Cartilage Oligomeric Matrix Protein (COMP), Thrombospondin-4 (TSP-4) and type I and III Collagens in tendon

An immunohistochemical and biochemical study

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Hymyä huuleen

Vaikka kusetkin vastatuuleen

(*old finnish saying*)

Abstract

Södersten F. 2006. *Cartilage Oligomeric Matrix Protein (COMP), Thrombospondin-4 (TSP-4) and type I and III Collagens in tendon – An immunohistochemical and biochemical study.*
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Injury to digital flexor tendons leading to partial or total rupture is a common cause of lameness in athletic horses. Degeneration to the tendon extracellular matrix (ECM) often precedes rupture and a high cyclic load is most likely an important factor explaining its high incidence in racing horses.
Tendon is composed of tenocytes that produce and support an abundant ECM responsible for the physical properties of the tendon. Type I collagen, the most abundant protein in the ECM, forms long fibrils and is oriented along the tensional axis of the tendon. Other matrix molecules include type III collagen, proteoglycans, glycoproteins (COMP and TSP-4). These molecules contribute to the strength of the tendon and are involved in regulating extracellular growth of the collagen fibrils.
The ultrastructural distributions of COMP were delineated with high immunolabeling in healthy three-year-old horses. In the same material, collagen fibril size and the relative distribution of thin, medium and thick fibrils were examined and the highest levels of thin fibrils were found in the three-year-old horses. Achilles tendons from rats were analysed and an active-related decrease in COMP immunolabelling was found. The immunolocalization of TSP-4 was clearly elevated in the pericellular compartment. In injured equine tendons, immunolabelling of COMP and type III collagen was present in all repair structures, described as organized and disorganized fibroblastic. TSP-4 was purified from equine tendon and a heterooligomer between COMP and TSP-4 was displayed.
This Thesis shows that COMP increases in equine tendons during skeletal maturation and is related to greater amounts of thin fibrils. Furthermore, COMP is present in tendon repair tissue and. these findings indicate a role during repair/remodelling where the fibrils are newly synthesised. The decrease in COMP found in active rat tendons can be caused by activity-induced degradation. The heterooligomers of COMP and TSP-4 and the immunolocalization of TSP-4 indicate a function as an adhesion and/or signal molecule between the cell membrane and the extracellular matrix. These results indicate that COMP is involved in activity and aging processes, in repair processes in combination with type III collagen, and in heterooligomer formation with TSP-4 in equine tendon.

Keywords: Tendon, extracellular matrix, COMP, TSP-4, equine, activity, ageing, type I and III collagen.

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Appendix

Papers I-IV

This thesis is based on the following papers which are referred to in the text by the Roman numerals as set below:

I. Södersten, F., Ekman, S., Eloranta, M-L., Heinegård, D., Dudhia, J., Hultenby, K. (2005) Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in relation to collagen fibrils in the equine tendon. *Matrix Biology* 24, 376-385

II. Södersten, F., Ekman, S., Schmitz, M., Paulsson, M., Zaucke, F. (2006) Thrombosponding-4 and cartilage oligomeric matrix protein form heterooligomers in equine tendon. *Connective Tissue Research* 47, 85-91

III. Södersten, F., Ekman, S., Niehoff, A., Zaucke, F., Heinegård, D., Hultenby, K. Ultrastrcutural immunolocalization of Cartilage oligomeric matrix protein (COMP), Thrombospondin-4 (TSP-4) and collagen fibril size in the rodent Achilles tendon in relation to exercise. *In manuscript.*

IV. Södersten, F., Hultenby, K., Heinegård, D., Johnston C., Ekman, S. Immunolocalization of collagens (I and III), and cartilage oligomeric matrix protein (COMP) in normal and injured superficial digital flexor tendon. *In manuscript.*

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Abbreviations

BAPN	beta amino propionitril fumarate
CDET	common digital extensor tendon
COMP	cartilage oligomeric matrix protein
DDFT	deep digital flexor tendon
DEAE	diethylamino ethanol
DIG	digoxigenin
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FGF	fibroblast growth factor
GAG	glucosaminoglycans
H&E	haematoxylin eosin
IGF	insuline-like growth factor
MED	multiple epiphyseal dysplasia
MS MALDI-TOF	mass-spectrometry matrix-assisted laser desorption / ionisation time of flight
MMP	matrix metallo proteinase
NSAID	non-steroidal anti inflammatory drug
PG	proteoglycan
PSACH	pseudoachondroplasia
PS-GAG	polysulphated glycosaminoglycans
SDFT	superficial digital flexor tendon
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SL	suspensory ligament
TIMP	tissue inhibitors of metalloproteinase
TSP	thrombospondin
UBM	urinary bladder matrix
VEGF	vascular endothelial growth factor

Introduction

Background

Tendons and ligaments are fibrous structures prone to injury in athletic horses. A survey in Britain covering thoroughbred horses, competing in both flat racing and national hunt, found the most common cause of lameness was damage to the flexor tendons and suspensory ligaments (Williams *et al*., 2001). These injuries varied from mild strains to partial or total rupture most often affecting the superficial digital flexor tendon (SDFT) in the forelimb. Injuries in tendons usually require long rehabilitation and recurrence is common (Goodship, Birch & Wilson, 1994; Dyson, 2004; Davis & Smith, 2006). Prognosis is often based on ultrasound and clinical findings and the severity of the initial injury is naturally significant in this. Repair should be monitored with repeated ultrasound scanning of the tendon to avoid early setbacks in rehabilitation (Dyson, 2004). In trotters, the suspensory ligament (SL) (hind limbs) is more affected (Goodship *et al*., 1994). Although the suspensory ligaments are to some extent muscular, their function is similar as the flexor tendons and the prognosis and treatment of such injuries is comparable (Goodship *et al*., 1994). Common treatment forms involve rest followed by slowly increasing, controlled exercise. There are several methods described involving injecting various substances in the tendon to enhance healing. These methods however, are based on empirical knowledge and there are few scientific reports in the literature (Dyson, 2004; Davis & Smith, 2006). Increased understanding of the biochemical and morphological response in the tendon to various tensional and compressive loads is important in order to understand the pathogenesis of tendon injury and rupture. Changes to the structural integrity in the extracellular matrix of the tendon will differ between anabolic (healing and repair) and catabolic (degeneration) processes. Data on these molecular changes in relation to exercise induced tendon injuries can result in candidate molecular markers for tendon homeostasis.

Anatomy

Tendons and ligaments are important structures in the locomotor apparatus that transmits high force between muscles and bones. In addition, the equine flexor tendons and SL are loaded eccentrically during the weight bearing part of the stride (Stephens, Nunamaker & Butterweck, 1989; Riemersma *et al*., 1996). The anatomical counterpart of the SDFT is the common digital extensor tendon (CDET), which is loaded only with muscular contraction. The CDET acts as a positional tendon extending the limb before it hits the ground during the stride, and is rarely injured due to overuse (Alexander, 1988; Kear & Smith, 1975). Moreover, the CDET responds to increased load or activity with lateral growth. The flexor tendons however, do not have the same capability to react and only minor changes in the width have been recorded in similar conditions (Birch *et al*., 1999b).

In particular, the SDFT stores kinetic energy through its elastic properties. Most of this energy is returned in the later phase of the stride improving the efficiency of

movement. The lost kinetic energy is transformed into heat (Goodship *et al*., 1994).

