normal tendon (1998). In human patellar tendinopathy, the content of monosulfated disaccharides including ΔDi-4S and ΔDi-6S was increased (Fu et.al. 2007). Besides wound healing, there are similar proteoglycan or GAG changes in human genetic disease. In Ehlers-Danlos syndrome which is characterized as defect in collagen synthesis in most patients, a substitution of one amino acid in galactosyltransferase I results in longer GAG chain, less iduronic acid, and higher level of protein core of decorin (Seidler et.al. 2006). Although the longer GAG chain of decorin is common in wound healing and genetic diseases, the shortening of decorin dermatan sulfate in later healing stages was not shown to occur in genetic diseases (Kuwabe et.al. 2002, and Quentin et.al. 1990). Dermatan sulfate of ESPA decorin in this study also showed aberrant pattern such as longer chain, lower content of iduronic acid, and increased 6-O-sulfation suggesting the possibility of genetic disease.
In the current study, we have identified another overexpressed proteoglycan in addition to decorin in ESPA tissues: hyaluronan and proteoglycan link protein 1 precursor (HAPLN1). This protein stabilizes the interaction of aggrecan with hyaluronic acid, expressed mostly in the cartilage (Binette et al. 1994). The overexpression is consistent by our previous findings of small foci of cartilage dispersed among collagen fibers in ESPA affected tendon and ligament and aggrecan detected by immunostaining in these spot (Halper et al. 2006).

In conclusion we have identified several novel processes participating in the pathogenesis of ESPA. Our results indicate that decorin and fibromodulin undergo changes in glycosylation of their glycosaminoglycans chains. Future experiments will show whether these changes are due to genetic changes in one of the glycosylation enzymes. It is likely that the differences in changes of decorin glycosylation are due to several different mutations affecting one or more of the genes encoding for glycosyltransferases. We hypothesize that the low binding affinity of these proteoglycans for TGFβ1 leads to changes in TGFβ expression and has a direct effect on collagen and proteoglycan activity. Future experiments will also show whether the changes in glycosylation lead to pathological fibrillogenesis and impaired biomechanical function.

REFERENCES


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Yanagishita M. 2001 Isolation of proteoglycans from cell cultures and tissues. *Methods in Mol. Biol.* **171**, 3-8


