The human Achilles tendon

Innervation and intratendinous production of nerve signal substances - of importance in understanding the processes of Achilles tendinosis

by

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Cover image: Achilles tendinosis tendon specimen processed for the NPY receptor Y1.
Marked immunoreactions are seen in the exteriors of the frequently occurring tendon cells (tenocytes).
Figs. 1-**3**: Reproduced from Wikipedias` Wikimedia Commons.
Fig. 4: By Gustav Andersson, adapted from Martin Fahlström, Fig. 7, Badminton and the Achilles tendon, Thesis, 2001.
Fig. 5: By Gustav Andersson, adapted from J Kastelic, A Galeski, E Baer. The multicomposite structure of tendon. Connective tissue research, 6: 11-23, 1978.
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"What is life without pain?"
(Professor Sture Forsgren)

Dedicated to my family Åsa, Jennifer and Josefine

Contents

ABBREVATIONS

ACh	acetylcholine
AChE	acetylcholine esterase
α_1- AR	α_1 adrenoreceptor (adrenergic receptor subtype α_1)
αSMA	alpha smooth muscle actin
β_1- AR	β_1- adrenoreceptor (adrenergic receptor subtype β_1)
BSA	bovine serum albumin
CGRP	calcitonin generelated peptide
ChAT	choline acetyltransferase
FITC	fluorescein isothiocyanate
htx	hematoxylin
-LI	-like immunoreactions
mAChR	muscarinic acetylcholine receptor
M_2R	M_2 receptor (mAChR, subtype M_2)
MRI	magnetic resonance imaging
NeuF	neurofilament
NK-1 R	neurokinin-1 receptor
NPY	neuropeptide Y
PAP	peroxidase-antiperoxidase
PBS	phosphate-buffered saline
PGP 9.5	protein gene product 9.5
SP	substance P
TH	tyrosine hydroxylase
TRITC	tetramethylrhodamine isothiocyanate
VAChT	vesicular acetylcholine transporter
Vim	Vimentin
Y1R	Y1 receptor (NPY receptor subtype Y1)
Y2R	Y2 receptor (NPY receptor subtype Y2)

ABSTRACT

Tendinopathies are painful tendon conditions of presumably multifactorial genesis. In tendinosis, as in Achilles tendinosis, there is apart from pain also morphological changes which are described as degenerative with no signs of inflammation. The exact mechanisms behind these conditions are still, to a large extent, unknown. Pain, being the foremost impairing symptom, leads us to the hypothesis that nerves are deeply involved in the symptoms and processes of Achilles tendinosis. Locally produced nerve signal substances may also be involved in the processes. Knowledge of the innervation patterns within the tendon and knowledge on a possible local nerve signal substance production are therefore of utmost importance. There is a lack of information on these aspects.

The specific aims of this thesis were 1) to investigate the innervation patterns regarding general, sensory, cholinergic and sympathetic innervations, and 2) to examine for the possible occurrence of a production of nerve signal substances and a presence of receptors related to these in the tendon cells, the tenocytes. Painfree normal and tendinosis Achilles tendons were examined.

Immunohistochemistry, using antibodies against the general nerve marker PGP9.5, the synthesizing enzymes for acetylcholine (choline acetyltransferase; ChAT), and catecholamines (tyrosine hydroxylase; TH), the vesicular acetylcholine transporter (VAChT), neuropeptide Y (NPY), substance P and calcitonin gene-related peptide, was applied. Immunohistochemistry was also used for the delineation of muscarinic (M_2R), adrenergic (α_1-AR) and NPY-ergic (Y1 and Y2) receptors. To detect mRNA for TH and ChAT, in situ hybridization was used.

In normal Achilles tendons, as well as in the tendinosis tendons, there was a very scanty innervation within the tendon tissue proper, the main general, sensory and sympathetic innervations being found in the paratendinous loose connective tissue. Interestingly, the tenocytes showed immunoreactions for ChAT, VAChT, TH, M_2R, α_1-AR and Y1R. The reactions were clearly more observable in tendons of tendinosis patients than in those of controls. The tenocytes of tendinosis patients also displayed mRNA reactions for ChAT and TH. Nevertheless, all tenocytes in the tendinosis specimens did not show these reactions. Immunoreactions for α_1-AR, M_2R and Y1R were also seen for blood vessel walls.

The present thesis shows that there is a very limited innervation within tendon tissue proper, whilst there is a substantial innervation in the paratendinous loose connective tissue. It also gives evidence for an occurrence of production of catecholamines and acetylcholine in tenocytes, especially for tendinosis tendons. Furthermore, that ACh, catecholamines and NPY can have effects on these, as well as on blood vessels, via the receptors observed.