The SDFT originates at the end of the superficial digital flexor muscle (*musculus flexor digitorum superficiali,),* a rather small muscle, originating on the medial epicondyle of humerus. The digital flexor muscles contribute little or minimal to the normal movement, but are important in damping high-frequency vibrations in the limb during high-speed galloping (Wilson *et al*., 2001). The muscle has a second origin directly attached to the caudal part of the radius with a fibrous band having minor contractive capabilities. This band fuses to the proximal part of the tendon just above the carpus. In the carpal channel, the tendon is invaginated and surrounded by synovial fluid (decreasing the friction over the joint). It runs superficially under the skin along the metacarpal bone where it is enclosed only by a loose network of connective tissue. Proximal to the fetlock, the SDFT passes into another tendon sheet where it envelops the deep digital flexor tendon (DDFT), then dividing and continuing as two branches each inserted on the proximal lateral eminences of the middle phalanx. Above the fetlock, the DDFT and SL are located between the SDFT and the limb (Fig. 1). Below the fetlock, the DDFT continues to the distal phalanx, where it inserts and the SL attaches laterally on the sesamoidean bones and continues dorsally, fusing to the long extensor tendon (Sisson & Grossman, 1953).

The extended length of the SDFT and that it passes two major joints, adds shearing and compressive forces to parts of the tendon. In these areas, the composition of the tendon is different than regions experiencing predominantly tensional forces (Vogel & Koob, 1989; Benjamin & Ralphs, 1997).

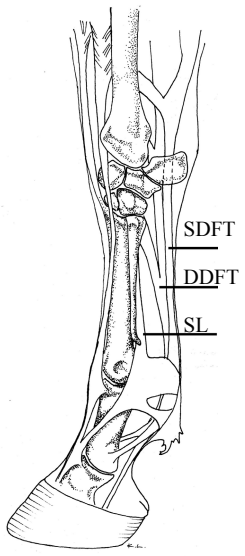

Figure 1. *Schematic, anatomical drawing displaying the weight bearing tendons in the distal front limb. Superficial Digital Flexor Tendon (SDFT), Deep Digital Flexor Tendon (DDFT), Suspensory Ligament (SL).*

The tissue

Tendons consist of relatively few cells (tenocytes) that produce, support and assemble several extracellular structural proteins, all of which contribute to the properties of the tendon. Of these, strength and elasticity are the most important. The most prominent extracellular matrix (ECM) protein is type I collagen, forming fibrils (Fig. 2). There are other structural molecules between the collagen fibrils (Jozsa & Kannus 1997a; rev Wang, 2006) including proteoglycans (such as aggrecan, versican, lumican, decorin, biglycan, fibromodulin) glycoproteins (COMP, TSP-4) and other proteins (collagen type III, V, IX, XI, XII, XIV). The cells also release proteolytic compounds and enzymes such as matrix metalloproteinase (MMP:s). Their main purpose is to degrade worn out components that are replaced by new tissue within the ECM. However, MMPs can be released by a stressed or damaged cell starting an uncontrolled degradation of the ECM (Kjaer *et al*., 2005; Sharma & Maffulli, 2005).

Type I collagen

The major protein in tendon ECM (70-80 % of the dry weight) is type I collagen, a 300 nm long (1.5 nm wide) triple-stranded helical molecule (tropocollagen) that merge and form long thick fibrils (mm long, up to 300 nm wide) (Fig. 2) (Kadler *et al* 1996).

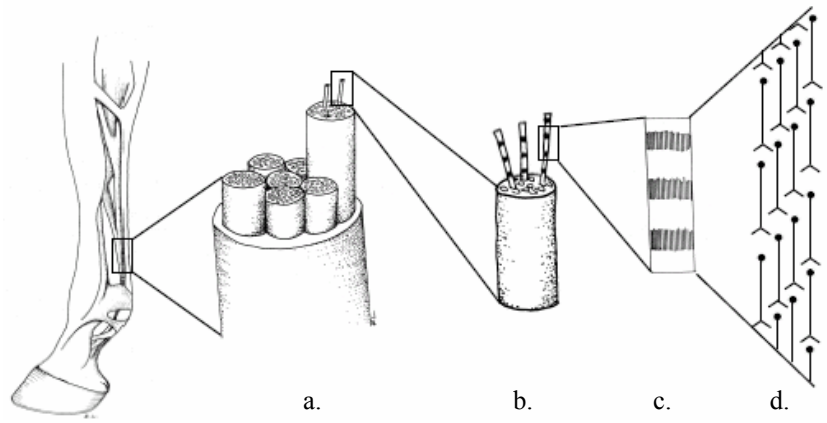

Figure 2. *Illustration of the different structures/levels present in the tendon.*

a) Transverse section of tendon with fascicles (fibrebundles) separated by thin endotenon.

b) Fibre with striated collagen fibrils.

c) Collagen fibril with characteristic dark and bright striation with 67 nm periodicity.

d) Tropocollagen assembled to a fibril in a staggered pattern.

The type of collagen is determined by the type of polypeptide chains incorporated into the triple helix. In type I collagen, there are two $\alpha 1(1)$ chains and one $\alpha 2(1)$ (Kadler *et al* 1996). Before the triple-helix formation proline- and lysine-residues within each chain are hydroxylated. These modified amino residues (hydroxylysine and hydroxyproline) are necessary in the later extracellular formation of covalent cross-links within and between the tropocollagen molecules (Myllyharju, 2003). With advancing age nonenzymic glycation with reducing sugars from circulation adds cross-links making the fibril more resistant to degradation. The ultrastructural cross-striation pattern with 67 nm periodicity present in collagen fibrils is a result of a staggered array of the tropocollagen (Fig. 2 and 3b) (rev Kadler *et al* 1996; Orgel *et al* 2006). On light microscopic examination, the collagen fibrils appear in a wavy pattern that is called crimp (Fig. 3a). When the tendon is loaded, the crimp is stretched and the fibrils are aligned. The stretching of the crimp waves is responsible for approximately 1-2 percent of extension that takes place during loading (O'Brien, 1992). When the tendon is unloaded, the crimp reappears and this is, of course, an important component in the elastic properties that the tendon possesses. The collagen fibrils form fibres, and fibre bundles (fascicles) that are arranged longitudinally in the direction of the tensional load. The fascicles are surrounded with a thin layer of loose connective tissue also containing vessels and nerves. This loose connective tissue allows limited sliding between the fascicles, which adds to the elasticity of the tendon.

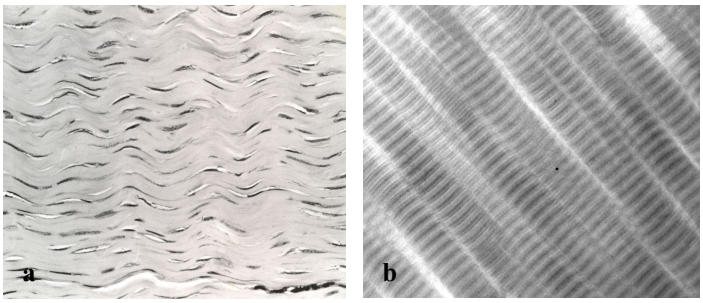

Figure 3. *Longitudinal sections of tendon at microscopic (a) and ultrastructural level (b).*