FIGURES AND TABLES

Table 3-1. Characteristics of horse tendons used for biochemical analysis

<table>
<thead>
<tr>
<th>Horse designation</th>
<th>Breed</th>
<th>Sex</th>
<th>Age</th>
<th>Onset of Symptom</th>
<th>Tissue (SDFT)</th>
</tr>
</thead>
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<tr>
<td>Control1 (C1)</td>
<td>Percheron</td>
<td>Male</td>
<td>5 months</td>
<td>-</td>
<td>Right forelimb</td>
</tr>
<tr>
<td>Control2 (C2)</td>
<td>Quarter</td>
<td>Female</td>
<td>8 years</td>
<td>-</td>
<td>Right forelimb</td>
</tr>
<tr>
<td>ESPA1 (E1)</td>
<td>Peruvian Paso</td>
<td>Male</td>
<td>18 Months</td>
<td>6 Months</td>
<td>Right forelimb</td>
</tr>
<tr>
<td>ESPA2 (E2)</td>
<td>Peruvian Paso</td>
<td>Male</td>
<td>3 years</td>
<td>1 Years</td>
<td>Left forelimb</td>
</tr>
<tr>
<td>ESPA3 (E3)</td>
<td>Peruvian Paso</td>
<td>Female</td>
<td>3 years</td>
<td>6 Months</td>
<td>Left forelimb</td>
</tr>
<tr>
<td>ESPA4 (E4)</td>
<td>Appaloosa</td>
<td>Female</td>
<td>5 years</td>
<td>6 Months</td>
<td>Right forelimb</td>
</tr>
<tr>
<td>ESPA5 (E5)</td>
<td>Peruvian Paso</td>
<td>Male</td>
<td>21 years</td>
<td>Many years</td>
<td>Right forelimb</td>
</tr>
</tbody>
</table>
Figure 3-1. Two step purification chromatography protocol. Tendons were homogenized and extracted with 4M guanidine HCl buffer. Sepharose CL-2B gel filtration column was used for separating proteoglycans from other ECM components. Individual proteoglycans purified by Q-sepharose anion exchange chromatography resolved into distinct peaks using a stepwise elution with 0M-0.7M NaCl buffer.
Figure 3-2. Fractionation of CL-2B gel filtration chromatography. Control and ESPA SDFTs were first homogenized and extracted with 4M guanidine HCl buffer. Three quarters of total extract (1.5ml) were loaded on CL-2B column and were separated with 4M guanidine buffer at flow rate of 2ml/20min/tube. The major peak (fractions 67-90) was divided into 4 fractions (I-IV), each fraction was concentrated to 2ml. Aliquots were separated by 10% SDS-PAGE (50µl each from fraction I and IV and 25µl each from fraction II and IV was loaded). A: chromatograph of control extract, B: Coomassie blue staining of control fractions separated on 10% SDS polyacrylamide gel, C: chromatograph of ESPA extract, D: Coomassie blue staining of ESPA fractions separated on 10% SDS polyacrylamide gel. High molecular weight proteins were eluted in early fractions while low molecular weight proteins eluted in late fractions. Lane T was loaded with 50µg of combined total proteins from the main peak. Additional band (*) was shown in D.
Figure 3-3. LC-MS spectrometry of overexpressed proteins. A: Coomassie blue staining of 10% SDS polyacrylamide gel. Lane 1 and 2 are control and lane 3-7 are ESPA samples. Fifty µg protein aliquots were loaded into each lane. Four different overexpressed bands were chosen and applied to Nano-LC tandem mass spectrometer. B: the LC-MS spectrograph of #1 band eluent. No. 1 and 2 were identified as decorin. C: LC-MS spectrograph of #3 band eluent. No. 3 and 4 were identified as hyaluronan and proteoglycan link protein 1 precursor (HAPLN1).
Figure 3-4. Immunohistochemistry of superficial digital flexor tendon for PGs. A: control tendon stained for decorin, B: ESPA tissue stained for decorin, C: control stained for fibromodulin, D: ESPA tissue stained for fibromodulin, E: control stained with hematoxylin & eosin, F: ESPA tissue stained with hematoxylin & eosin. Immunostaining for both proteoglycans in control tendons is more evenly distributed, and with less intensity in the healthy tendon than in ESPA-affected tendon. Magnification x 200.
Figure 3-5. Western blot for proteoglycans in CL-2B Sepharose fractions. An aliquot of 25 µg of total protein from the main peak was separated on 10% SDS-polyacrylamide gel and was subjected to immunoblotting. A: Western blot for decorin in untreated samples, B: Western blot for decorin in ChaseABC treated samples, and C: Western blot for fibromodulin in untreated samples. A: The level of high molecular decorin of ESPA (E1-E5) was increased, and its travel time through the gel was slower. B: Treatment with Chase ABC led to marked increase in the size of high molecular weight decorin to about 140kD (*) even though the glycosaminoglycans chain(s) was removed (=, decorin core protein). C: Glycosylation of fibromodulin in ESPA samples also led to increased size of the proteoglycan.
Figure 3-6. Two-dimensional gel electrophoresis and western blot for decorin. A and B are control and C and D are ESPA samples. A and C are Coomassie blue stained 10% SDS polyacrylamide gel after isoelectric focusing in pH 3-10. B and D are western blot for decorin of A and C. Fifty µg protein aliquots were loaded on each gel. In B and D, the decorin in ESPA samples showed slower mobility (arrow) and there is an additional high molecular weight band about 140kD at pH 10 (*) present only in ESPA, but not control samples.
Figure 3-7. Western blot for decorin and silver staining of 0.7M NaCl fractions from Q-sepharose chromatography. Each lane had 0.5ml of 0.7M NaCl fractions loaded. A: Western blot of non-treated samples, B: Western blot of Chase ABC treated samples, C: Silver stained gel of Chase ABC treated samples. A: 0.7M NaCl fractions show enhanced glycosylation of decorin in ESPA samples (E1-E5) than in controls (C1, C2). In B, decorin doublet (=) about 45kD was shown after treatment with Chase ABC.
Figure 3-8. Western blots for fibromodulin in Q-sepharose fractions. A: untreated 0.1M NaCl fractions probed with anti-fibromodulin antibody, B: untreated 0.5M NaCl fraction probed with anti-fibromodulin antibody, C: non-treated blot, D: immunoblot of keratanase-treated samples, and E: immunoblot of keratanase II-treated samples. Glycosylated fibromodulin was eluted in 0.5M NaCl fraction, and the amount of fibromodulin was higher in ESPA samples (E1-E5) than in controls (C1 and C2). Keratanase II cleaved keratan sulfate in fibromodulin (E) while keranase did not (D).
Figure 3-9. HPLC analysis of disaccharides present in Q-Sepharose 0.7M fractions treated with Chase ABC from control and ESPA samples. A: a chromatograph of control sample, B: a chromatograph of ESPA sample. Peaks correspond to $\Delta$Di-0S (1), $\Delta$Di-4S (2), $\Delta$Di-6S (3). Absorbance was measured at 230 nm. Sulfation at 6-position of GalNac is increased in ESPA samples.
Table 3-2. 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.