The observations suggest that Achilles tendon tissue, whilst containing a very scarce innervation, exhibits autocrine/paracrine cholinergic/catecholaminergic/NPY-ergic effects that are upregulated in tendinosis. These findings are of great importance as the results of such effects may mimic processes that are known to occur in tendinosis. That includes effects related to proliferation and angiogenesis, and blood vessel and collagen regulating effects.

In conclusion, within the Achilles tendon there is a very scarce innervation, whilst there appears to be a marked local production of nerve signal substances in Achilles tendinosis, namely in the tenocytes, the cells also harbouring receptors for these substances. The observations give a new insight into how the tendon tissue of the Achilles tendon is influenced by signal substances and may give options for new treatments of Achilles tendinosis.

ORIGINAL PAPERS

I. **The innervation pattern of the human Achilles tendon – Studies on the normal and tendinosis tendon using markers for general and sensory innervations**
Dennis Bjur, Håkan Alfredson and Sture Forsgren
Cell and Tissue Research, 320:201-206, **2005**.

II. **Presence of a non-neuronal cholinergic system and occurrence of up- and down-regulation in expression of M2 muscarinic acetylcholine receptors: new aspects of importance regarding Achilles tendon tendinosis (tendinopathy)**
Dennis Bjur, Patrik Danielson, Håkan Alfredson and Sture Forsgren
Cell and Tissue Research, 331: 385-400, **2008**.

III. **Immunohistochemical and *in situ* hybridization observations favour a local catecholamine production in the human Achilles tendon**
Dennis Bjur, Patrik Danielson, Håkan Alfredson and Sture Forsgren
Histology and Histopathology, 23: 197-208, **2008**.

IV. **Presence of the neuropeptide Y1 receptor in tenocytes and blood vessel walls in the human Achilles tendon**
Dennis Bjur, Håkan Alfredson and Sture Forsgren
British Journal of Sports Medicine,43, **2009**. Epub ahead of print.

1. INTRODUCTION

1.1 The human Achilles tendon

1.1.1 History, historic terminology

The Achilles' name originates from ancient Greek mythology and the word itself can be analyzed as a combination of ἄχος (akhos) "grief" and λαός (Laos) "a people, tribe, nation". In other words, Achilles is an embodiment of the grief of the people, grief being a theme raised numerous times in the Iliad.

In the Greek mythology a boy named Achilles, son of king Peleus and a goddess, the Nereid Thetis, was to be a hero. Thetis expected her son to be invulnerable and strong. Two mytholocical stories have addressed this. In an early version, Thetis anointed Achilles with ambrosia (Figure 1), a drink of the gods that reinforced their immortality. Then she put him in a fire so that all his mortal parts would burn away, leaving only his immortal anointed parts. However, king Peleus interrupted her and pulled Achilles out of the fire before his heel was burnt, why it remained vulnerable. In a later version Thetis dipped Achilles in the river Styx in Hades (Figure 2), believing he should become safe from all harm and weapons in the future to come. Achilles was held by his foot (heel) when mother Thetis dipped him. Just his heel remained dry and was therefore still vulnerable.

Figure 1. Thetis anoints Achilles with ambrosia in 17th - 18th century engraving-etching by Johann Balthasar Probst (1673 - 1748), Fine Arts Museums of San Francisco.

Figure 2. The Goddess Thetis dipping Achilles in the river Styx.
Donato Creti (1671 - 1749) painting around 1710.
Museum: Pinacoteca Nazionale, Bologna, Italy.

Achilles was later in his life wounded in his right foot by an arrow shot by the Trojan prince Paris during the Trojan War (Figure 3). He eventually died from this wound. The story gave rise to the expression "Achilles heel", meaning a persons principal weakness (Edwards 1985, 1988, Hedreen, 1991, Nagy, 1994).

Figure 3. Achilles is wounded in his right heel by Paris during the Trojan War, subsequently leading to his death. Peter Paul Rubens (1577-1640), painting from 1630-32.
Museum Boijmans Van Beuningen, Rotterdam.

Another mythological story claims, according to Homer, that Achilles killed the Trojan hero Hector in the Trojan War, pierced his heel tendons and dragged his corpse around the city walls for twelve days (Grimal, 1986a, Martinelli, 2000a). Historically, there have thus been

discussions about whether to use the term “Hectors` tendon” or the nowadays used “Achilles` tendon” (Grimal, 1986b, Martinelli and Maffulli 2000b, Shalabi, 2004a) when depicting the heel tendon.