Type III collagen

Type III collagen is present to some extent in normal tendon. It is found mainly in the endotenon surrounding the type I collagen fibre bundles (Benjamin & Ralphs, 1997, rev Abrahamson, 1991) but also incorporated in the fibril structure where it is believed to inhibit lateral growth of the type I fibril (Benjamin & Ralphs, 1997; Birk & Mayne, 1997). Type III collagen is, as with type I collagen, fibrillar in basic structure. The fibrils of type III collagen have a generally thinner diameter compared with type I collagen fibrils and the tropocollagen contains three $\alpha 1(3)$ chains (Lapiere *et al*, 1977; rev Kadler *et al* 1996). In the early repair of the injured tendon, type III collagen fibrils are quickly synthesized to restore some

strength and elasticity (Williams *et al.,* 1984; Dahlgren, Brower-Toland & Nixon, 2005). However, the fibrils do not have the same tensile strength quality as type I collagen and so lack the functional properties needed in a tendon experiencing maximal load. The repair processes continues with type III fibrils slowly being replaced by type I collagen fibrils in an attempt to normalize the properties of the tendon (Williams, McCullagh & Silver 1984; Dahlgren, Brower-Toland & Nixon, 2005).
In studies on newly ruptured human Achilles tendons, higher levels of type III collagen were found in the area surrounding the rupture (Eriksen *et al*., 2002). In clinically non-lame horses, macroscopic and microscopic changes have been found in flexor tendons. These tendon changes showed increased levels of type III collagen compared to morphologically normal tendons (Birch, Bailey, & Goodship, 1998).

Type V collagen

Type V collagen, another fibril forming collagen, is found in small amounts inside the fascicles and in the endotenon. Type V collagen binds to type I collagen and regulates the lateral growth of the fibril. In tendons from rabbit, type V collagen has been linked to high amount of thin fibrils, suggesting inhibition of fibril assembly (Dressler *et al*., 2002). Deficiencies in type V collagen (genetic disorders) give symptoms of joint laxity and poor wound healing (Schwarze *et al*., 2000).

Proteoglycans (PGs)

The tendon also contains different proteoglycans, which are large molecules consisting of a protein core (backbone) that attach glycoseaminoglycans (GAGs) groups. GAGs are long unbranched polysaccharides made of repeating units containing an amino sugar derivative. The PGs are negatively charged, attracting positive ions and water, which are important for the extracellular matrix. Versican and aggrecan are two large PGs, with hydrophilic properties responsible for holding water in the tendon (rev Abrahamson *et al*., 1991; Benjamin and Ralphs 1997).
Decorin, biglycan, fibromodulin are three small proteoglycans found in the tendon. They contain one or two GAG-side chains, are collagen binding, act as regulators of the fibrillogenesis and also interact with collagen and other macromolecules (Watanabe *et al*., 2005; Zhang *et al*., 2006). Fibromodulin-deficient mice were characterized by irregular and disorganized type I collagen fibre bundles (Svensson, Oldberg & Heinegård, 2001). A more irregular and often smaller collagen fibril population was also reported in fibromodulin-deficient, biglycan-deficient and fibromodulin/biglycan-deficent mice compared to wild types (Ameye *et al*., 2002).
The expression of proteoglycans in tendon changes with location and type of load. An increase of aggrecan is found in parts of the tendon subjected to compression load (Vogel *et al*., 1994). In chronic painful Achilles tendinopathy, an increase in the expression of aggrecan and biglycan has also been shown in the area subjected to tensional load (Corps *et al*., 2006), suggesting differences of the ECM between normal and injured tendons.

COMP

Cartilage Oligomeric Matrix Protein (COMP), also called TSP-5, is a glycosylated protein in the thrombospondin family. COMP contains five identical subunits (Mörgelin *et al.*, 1992) linked together with a coil in the N-terminal forming a five-armed pentamer structure. There are collagen-binding domains on each arm (type I and II collagens) allowing a single pentamer to bind to more than one collagen molecule at the same time (Rosenberg *et al.,* 1998). COMP, first discovered in cartilage (Hedbom *et al.,* 1992), has also been found in tendon (DiCesare *et al.,* 1994) tissue where it is one of the most abundant non-collagenous proteins. The function of COMP is not fully understood, but it is related to fibrillogenesis and binds to several proteoglycans including fibromodulin (Hedlund *et al*., 1994). COMP levels in tendon has been correlated to activity (Smith *et al*., 1997), indicating that COMP is important in the physiological adaptation of the connective tissue to increased load. Deletions and mutations in the COMP genome are responsible for the inherited diseases Pseudoachondroplasia (PSACH) and Multiple epiphyseal dysplasia (MED) (Briggs *et al*., 1995). These conditions result in COMP not being released from the cells so the protein is stored within the cytoplasm, which ultimately impairs normal cell function (Hecht *et al*., 1999; Thur *et al*., 2001; Dinser *et al*., 2002). This results in symptoms of arthritis, deformed bones and laxities in the tendons.

TSP-4

Thrombospondin-4 (TSP-4) is, like COMP, a glycosylated protein in the thrombospondin family. Structurally, it is similar to COMP having five subunits that are linked together in the N-terminal. Each subunit that forms an arm in the pentamer has collagen binding domains. Unlike COMP, TSP-4 has a heparin-binding domain in the N-terminal allowing it to bind to cell surfaces (Lawler *et al*., 1995). TSP-4 has been identified in tendon (Hauser *et al*., 1995) and in heart and brain during embryogenesis but its ultimate function is not yet known (Lawler *et al*., 1993; Narrouz-Ott *et al*., 2000). The primary and secondary structural resemblance to COMP suggests a similar function involving regulatory action during fibrillogenesis. The additional heparin-binding domain indicates that TSP-4 can act as an interface between extracellular collagen fibrils and the tenocytes.

Matrix metalloproteinases

Most tissues have periods of normal breakdown in the ECM, during development, morphogenesis, resorption and remodelling. MMPs are involved in these processes. MMPs are synthesized in the cell and secreted as inactivate pro-enzymes into the extracellular matrix. The expression of MMPs can be regulated by growth factors, hormones and cytokines as well as cellular transformation. They have several functions and are divided into type according to the substrate they digest, such as interstitial collagenases (MMP-1, 8, and 13), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10 and 11), matrilysins (MMP-7), and membrane-type MMPs such as MMP-14. After release, their function is controlled by endogenous inhibitors where TIMPs (tissue inhibitors of metalloproteinases)

are an important group (rev Nagase & Woessner, 1999). In a study where the expression of MMPs and TIMPs was compared between normal, degenerated and ruptured human Achilles tendon, MMPs 3 and 7 and TIMPs 2, 3 and 4 were down regulated in the ruptured tendons. In addition, there was higher expression of MMPs 1, 9, 19 and 25 and TIMP-1 in ruptured tendons compared to normal tendon (Jones *et al*., 2006). In painful but not ruptured tendons, an up regulation of MMP-23 and a down regulation of MMPs 3 and 10 and TIMP-3 was found (Jones *et al*., 2006). In another study, MMP activity and their relationship with collagen remodelling in tendon pathology was investigated. Briefly, the enzyme activity of MMPs 1, 2, 3, 9 and 13, measured in extract from ruptured human supraspinatus tendons, was compared to MMP activity in extracts from normal supraspinatus tendons and biceps brachii tendons (Riley *et al*., 2002). In addition, collagen denaturation was measured. MMP-1 activity was higher in the ruptured tendons where an increased amount of denaturated collagen was also found. But the activity of MMP-2 and MMP-3 was lower in the ruptured tendons (Riley *et al*., 2002). These findings emphasize the role of MMP during breakdown and turnover of the tendon ECM.