<table>
<thead>
<tr>
<th>Samples (0.5ml)</th>
<th>ΔDi-4S (ng)</th>
<th>ΔDi-6S (ng)</th>
<th>4S/6S Ratio</th>
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<tr>
<td>C1 (3.26µg)</td>
<td>164.78</td>
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<td>2.50</td>
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<td>C2 (3.02µg)</td>
<td>116.50</td>
<td>34.79</td>
<td>3.35</td>
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<tr>
<td>E1 (3.19µg)</td>
<td>386.00</td>
<td>241.18</td>
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<td>E2 (3.70µg)</td>
<td>320.30</td>
<td>192.49</td>
<td>1.66</td>
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<td>E3 (5.00µg)</td>
<td>612.33</td>
<td>1375.62</td>
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</tr>
<tr>
<td>E4 (4.12µg)</td>
<td>467.91</td>
<td>923.73</td>
<td>0.51</td>
</tr>
<tr>
<td>E5 (3.60µg)</td>
<td>254.39</td>
<td>215.12</td>
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Figure 3-10. GC/MS of Q-Sepharose 0.7M NaCl fractions. A: 25µg protein aliquot of control sample; B: 50µg aliquot of ESPA fraction. 1: Iduronic acid (IdoA), 2: Glucuronic acid (GlcA), 3: N-acetyl galactosamine (GalNAc), 4: Mannose (Man), and 5: N-acetyl glucosamine (GlcNAc). In ESPA fraction, GlcA and GlcNAc are increased while Man and IdoA are decreased.
Table 3-3. GCMS analysis of Q-Sepharose 0.7M NaCl fraction. GlcA/IdoA ratio is increased in ESPA fractions indicating that there might be a change in glycosylation of dermatan sulfate of decorin. Decrease in mannose and increase in N-acetyl glucosamine suggest change in N-linked glycosylation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glycosyl residue</th>
<th>Mass (µg)</th>
<th>Mole %</th>
<th>Ratio (GlcA/IdoA)</th>
</tr>
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<tbody>
<tr>
<td>C1(25µg)</td>
<td>Glucuronic Acid (GlcA)</td>
<td>1.2</td>
<td>12.3</td>
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Figure 3-11. Direct enzyme linked immunosorbent assay (ELISA) for binding ability of purified decorin from Q-sepharose 0.7M NaCl fraction to antibody. A: As amount of coated decorin is increased, binding ability increases in small increments. Binding is saturated at > 0.2μg decorin/well in both control and ESPA samples. B: Binding affinity of anti-decorin antibody for ESPA decorin (E1-E5) is statistically decreased compared to affinity of the same antibody to control decorin (C1, C2) (*, n=3, P < 0.05).
Figure 3-12. Binding assay of purified decorin to TGFβ1 by ELISA. The bottom of the well was coated with decorin (100ng). TGFβ1(10ng, in blocking buffer /tween 20) was added to replicate wells. TGFβ1 bound to decorin was detected by polyclonal TGFβ1 antibody. Total binding of TGFβ1 to decorin was decreased in ESPA samples (*, n=3, P < 0.05).
Figure 3-13. In situ hybridization of flexor tendon for TGFβ1. A: section of SDFT from a control horse. B: section of SDFT from an ESPA horse. TGFβ1 expression in ESPA tissue is more prominent, especially in septa (*) than in control tissue. Magnification x100.
CHAPTER 4