The oldest known written record of the tendon inserting into the calcaneal bone being named for Achilles tendon, is in 1693 by the Flemish/Dutch anatomist Philip Verheyen. In his widely used text *Corporis Humani Anatomia*, Chapter XV, page 328, he described the tendon's location and termed it "the cord of Achilles" ("quae vulgo dicitur chorda Achillis").

1.1.2 Background for the development of chronic pain and for the current thesis

Lifestyle in the economically growing parts of the world has driven humans to live stressed in their minds but physically sedentary. Many studies have shown the benefits from physical activity in different diseases, not least the endemic metabolic syndrome inducing cardiovascular diseases and diabetes (Pedersen and Saltin, 2006). When it comes to the musculoskeletal system our self-selected stressful life puts the biologically prerequisite of physical activity at hold, actually decreasing the well-being. This leaves physical activity to be irregularly performed, and when implemented, short and maybe too intense. This activity, in turn, leads to the risk of overuse problems.

The Achilles tendon is one part of the musculo-skeletal system that is prone to give symptoms. The Achilles tendon has actually been reported to be one of the most injured tendons in the body (Kvist, 1994, Józsa and Kannus, 1997, Alfredson and Lorentzon, 2000a, Paavola et al., 2000). The symptoms are, however, not always derived from extensive or abrupt changes in physical activity. They also occur among subjects reporting a rather sedentary to moderate physical activity lifestyle (Rolf and Movin, 1997).

Pain is the most common symptom occurring in the Achilles tendon, and the far most impairing one. The etiology and pathogenesis for chronic tendon pain are still not fully understood. Although tendon research has progressed reasonably well during the last few decades, the molecular and morphological fundamentals of chronic Achilles tendon pain are yet to be revealed. A contributing factor is that there still is a scarce knowledge about the innervations of the Achilles tendon. This lack of information of how the Achilles tendon is innervated is surprising. Many researchers have asked where the pain is coming from and have suggested biochemical, and thus not only structural, changes, as pain does not correlate that convincing with either collagen rupture or radiologic findings (Adriani et al., 1995, Gotoh et al., 1998, Kiss et al., 1998, Khan et al., 1999b). It would therefore be of interest to know what nerve signal substances that might be involved in these biochemical changes.

1.1.3 Anatomy, fiber typing, tendon insertion

The details of Achilles tendon anatomy have been described elsewere (Józsa and Kannus, 1997, Maffulli and Almkinders, 2007). In this thesis, the anatomy of the tendon will therefore mainly be described in overview terms. Despite the fact that an “Achilles heel” reflects “a weakness”, the Achilles tendon is by necessity the largest, toughest and strongest tendon in the human body. Its function is thus to lift the entire body weight, sometimes implying a heavy load. The Achilles tendon is also called the calcaneal tendon (tendo calcaneus) or the triceps surae tendon (Harris and Peduto, 2006), and originates from the two tendon portions

formed by the extension of the gastrocnemius, with its medial and lateral head originating from the condyles of the femur, and the soleus muscle, and inserts into the calcaneal bone (Figure 4). The three muscle parts are together referred to as the triceps surae muscle. The gastrocnemial parts of the tendon, which range from 11-26 cm in length, are broad and flat near their origin, and become more round and narrow distally, while the soleus part, which ranges from 3-11 cm, begins as a band proximally on the posterior surface of the soleus muscle and becomes the anterior part of the tendon, ending medially at the insertion (Józsa and Kannus, 1997, Jones, 1998, Maffulli, 1999).

The gastrocnemius muscle is activated when jumping and running and is composed predominantly of type II muscle fibres (Fugle-Meyer et al., 1979). In contrast, the soleus muscle has more of a stabilizing function on the foot, especially when standing, and consists foremost of type I muscle fibres (Garret et al., 1984). The triceps surae muscle is a stance-phase muscle that undergoes both eccentric (lengthening) and concentric (shortening) contractions during walking and running (Teitz et al., 1997).

The thinnest part of the Achilles tendon, with a crossection of 0.4-1.4 cm^2 (Kvist, 1994, Magnusson and Kjaer, 2003), is located in the midportion of the tendon, 2-6 cm from the inserton of the tendon into the calcaneal bone.