Molecular biology

All these extracellular molecules are synthesised and secreted by relatively few cells (tenocytes) located in the area between the fibres with protruding extensions into the fibres (Fig. 4). Tenocytes originate in the mesenchymal and have features similar to fibrocytes.

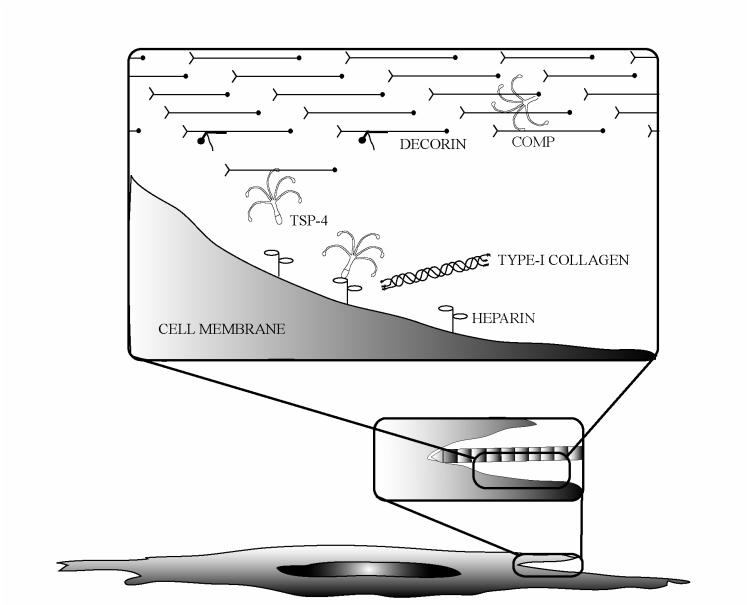

Figure 4. *Schematic figure of the tenocyte during collagen fibril synthesis, with additional molecules COMP, TSP-4, heparin and decorin involved in the fibrillogenesis/fibrilassembly and linking between the cell and the extracellular matrix.*

They are suggested to be a differentiation of fibrocytes/blasts (Junquiera *et al*., 1991; Towler & Gelberman, 2006). Endotenon is comprised of fibrocytes/blasts and vessels. They show low metabolic activity in mature tendon, which only allows slow and small adjustment of the ECM.The turnover rate of type I collagen in the tendon is similar to that in bone. The hydroxylation of proline and lysine stabilize the molecule and, accordingly, decrease the turnover rate even more. The hydroxylation processes are not evenly distributed in the tendon, hence the turnover rate can differ within the tendon (rev Kjaer, 2004).The macromolecular and cellular composition of tendon changes with age (Vogel & Koob 1989; Smith *et al* 1997; Birch *et al* 1999a). The elastic modulus decreases with age and the density of tenocytes decreases. The diameters of the collagen fibrils decrease (rev Benjamin & Ralphs, 1997) and the amount of proteoglycans and glycosaminoglycans (GAGs) in avian tendon increases during maturation and with exercise (Yoon *et al.*, 2003). An increase of hydroxylation between the fibrils in horses has also been reported with age (Patterson-Kane *et al*., 1997). Some of these changes can be physiological and hence normal in the maturation process, but many of the alterations decrease the strength and/or the elasticity of the tendon and it is probable that they are more degenerative in nature.

The cells in the tendon support and produce new extracellular matrix and adapt the tendon to load (Langberg *et al*., 2001; Smith *et al*., 2002). For example, when mechanical activity increases, the flexor tendon does not grow in width in the same proportions as muscles do. Instead, ECM composition is altered with a change in the collagen fibril structural pattern and macromolecular alteration with an increase in the amount of thinner collagen fibrils (Cherdchutham *et al*., 1999).

When the tendon is loaded with tensional forces the ECM and the tenocytes within is stretched and elongated. Cells can detect mechanical stimuli in different ways, such as stretch activated ion channels, calcium channels (Ca^{2+}), gap-junctions and integrin interaction with the matrix (Banes *et al*., 1995; rev Wall & Banes, 2005). In human tendon cells an early response with an increase of intracellularly Ca^{2+} has been reported (rev Wall & Banes, 2005). Calcium-ions act as an intracellular messenger in many signal-transducing pathways, which induces transcription of certain genes. In addition, calcium-ions can pass through Gap-junction into other cells propagating the signal and response in the tissue. The activated/stimulated tenocytes start to synthesize new matrix components for the ECM and DNA-synthesis indicating mitotic activity (Banes *et al*., 1995). Inactivity, immobilization or stress-shielding reduces the tendon strength and give a deterioration of the matrix (Yasuda & Hayashi, 1999). This indicates that loading is necessary to stimulate the tenocytes to sustain the tendon-ECM. However when the load exceeds a certain threshold, the wear (represented by breaking of links and bonds within the matrix) cannot be compensated.

Tendon injuries

The terminology of tendon disorders and tendon injuries is confusing. Tendinopathy, tendinosis, tendonitis, paratenonitis, tenosynovitis, tenovaginitis and peritendinitis are all labels used for diagnoses, though the definition of each

remains unspecified (Josza & Kannus, 1997). They appear to be used differently by equine practitioners, radiologists, and pathologists. To summarize;

Tendinopathy - is a diagnosis that includes all non-specific pathologic changes in the tendon, and has been used when degeneration of the ECM is found in the tendon without inflammation (Maffulli, Khan & Puddu, 1998; rev Kjaer, 2004).

Tendinosis - (previously defined as tendinits) is used similarly to tendinopathy, for degeneration showing no clinical or histological signs of intratendinous inflammation (Josza & Kannus, 1997a; Maffulli, Khan & Puddu; 1998, Kahn *et al* 2002).

Tendinitis - can be defined as manifest inflammation in the tendon including inflammatory cells. An inflammation will always be present in connection with acute rupture (total or partial) characterized by collagen necrosis, haemorrhaging and inflammatory oedema (Josza & Kannus, 1997a).

Tenosynovitis, tenovaginitis and peritendinitis all come under the definition of paratenonitis. These are characterized by the presence of inflammatory cells in paratenon or peritendinous areolar tissue.

Tendon injury ranges in severity from small partial fibre rupturing to complete rupture of the tendon (Goodship, 1993). The rupture is caused by overstrain or percutaneous insult. Overstrain injuries have two aetiologies: 1) sudden impact or load that exceeds the resistive strength of the tendon tissue, or 2) degenerated tendon tissue that has lost its normal tensile strength with rupture occurring under a 'normal' load (Davis & Smith, 2006).

High cyclic load alone has been suggested to cause collagen fibril degeneration. That is, repeated high load causes small ruptures in the fibril structure, tenocytes cannot replace the ruptured tissue, leading to slow degradation (wear and tear) of the tissue. During the stride, tendons experience load so the tenocytes respond with increased metabolic activity (Langberg *et al*., 2001). Tensional loading (defined as running) stimulates the cells to repair and restore injured and damaged structures in the ECM of the Achilles tendon (Langberg *et al*., 2001). When this wear and tear proceeds faster than the repair and replacement processes, the tendon will lose structural integrity; it will degenerate and, therefore can rupture (Fackelman, 1974; Benjamin & Ralphs, 1997).

During cyclic load, as in galloping, some of the kinetic energy is lost and transformed into heat. This raises the temperature (42-44° C) in the tendon, which cannot be transported away due to a relatively limited blood supply (Wilson & Goodship, 1994). High temperatures affect the tenocytes, altering metabolic activity, and so decreasing their ability to repair ECM. In addition, stress promotes an uncontrolled release of proteolytic enzymes, such as MMPs. Under normal conditions these remove damaged or old structures in the ECM. The uncontrolled enzyme release degrades the ECM regardless whether it is damaged or not. The cells are unable to compensate for this increased tissue damage and the ECM starts to degenerated (early aging).