CONCLUSION AND DISCUSSION

The pathogenesis of DSLD is incompletely understood. Degenerative suspensory ligament desmitis (DSLD) is a heritable, debilitating syndrome recognized in Peruvian Pasos, Peruvian Paso crosses, Arabians, American Saddlebreds, American Quarter Horses, Thoroughbreds, and some European breeds (Young, 1993). Historically, the pathology associated with DSLD has been thought to involve collagen fibers and to be limited to the suspensory ligaments of the distal limb of horses. This project was designed to identify the histological characteristics and biochemical mechanism of DSLD because the pathogenesis of this disease was not understood.

PRELIMINARY DATA - Degenerative suspensory ligament desmitis as a systemic disorder characterized by proteoglycan accumulation: The findings of this study suggest that DSLD is in fact a systemic disorder involving many tissues and organs with a significant connective tissue component. Tissues with histological lesions in addition to the suspensory ligament documented in this study include deep and superficial digital flexor tendons, patellar ligaments, aorta, coronary arteries, nuchal ligaments, and ocular sclerae. In light of these observations, a more appropriate term for this disease process may be equine systemic proteoglycan accumulation (ESPA).

In our observations, abnormal accumulations of proteoglycan between collagen fibers, within the tendon matrix, and between elastic fibers in the blood vessels are the most consistent
and prominent histological feature associated with DSLD affected horses. This result suggested that marked increase in small collagen fibrils in DSLD tendons, observed by us in electron microscopic examination, is most likely secondary to qualitative changes in the synthesis of PG(s) because PGs, especially SLRPs, are involved in regulation of collagen fibrillogenesis (Iozzo, 1998). We were interested in identification of the individual proteoglycan(s) in accumulated DSLD lesions.

The proliferative tendon lesions found in three horses with DSLD are significant as they represent in all likelihood early lesions which eventually progress to a less cellular stage characterized by increasing PG accumulation. Characteristically, no inflammatory or fibrotic changes accompanied these deposits or proliferative lesions. We hypothesize that the proliferating fibroblasts secrete PGs which then accumulate in tissues. The proliferation of fibroblasts and growth of the exuberant connective tissue may explain the presence of pain in the early stage of the disease.

All 28 horses with DSLD had histopathological lesions in all SDFTs, DDFTs and SLs. It is of interest that in our study many control SDFTs and DDFTs had signs of old microinjuries consisting mostly of small foci of fibrosis, i.e., regular scar tissue and small deposits of PGs in the midmetacarpal (and proximal) region. This is different from other studies claiming the sparsity of injuries to DDFT in normal horses (Williams et.al. 2001, Schultz, 2004). The lack of systemic involvement in control horses is also indicative that they did not have DSLD.

It is not clear whether the changes observed in the blood vessels and eyes progress over time and lead to clinical manifestations. Several horse breeders brought to our attention that they have encountered horses with a record of clinically diagnosed DSLD dying suddenly without a precipitating disease. Abnormalites in connective tissues components of the aorta or major
vessels may predispose to rupture of aortic aneurysm and sudden death (Robinson et al. 2000, Hausladen et al. 2004). Further epidemiologic studies are necessary to determine the clinical significance of these findings.

DSLD is thought to run in families and bears some similarity to several hereditary diseases, such as Ehlers-Danlos syndrome or Marfan syndrome, afflicting connective and musculoskeletal tissues in people. Because most of the mutations occur in genes encoding for structural components of tendons and blood vessel walls (e.g., collagen, elastin, fibrillin) that contribute to biomechanical integrity of these tissues, the diseases share certain similarities in their presentation.

Current experiments in our laboratory are directed at the characterization and identification of the specific proteoglycans involved in DSLD. Further characterization and understanding of the pathogenesis of DSLD will allow us develop diagnostic tests to identify asymptomatic horses and help prevent the propagation of the syndrome through a selective breeding program.

The findings of this study demonstrate that so called degenerative suspensory ligament desmitis (DSLD) thought to be limited to suspensory apparatus is actually a systemic disorder affecting tissues with a high content of connective tissue, such as tendons and other ligaments, blood vessels and sclerae. Due to the systemic nature of the disease we feel that the term equine systemic proteoglycan accumulation or ESPA is more appropriate than DSLD.

CHAPTER 3 - Purification and characterization of proteoglycans altered in equine systemic proteoglycan accumulation: We have identified some of the biochemical changes occurring in ESPA, a chronic equine disease characterized by abnormal accumulation of proteoglycans in tendons, ligaments and other connective tissues. First of all, the accumulations
of proteoglycans contained high amount of an abnormal, high molecular weight isoform of decorin. We attribute the presence of this form of decorin to abnormal sulfation and glycosylation of its glycosaminoglycan. These changes in decorin structure also led to decreased binding affinity of decorin to TGFβ1. Second, another proteoglycan, fibromodulin, also underwent changes in sulfation and O-glycosylation. Third, these biochemical changes were reflected in abnormal distribution of decorin and fibromodulin as documented by immunostaining of SDFT and in overexpression of TGFβ1 revealed by in situ hybridization in affected tissues.

Decorin synthesized in ESPA tissues undergoes biochemical changes affecting its glycosaminoglycan chain but not the protein core. Interestingly, those modifications varied among horses affected with ESPA. The rates of higher molecular weight, increased 6-O-sulfation, and binding affinity to antibody in ESPA affected samples are varied according to their age and onset time. The change in the expression of decorin was not unexpected because this chondroitin/dermatan sulfate proteoglycan is a well known regulator of collagen fibrillogenesis and inhibitor of TGFβ1 activity. The decorin identified in ESPA affected tendons had higher molecular weight, about 140kD, and was resistant to treatment with Chase ABC. Several explanations are possible. One is that this alteration is an experimental artifact as polymerization of decorin protein cores can exist at high molar ratio of cross-linker or after dialysis against water and lyophilization (Goldoni et.al. 2004). Molecular weight (about 140kD) and pI(10) of this overexpressed decorin is similar to a trimer of decorin core protein (45kD). This presumptive trimer could not work properly as the normal monomer because the monomer is regarded to be the biologically active decorin in a recent paper (Goldoni et.al. 2004).
Another, biologically more relevant and plausible explanation is that glycosaminoglycan chains attached to decorin synthesized by ESPA tissues are abnormally produced due to a mutated gene(s) encoding for one of the enzymes participating in glycosylation. The chain of dermatan sulfate of decorin undergo sulfation at 4-position or 6-position of N-acetyl galactosamine or at 2-position of iduronic acid (Trowbridge et al. 2002). Our HPLC results showed increased sulfation of ESPA decorin, specifically at 6-position of GalNAc. The ratio of 4-sulfation over 6-sulfation in ESPA decorin was decreased in ESPA affected samples showing the similar pattern to molecular weight pattern. The presence of IdoA in dermatan sulfate distinguishes it from chondroitin sulfate and high amount of IdoA inhibit the proliferation of normal fibroblast (Westergren-Thorsson et al. 1991). Our GC/MS data showed the ratio of GlcA over IdoA in ESPA affected sample is 4 times higher than in control sample indicating most of GlcA was not converted to IdoA. The decreased IdoA content may be responsible for an increase in 6-O-sulfation because IdoA enhances 4-O-sulfation of GalNAc in the course of the biosynthesis of dermatan sulfate chains (Eklund et al. 2000).

The other proteoglycan which underwent changes in glycosylation was fibromodulin. It is interesting that the biochemical changes affecting glycosaminoglycans attached to fibromodulin were more consistent from one case of ESPA to another. Highly glycosylated fibromodulin was identified in all extract from ESPA tendons, including the samples from the 21 year old but not in the 8 year old control. The amount of fibromodulin core protein (i.e., without keratan sulfate chains) decreased with age in both controls and ESPA affected samples.