Microscopic degenerative changes have been reported in tendons from horses without any clinical sign of tendon injury (Webbon, 1978). The same type of histological changes can be seen near the rupture in newly ruptured human Achilles tendons (Eriksen *et al*., 2002; Jozsa & Kannus, 1997b). This suggests that degeneration precedes the rupture. The degradation process is probably an inevitable aging process, but there are reports suggesting that the speed of the

degeneration can be altered (increased/decreased) by using certain types of training regimes (Smith *et al*., 2002). Furthermore, training during growth should be optimized to obtain the highest possible physiological properties in the tendon (Smith *et al*., 2002).

After rupture, most often partial, an acute bleeding with oedema and an inflammatory response follows. The repair process starts with fibroblasts becoming activated and migrating into the damaged area. Fibroblasts produce type III collagen fibrils to bridge the rupture. These fibrils are then slowly replaced by collagen type I fibrils, but the orientation of these fibrils is often not strictly linear, thereby reducing the tendon's elasticity (Davis & Smith, 2006).

Both increased vascularization and loss of vessels might be involved in the pathogenesis of equine flexor tendon and human Achilles tendon pathology (Strömberg & Tufvesson 1969; Pufe *et al*., 2005). In the Achilles tendon, degenerative changes most commonly emerge in areas that have poor vascularization (Josza & Kannus, 1997b; Petersen *et al*., 2005). The avascular tendon tissue is thought to be predisposed to degenerative tendon disease after findings of increased lactate levels during exercise in human achilles (Alfredsson *et al.*, 2002) and/or the hypothermia (42-44°C) reported in horse tendons during high-speed training (Wilson & Goodship, 1994). In contrast to this, neovascularization (detected with Doppler and ultrasound) has been reported in Achilles tendons with chronic pain (Öhberg, Lohrentzon & Alfredson, 2001). Similar vascular changes have been found in horses with chronic tendon problems (Kristoffersen *et al*., 2005). Two of the growth factors that stimulate endothelial cell proliferation are vascular endothelial growth factor-1 and 2 (VEGF-1 and 2). VEGF-1 and 2 also stimulates both endothelial cells and tenocytes to produce and release MMPs that degrade the ECM further.

The mechanism of neovascularization is not clear but it is induced by several growth factors and cytokines released from the hypoxic/hyperthermic/avascular tissue. These stimulate the endothelial cells to proliferate and migrate into the degenerative area where neovascularization is triggered by VEGF-1 and VEGF-2 (Pufe *et al.,* 2005).

Treatment of equine tendon injury

In current therapies of tendon injuries (ruptures) the primary object is to minimize the haemorrhages and inflammation using pressure, cold bandages and anti-inflammatory drugs (Dyson 2004; Davis & Smith 2006). Then focus is on the repair and remodelling period optimize the type I collagen synthesis and alignment of the fibrils without creating an irregular vascularized scar tissue. Several strategies have been proposed including intratendinous injections with hyaluronan, polysulphated glycosaminoglycans (PS-GAG), beta-aminopropionitrile fumarate (BAPN) and the almost ancient surgical splitting of the tendon (Davis & Smith 2006).

PS-GAG has been proposed mainly because of its ability to inhibit collagenase and macrophage activation (Dowling *et al*., 2000). In addition, PS-GAG has been suggested to stimulate the synthesis of collagen in the tendon (Dowling *et al*., 2000). Hyaluronan treatment of acute tendon is thought to have similar functions as PS-GAG with anti-inflammatory properties and to reduce adhesion between the

injured tendon and the surrounding tissue. However, scientific results confirming these theories are absent or weak and collagenase-induced tendon lesions treated with hyaluronan showed no significant benefit from this treatment (Foland *et al*., 1992).

BAPN is a substance inhibiting the hydroxylation of lysine by binding to the lysyloxidase enzyme (Stryer, 1988). Lysyloxidase is an essential enzyme in the formation of intra- and intermolecular cross-links in the collagen fibril. This makes the collagen fibrils less resistant to degradation, which theoretically increases turnover. This facilitates the remodelling of the repair tissue, and results in better aligned collagen fibre structures.

Tendon splitting is another treatment of tendon and ligament injuries in horses first described by Gerhard Forssell, later modified by Åsheim (Åsheim, 1964; Strömberg, Tufvesson & Nilsson 1974). The injured part of the tendon/ligament is divided by a single longitudinal incision, which allows in growth of vessels. These vessels were thought to promote healing and connective tissue synthesis (McCullagh, Goodship & Silver, 1979). Nowadays, tendon splitting is used in acute core lesions with presence of hematoma or oedema (Dowling *et al.,* 2000) to reduce the pressure and hamper compartment formation (Davis & Smith 2006).

In a study by Dyson (2004) BAPN, PS-GAG and hyaluronan injections were compared with the conservative treatment, including acute NSAID treatment followed by a rehabilitation programme with slowly increasing activity and repetitive monitoring with clinical examination and ultrasound. BAPN treated tendons had a better alignment of the ultrasound structure and a lower reinjury rate compared with PS-GAG and the conservative method. However, when reinjury to the untreated limb was considered the rate of reinjury rose to ~40% in the BAPN treated horses, and the four treatments did not differ significantly.

BAPN-, PS-GAG, hyaluronan-injections as well as tendon splitting all aim at a repair- and remodelling-process with the end result of a functional scar. However, the difference between the treatments and conservative management is minimal (Dyson 2004).

In recent years, another approach has been introduced on tendon injuries in order to optimize the repair; growth factors and stem cells are used to induce normal tendon tissue formation in the lesion area. Growth factors such as insulin-like growth factors 1 and 2 (IGF-1, 2) and fibroblast growth factor (FGF) have been used experimentally (*in vitro*) on tendon explants. An increased synthesis of type I collagen and other ECM -compounds was observed. In a study performed on collagenase induced tendon injuries in horses, treatment with IGF-1 decreased the soft tissue swelling and increased the DNA and collagen synthesis (Dahlgren *et al*., 2002).

Clinically, different “mixtures / brands” are injected into the injured tendon (such as; urinary bladder matrix (UBM) also known as A-cell) which, according to the manufacturer, contains several important factors that promotes a production of normal ECM.

Adding stem cells to the injured tendon has also become a treatment that is used today (Smith *et al* 2003; Smith & Webbon 2005). Experimental studies on acute injury rabbit Achilles tendon (Young *et al*., 1998) and patellar tendon (Awad *et al*., 1999) showed new tendon-like tissue after stem-cell injection. However, there is little scientific validation of the treatment results on equine tendon injuries

(Smith *et al*., 2003; Smith & Webbon 2005). In brief, sternal bone marrow fluid is collected from the patient and then injected into the lesion. To reduce the risk of injecting harmful substances, such as bone fragments, a refined method has been developed, where stem cells are collected from the bone marrow and cultured for 4-6 weeks. The cell suspension of fibroblastic cells is injected into the lesion (Smith *et al*., 2003; Smith & Webbon 2005).

Aims of the thesis

This thesis is designed to investigate the ultrastructural distribution of certain macromolecules (COMP, TSP-4, collagen I and III) within normal tendon with respect to their concentration in various extracellular compartments. More specifically, the study was also designed to evaluate the influence of age and physical activity on these molecules in the midmetacapal part of the equine superficial digital flexor tendon and rodent Achilles tendon. Finally, the thesis intended to describe the microscopic alterations and immunolocalization of COMP, TSP-4, type I and III collagens in equine tendons with chronic injuries.
Increased understanding of tendon ECM and its response to activity, ageing, and injury is important in designing training programmes and treatment regimes for athletic horses, and for other active species.

Hypothesis

The hypotheses were:

* COMP levels increase in the SDFT from birth up to skeletal maturity, and increasing COMP levels are correlated to a higher amount of thin type I collagen fibrils.
* TSP-4 is present in the equine tendon and has a similar distribution pattern as COMP.
* Running activity in rats increases COMP and TSP-4 in the Achilles tendon ECM. Activity also affects fibril thickness of type I collagen.
* Scar tissue in chronic tendon injuries contains more type III collagen than normal tendons and additionally, COMP and COMP mRNA levels are higher in these regions.

Material and methods

The first study (I) sampled from the superficial digital flexor tendon and deep digital flexor tendon in active horses (standard bred and thoroughbred) that had been euthanized for reasons other than tendon injury. Horses with no history of tendon problems showing no macroscopic or histological sign of tendon injuries were included in the study. Three mm cubes were collected from the midmetacarpal region of the tendons within 4 hours post mortem. The samples were fixed in a mixture of 3% paraformaldehyde and 0.1% glutaraldehyde in 0.15 M cacodylate buffer with 3 mM $CaCl_2$ pH 7.4 and stored at 4 °C. Specimens were then cryosectioned longitudinally as described by Hultenby and co-workers (1993) and embedded transversally for routine electron microscopy according to standard protocol (Gross *et al*., 2000). Parallel samples, 5 mm thick, were fixed in 4% phosphate buffered formaldehyde solution, pH 7.4 and used for light microscope evaluation and *in situ* hybridisation.

The ultrathin cryosections were incubated with rabbit polyclonal antiserum to bovine COMP (1:400) (Hedbom *et al* 1992). The bound antibodies were detected with protein-A coated with 10 nm gold (Amersham, UK). Specimens were examined in a calibrated Leo 906 transmission electron microscope (TEM) (Oberkochen, Germany) at 80 kV. Five micrographs (approximately 67000 x magnifications) from both compartments (pericellular and interterritorial) in each specimen were analysed. The number of gold markers were examined in a Leo 906 microscope, calculated on digital images using an automatic calculation program, KS 4.00 Software (Kontron, Germany) and expressed as gold markers per square micrometer ($Au/\mu m^2$). In addition, the distance from each gold marker to the closest dark-band was measured manually on printed micrographs (100,000 x magnifications). The gold markers were grouped at distances of 0-16, 17-32, 33-48, 49-64 nm, and displayed in a diagram.
Fibril thickness was measured manually in each compartment using electron micrographs (three micrographs from each compartment) (x101000) on transversally cut specimens from every site. The fibrils were pooled into three groups according to thickness (0<60 nm, 60-150 nm and >150 nm) and displayed on histogram as percentage of the total number of fibrils.
For *in situ* hybridisation, a cocktail of three oligonucleotide probes (32-35 mers) complementary to equine COMP mRNA was used. The probes were labelled at their 3´ end with digoxigenin-11-dUTP by using the DIG Oligonucleotide Tailing Kit (Roche, Mannheim, Germany). Sections from the paraffin embedded material were dewaxed, rehydrated and treated with Proteinase K (5 µg/ml; Roche, Mannheim, Germany). After acetylation with 0.25% acetic anhydride (Sigma) and prehybridisation the sections were overlaid with hybridisation solution containing 5 pmol/ml of each probe and incubated overnight. Immunological detection of the bound probe was performed as described in the Non-radioactive *in situ* hybridization application manual by Boehringer Mannheim with slight modifications. The sections were examined with light microscopy and the intracellular marking was compared between the different age-groups.

In the second study (II), fresh, front limb, midmetacarpal SDFTs were collected immediately after euthanasia from adult horses killed for non-orthopaedic reasons and stored at –20 °C until further processing. The tendon tissue material (200 g) was thawed, homogenized and extracted with a buffer containing EDTA. The extract was loaded to a DEAE-sepharose (Pharmacia) ion-exchange column. The protein rich fractions were collected and pooled. The pooled fractions were then loaded to a heparin-sepharose column. The bound proteins were eluted with a linear gradient of 0-1 M NaCl. The protein rich fractions were pooled and examined with SDS-PAGE and Western blot with antibodies against COMP and TSP-4. In addition, bands from a non-reduced gel were cut out and trypsin digested. The peptides in the digests were analysed with MS-MALDI TOF. The fractions were also subjected to two-dimension gel electrophoresis with non-reducing conditions in the first gel followed by reduced conditions on the second. In addition, the sample was loaded to a protein-A agarose coupled to antibodies against COMP or TSP-4. After washing, the bound proteins were eluted and analysed with SDS-PAGE and Western blot.

The third study (III) comprised Achilles tendons from 14 seven-week-old Sprague-Dawley female rats (Niehoff *et al.,* 2004). The control group of five rats was housed in pairs in cages without treadmills. The other nine were housed individually in similar cages with a treadmill. Exercise was done voluntarily and the activity of each treadmill was monitored by a computer and the distances were displayed as kilometre. The active rats were divided into two groups, low running distance 27-36 km/week and high 56-92 km/week. After four weeks of activity the rats were anaesthetized, weighed and euthanized before the left Achilles tendon were collected and fixed in a 3% paraformaldehyde and 0.1% glutaraldehyde in 0.15 M cacodylate buffer with 3 mM $CaCl_2$ pH 7.4 and stored at 4 °C.
The tendons were trimmed into ~1 mm cubes and subjected to cryo-sectioning as described by Hultenby and co-workers (1993). Briefly, the specimens were infiltrated with 2.3 M sucrose and frozen in liquid nitrogen. Sectioning was performed at –95 °C. Ultrathin longitudinal sections (100 nm) were placed on carbon coated formvar nickel grids, thawed and placed on drops on 0.15 M cacodylate buffer containing 0.1 M glycine followed by drops of 10% bovine serum albumin (BSA) to reduce non-specific background. Parallel samples were embedded transversally for routine electron microscopy according to a standard protocol (Gross *et al.*, 2000).
Parallel longitudinal ultrathin sections were incubated with a rabbit polyclonal serum against bovine COMP (1:100) (Hedbom *et al*., 1992) and a guinea-pig polyclonal serum against recombinant rat TSP-4 (1:50) (Narouz-Ott *et al*., 2000). The sections were thoroughly washed in the same buffer and the bound antibodies were detected with protein-A coated with 10 nm gold (Amersham, UK). The total gold markers was calculated in a Tecnai 10 microscope (FEI company, The Netherlands). Digital images were photographed with a Megaview III camera (Soft Imaging System, Münster, Germany) and calculations were done manually in Adobe© Photoshop 5.0.2 and expressed as gold markers per square micrometer (Au/µm2). The distance from each gold marker to the closest dark band was measured manually in Adobe© Photoshop 5.0.2 in a selected number of