Keratan sulfate chains of fibromodulin can be sulfated at 6-position of galactose and/or N-acetyl glucosamine (Funderburgh et al. 2000). In our study keratanase treatment of high molecular weight fibromodulin did not lead to changes in molecular weight. However, treatment
with keratanase II led to the appearance of a band with faster mobility on a Western blot. This indicated that keratan sulfate chains attached to high molecular weight fibromodulin synthesized in ESPA tissues are sulfated at 6 position of both monosaccharide because keratanase does not act on 6-sulfated galactose while keratanase II requires the sulfate at 6-position of the glucosamine for its activity.

Increase of sulfation in decorin and fibromodulin may affect the binding affinity of both proteoglycans due to the increase of negative charge provided by sulfation. Binding affinity of ESPA decorin to decorin antibody was decreased compared to control decorin, and this result suggests that the binding affinity of decorin to decorin antibody increases in inverse proportion to 6-O-sulfated dermatan sulfate.

Dermatan sulfate proteoglycans bind to specific site on collagen (Iozzo, 1997), growth factors, including TGFβ (Yamaguchi et.al. 1990, Tumova et.al. 2000, Penc et.al. 1998) and other ECM components (Schmidt et.al.1987, Elefteriou et.al. 2001). TGFβs form a large family of multifunctional growth factors that includes three isoforms of TGFβ (1-3). TGFβ1 activates proliferation of fibroblasts, elevates the tendinous type I and type III collagen production (Klein et. al. 2002 and Heinmeier et. al. 2003) and increases the expression and synthesis of other ECM proteins, e.g., proteoglycans (PGs) (Robbins et. al 1997). In the hypertrophic scar fibroblasts culture, however, recombinant human decorin inhibits cell proliferation and reduces TGFβ1 production (Zhang et.al. 2007). Binding affinity of decorin of ESPA affected sample to TGFβ1 was markedly decreased to one third of control activity suggesting that ESPA decorin may not be able to inhibit production and activity of TGFβ1. This low affinity may be due to the presence of aberrant glycosaminoglycans of ESPA decorin because normal dermatan sulfate chains attached to SLRPs can inhibit the interaction between SLRPs and TGFβs (Hildebrand et.al 1994).
Birch et al have noted that in degenerated equine superficial digital flexor tendon, total amount of sulfated GAGs was significantly increased in central region relative to peripheral region and normal tendon (1998). In human patellar tendinopathy, the content of monosulfated disaccharides including ΔDi-4S and ΔDi-6S was increased (Fu et.al. 2007). Besides wound healing, there are similar proteoglycan or GAG changes in human genetic disease. In Ehlers-Danlos syndrome which is characterized as defect in collagen synthesis in most patients, a substitution of one amino acid in galactosyltransferase I results in longer GAG chain, less iduronic acid, and higher level of protein core of decorin (Seidler et.al. 2006). Dermatan sulfate of ESPA decorin in this study also showed aberrant pattern such as longer chain, lower content of iduronic acid, and increased 6-O-sulfation suggesting the possibility of genetic disease.

In the current study, we have identified another overexpressed proteoglycan in addition to decorin in ESPA tissues: hyaluronan and proteoglycan link protein 1 precursor (HAPLN1). This protein stabilizes the interaction of aggrecan with hyaluronic acid, expressed mostly in the cartilage (Binette et.al. 1994). The overexpression is consistent by our previous findings of small foci of cartilage dispersed among collagen fibers in ESPA affected tendon and ligament and aggrecan detected by immunostaining in these spot (Halper et.al. 2006).

In conclusion we have identified several novel processes participating in the pathogenesis of ESPA. Our results indicate that decorin and fibromodulin undergo changes in glycosylation of their glycosaminoglycans chains. Future experiments will show whether these changes are due to genetic changes in one of the glycosylation enzymes. It is likely that the differences in changes of decorin glycosylation are due to several different mutations affecting one or more of the genes encoding for glycosyltransferases. We hypothesize that the low binding affinity of these proteoglycans for TGFβ1 leads to changes in TGFβ expression and has a direct effect on
collagen and proteoglycan activity. Future experiments will also show whether the changes in glycosylation lead to pathological fibrillogenesis and impaired biomechanical function.

REFERENCES


Funderburgh J.L. (2000) MINI REVIEW Keratan sulfate: structure, biosynthesis, and function Glycobiology, 10(10), 951-958