micrographs. The gold markers were grouped at distances of 0-17, 18-33, 34-50 and 51-67 nm and displayed in a diagram.
Fibril thickness was measured manually on digital images using analysis software (Soft Imaging System, Münster, Germany). Mean fibril thickness was measured in each specimen in the pericellular and interterritorial compartment. In addition, fibrils were pooled into three groups small (0>60 nm), medium (60-150 nm) and large fibrils (>150 nm) and displayed as a percentage of the total number of fibrils in each individual representing fibril size distribution.
In study IV, nine superficial digital flexor tendons were collected from horses (age 5-25 years) euthanized due to chronic tendon injuries defined and localised with ultrasound scanning. As controls, three superficial digital flexor tendons from high performing horses (age 3-13 years) sent to necropsy for non-orthopaedic reasons were used. Thin 5-mm-slices of the midmetacarpal areas of the normal and injured tendons were obtained and fixed in a standard phosphate buffered 4% formaldehyde solution. After dehydration and paraffin embedding the specimens were sectioned longitudinally in 6 µm thick sections. Parallel sections were made from each specimen (including controls) and incubated with antibodies against type I and III collagen and COMP. Staining with haematoxylin & eosin (H&E) and Masson-Trichrome was also performed and *in situ* hybridisation for COMP mRNA (described in paper 1) was done. Each specimen was morphologically described and the structures were classified into: normal aligned fibres, fibroblastic organized tissue, fibroblastic disorganized tissue, endotenon, necrosis and haemorrhages. The area of each structure was estimated using a morphometric grid (10*10) and displayed as percentage of the total section area. The immunostaining were graded between 0-3 where 0=no staining and 3=strong and widespread staining in every structure. The amount of cells with positive intracellular staining for COMP mRNA was calculated in the different structures, in each section, and graded as 0, 1, 2 and 3, with 0 representing no positive cells and 3, a majority of positive cells.

Results

COMP in SDFT from normal horses (papers I and IV)

Immunolabelling for COMP in the SDFT showed low levels in foetuses and foal and a more than two fold increase in three-year-old horses (paper I). These findings agreed with those for *in situ* hybridisation of COMP. The highest expression of COMP mRNA was confined to the tenocytes in the central part of the SDFT from three-year-old horses. The tendon from foetuses did not express any COMP mRNA (paper I). Immunollabeling for COMP and expression of COMP mRNA in SDFT from adult horses (3, 3, 13 years old controls, paper IV) under light microscopy were located exclusively in the fibre bundles with no staining in the endotenon.

COMP and TSP-4 in normal equine SDFT (paper II)

In an extract of normal equine superficial digital flexor tendons, COMP and TSP-4 could not be separated under non-reducing conditions. This close association between COMP and TSP-4 implicated further studies that revealed unique heterooligomersation between COMP and TSP-4 in the equine tendon.

Collagen type I fibril thickness in the SDFT from normal horses (paper I)

The distribution of fibrils into thin, medium and thick differed between age groups, showing an increase in the relative number of thin fibrils in tendons from older horses. In addition, the fibrils in tendons from foetuses had a homogenous fibril population, with few thick and thin fibrils.

COMP, TSP-4 and type I collagen fibrils in Achilles tendon from active rats (paper III)

The immunolabelling for COMP was significantly lower in Achilles tendon from rats in the two active groups compared to the control group. These tendons did not show any difference in COMP immunolabelling in the interterritorial as compared to the pericellular compartments.

The immunolabelling for TSP-4 was generally higher in the pericellular compartments compared to the interterritorial compartments of the Achilles tendons. However, no difference was recorded between the active and control groups. The location of gold markers for COMP and TSP-4 was accentuated at the dark bands of the collagen fibril, representing the gap region.
In the pericellular compartment, a higher amount of small fibrils was recorded in the tendon of active rats compared to the controls.

Immunolocalization of COMP, type I and III collagens in the injured equine tendon (paper IV)

In all the normally aligned structures including the controls, there was a clear immunostaining for type I collagen as well as COMP. Also, the expression of COMP mRNA was strong in the fibre bundles. The endotenon exhibited no or weak immunolabelling for type I collagen and COMP, instead immunoreactivity for collagen type III was evident.

Immunostaining for COMP was most prominent in the normal aligned structures in the injured tendons. The endotenon of the injured tendon was mostly unstained for COMP and lacked expression of COMP-mRNA in most cells. Fibroblastic, organized and disorganized structures showed a mild to moderate intensity of the immunolabelling for COMP in most of the tendons.
Most fibroblastic structures, both organized and disorganized, were immunostained for type I collagen. All sections of injured tendons were to some degree immunolabelled for type III collagen. However, the pattern differed between controls and injured tendons where immunolabelling in the controls was restricted to the endotenon areas. The injured tendon displayed immunostaining for type III collagen in the pathologic structures including the necrotic areas.

General discussion

The extracellular matrix in the equine tendons changes with age and activity with subsequent alterations of the physical properties present (Birch *et al*., 1999a, Birch *et al*., 1999b). These changes might be a physiological adaptation to load, but can result in a degenerative process (Smith *et al*., 2002) that weakens the tendon. The degeneration predisposes the tendon to micro tears, and partial and total ruptures with an acute inflammatory reaction followed by repair and remodulation. The aethiopathogenesis of this degeneration is not clear but local hyperthermia, hypoxia and cyclic high load alone or in combinations have been proposed as possible causes (Goodship, 1993; Wilson & Goodship, 1994).
In this thesis, COMP and its relation to TSP-4, type I collagen fibrils and variation with age and exercise is studied. The distribution of COMP, type I and III collagens in the different structures present in repair tissue of injured tendons are also investigated.

<u>COMP, TSP-4 and Collagen in normal horses</u>
Previous studies report an increase of COMP in equine flexor tendon with load and maturation (Smith *et al*., 1997). Changes in COMP related to activity have been reported, with a decrease in COMP after an 18-months exercise programme (Smith *et al*., 2002). COMP has also been proposed as a biomarker for tendon injury (Smith & Heinegård, 2000). The results from the first study (I) with high immunogold labelling in superficial digital flexor tendons from three-year-old horses support these data. In the same study an increase in the relative amount of thin type I collagen fibrils in tendons from the three-years-old horses were observed. This change in fibril pattern, also observed in other studies (Cherdchutham *et al*., 2001) can be an early sign of degeneration, fibril splitting, or an adaptation to load with increased synthesis of new, thin fibrils. This can be included in the normal maturation process or be interpreted as result of micro trauma. A decrease of the mean fibril thickness in the central core of the SDFT in horses after commencing an 18-months training programme has been reported (Patterson-Kane *et al*., 1997) and this decrease was interpreted as possible evidence of micro trauma.
The cause of the increase in COMP is not clearly stated. Either it could be a result of normal growth/ageing/maturation or it could be a result of the longer accumulated activity, present in the equine athletes. The results in Paper I suggest that COMP is related to the increase in amount of thin collagen fibrils. If the presence of small fibrils in the tendon is a sign of micro trauma, the connection with high immunolabelling for COMP may suggest COMP as a biomarker for overuse in tendons.
The presence of TSP-4 in bovine tendons was reported by Hauser and coworkers (1994). Its structural resemblance to COMP suggested a similar function. In Paper II the aim was to purify TSP-4 from equine tendon to produce antibodies for further ultrastructural investigations. However, TSP-4 could not be separated from

COMP in the equine tendon, under non-reducing conditions. *In vitro* studies on COMP and TSP-4 (Hecht *et al*., 1998) suggest a possible heterooligomerization between COMP and TSP-4. Our results revealed for the first time that COMP and TSP-4 form oligomers *in vivo* in the equine tendon. The addition of a TSP-4 subunit into the COMP -bouquet adds heparin-binding properties with abilities to interact with cell surfaces. Heterooligomeres of COMP/TSP-4 have not been found in other tissues, such as cartilage. Hence, this may be used to determine whether the COMP molecules found in serum originate from ECM of cartilage or tendon.

COMP, TSP-4 and Collagen fibrils in active rats

To further investigate the influence of activity on ECM-structural molecular composition rats were used in an experimental model, where the running distance in a treadmill of each rat was monitored (Paper III). After four weeks of voluntary running, the rats were sacrificed and the Achilles tendons were analysed for immunolcalization of COMP, TSP-4 and collagen fibril diameter at ultrastructural level. The results did not confirm our hypothesis that COMP would increase with activity; however, an alteration of molecular set up was found with lower immunolabelling of COMP in tendon from active rats compared to controls. This could either be explained with species differences (rat vs. horse), different tendons (Achilles vs. SDFT) or the short period of load combined with the use of skeletal immature individuals (the rats were only three weeks old when the experiment started).

There were significant higher amounts of thin fibrils in the pericellular area of the tendon from the active rats compared to the controls. This increase of thin fibrils might be an indication of synthesis, but can also be caused by splitting or degradation of collagen. In addition to COMP, we also delineated the immunolabelling of TSP-4 that is structurally related to COMP. TSP-4, like COMP, has collagen binding properties. However, unlike COMP it has an additional heparin-binding domain in the N-terminal. This heparin-binding domain adds cell-binding properties suggesting TSP-4 as an interface molecule between the tenocytes and the extracellular matrix. The ultrastructural immunolocalization of TSP-4 was highest in the pericellular compartment with a marked localization to the fibrils next to the cell membrane. Moreover, the majority of gold markers representing both COMP and TSP-4 were located at the dark-bands of the collagen fibril representing the gap-region. This suggests the same binding sites on the collagen fibril and/or co-localization (i.e. heterooligomerization).

COMP, type I and III Collagens in the injured equine tendon

In Paper IV, injured equine SDFT, where the lesions were defined and localized with ultrasound scanning, were examined on light microscopic level. The immunolocalization of COMP, and type I and III collagens was delineated with reference to normal and pathologic areas in the tendons. The sections were morphologically characterized and the areas of the different structures (normal and pathologic) were estimated. In addition, the immunostaining of the different structures were evaluated and scored form 0-3 (0=no positive staining at all and 3=strong positive staining).

The type III collagen fibrils have a thinner diameter than type I (Birk & Mayne, 1997) and lower tensile strength. Increase of type III collagen has been reported in the early phase of repair after experimentally induced tendon lesion (Dahlgren, Mohammed & Nixon, 2005). The presence of type III collagen in the fibroblastic areas (Paper IV) indicates such a repair process. In ruptured human Achilles tendons, an increase of type III collagen fibrils have been reported (Eriksen *et al*., 2002). Type III collagen were also found in acellular areas, which suggests that type III collagen is involved in the degenerative process as described in the human Achilles tendons (Eriksen *et al*., 2002; Mafulli & Kader, 2002).
Adult mesenchymal stem cells are present as reservoirs in various tissues of the body. Stem cells are found in the bone marrow but there are also tissue stem cells, located in different sites called niches outside the bone marrow (Kumar, Abbas & Fausto, 2005). Stem cells retrieved from the bone marrow injected into the injured tendon are thought to differentiate into tenocytes or fibroblasts with tenocytic properties (Smith *et al*., 2003). These cells could then aid in the repair of the lesion by production of ECM molecules that resembles the original ECM of a non-injured tendon. However, scientific data for this hypothesis are sparse. The results in Paper IV do not indicate a lack of fibroblasts/tenocytes as a key mechanism in tendon healing. The high number of fibroblastic cells found in the injured tendons (Paper IV) shows that cells are already present in the tendon. These cells, probably originating from the endotenon and surrounding paratenon, migrate into the degenerated areas where they proliferate and differentiate into more tenocyte-like cells. However, it is not possible to differentiate between the fibroblast and the tenocyte in the present study. Previous studies suggest that tensional load is necessary for a tenocytic differentiation of the mesenchymal cells (Banes *et al*., 1999; Tsuzaki *et al*., 2005). Irrespective of the origin of the cells in the lesion (implanted, migrated or other) load is necessary to stimulate the synthesis of ECM -molecules needed for restoring the tendon properties.

The clear presence of COMP (immunolabel and mRNA expression) in the fibroblastic areas indicates that COMP is involved in the repair and remodelling processes of the collagen fibrils in the ECM. The COMP molecule has been suggested to enhance the fibrillogenesis. In accordance to the present results with increased COMP in the repair/healing tendon, previous studies report increased levels of COMP in synovial fluid from horses with injured tendons (Smith & Heinegård, 2000). In addition, dermal fibroblasts from Scleroderma patients express COMP, which is not found in normal skin fibroblasts (Farina *et al.,* 2006). Scleroderma or systemic sclerosis is characterized by abnormal accumulation of fibrous tissue, dominated by type I collagen, in skin and other organs (Abbas, 2005). The presence of COMP indicates a role in the collagen fibrillogenesis in the repair process. In accordance with this and previous findings, COMP might be a possible biomarker for injury and repair in the equine tendon.

Conclusions

The ultrastructural immunolocalization of COMP together with the COMP mRNA expression in equine superficial flexor tendon suggest an increase in synthesis and presence of COMP during maturation (until three-years of age). In addition, the relative number of thin fibrils increases with age/maturation, up to three years of age, in the equine tendon. The higher quantity of thin fibrils indicates either novel synthesis or degradation of the collagen fibrils.

TSP-4 and COMP form heterooligomeres in the equine superficial digital flexor tendon. This unique molecule may be a marker candidate for tendon metabolism.

Four weeks of voluntary activity decreases immunolabelling of COMP in Achilles tendons from young rats and the collagen fibrils in the pericellular compartments are thinner compared with fibrils in the interterritorial compartments in the active rats indicating synthesis or degradation. Moreover, the ultrastructural immunolocalization of TSP-4 was clearly elevated in the pericellular compartments compared with the interterritorial compartment in tendons from rats in both the control and exercise groups, indicating a role as interface molecule between the cell surface and the ECM.

Immunolabelling of COMP and type III collagen in injured equine tendons was marked in the fibroblastic repair tissues. The marked presence in repair tissue suggests a role for COMP in the remodelling/organisation of the scar tissue.

Future perspectives

The present thesis has focused on some important extracellular matrix proteins present in the tendon. The relationship between COMP, thin fibrils and activity must be further investigated in the equine athlete. The presence of heterooligomers between COMP and TSP-4, i.e. the relation within the heterooligomer and whether there are pure pentamers of TSP-4 in the equine tendon needs further attention. The unique heterooligomeres of COMP and TSP-4 may have a specific function in the tendon and therefore, be important in tissue healing. The heterooligomer can also help distinguishing the origin of COMP-residues in serum.

In addition, injured tendons should be characterized with an extended battery of antibodies where antibodies should be used against VEGF-1 and –2, TSP-4, and small proteoglycans such as decorin, fibromodulin, biglycan, involved in the regulatory processes in collagen fibrillation.

These studies should be done with the aid of ultrasound imaging, light and electron microscopy to characterize the normal and injured tendon tissue. These types of studies should concentrate on tendon repair where complete healing is achieved, to find the key mechanisms behind healing. This knowledge will aid in better treatments of tendon injury without scar formation, which often has an impaired tensile strength and is prone to adhesions and reinjury.

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