The myotendinous junction is a highly specialized region were the tension generated by the calf muscles is transmitted from intracellular contractile proteins to extracellular connective tissue proteins, collagen fibrils, of the tendon. The collagen fibrils insert into deep recesses formed by the muscle cells. By this, the contact area increases by 10 to 120-fold reducing the force applied per suface unit during muscle contration (Józsa and Kannus, 1997). This arrangement is of utmost importance as great mechanical stress arise when the contractile force from the muscle is transmitted to the tendon.

The tendon insertion into the calcaneal bone is intimately related to the retrocalcaneal bursa and the collagen fibers are interspersed into the calcaneal bone forming a stiff fibrocartilaginous expansion (Józsa and Kannus, 1997) called the osteotendinous junction, also described as an enthesis (Frey et al., 1992). The enthesis is characterized by three distinctive fibrocartilages, two in the tendon (enthesial and sesamoid) and one on the heel bone (periosteal). Anteriorly to the horseshoe-shaped retrocalcaneal bursa, the Karger´s fat pad is protecting the bursa and tendon against the posterior tip of the calcaneal bone (Figure 4). The retrocalcaneal bursa contains synovial fluid which brings down the friction between the bursa walls and subsequently between the Achilles tendon and the calcaneal bone (Reinherz et al, 1991).

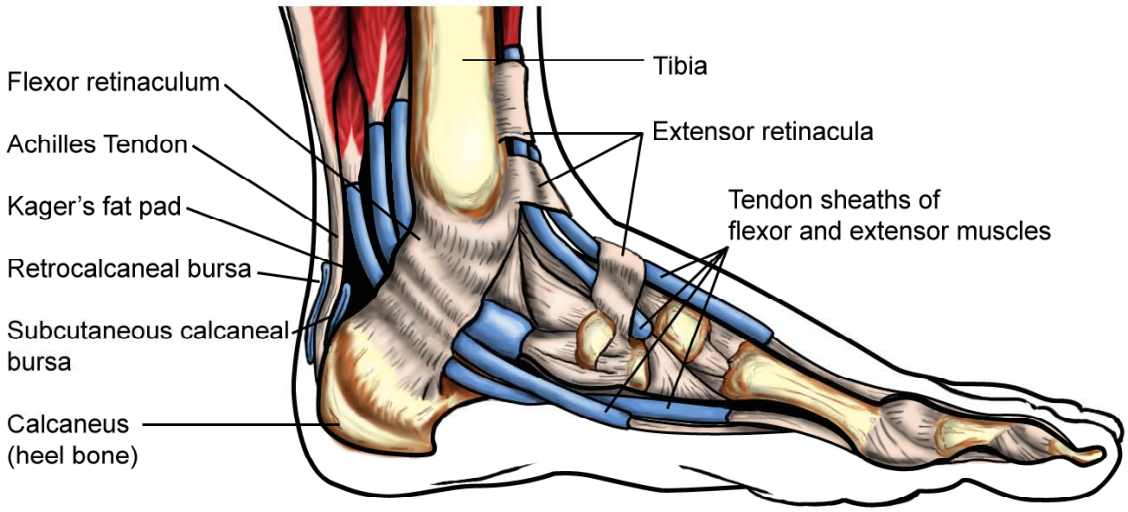


Figure 4. Medial view of the foot and ankle, left.

1.1.4 Tendon structure; aspects on architecture and molecular composition

1.1.4.1 Tendon overall structure

Macroscopically, a tendon is defined as a highly fibrous dense regular connective tissue, where the collagen fibers form bundles, and, when healthy, the tendon is known to have a brilliant white colour with a glistening appearance and to be fibro-elastic in its texture, withstanding considerable loading in working directions. Large human tendons such as the Achilles tendon are surrounded by a loose areolar connective tissue, called the paratenon (Tuite et al., 1997) and closest to the tendon tissue proper the tissue has the structure of a fine connective tissue sheath called the epitenon (Figure 5). This in contrast to smaller tendons in the hand and foot that are surrounded by a more dense connective tissue called a tendon sheath (Kannus, 2000).

Together the epitenon and paratenon are called the peritenon (Józsa and Kannus, 1997). The peritenon (also called paratendon) has both a visceral, inner layer continuous with the epitenon and a parietal layer, continuous with the deeper fascia (Salzman and Bonor, 1994). There is also a middle layer inbetween these two layers, called the mesotenon. The parietal layer and mesotenon thus, forming the paratenon. There are thus three layers consisting of fibrous connective tissue with fine blood vessels, lymphatic vessels and nerves, and forming the entity described as the peritenon (Gould and Korson, 1980). The interwoven fibre structure forms a tensile system and is working as an elastic sleeve allowing the paratenon to stretch several centimetres in length during tendon movement, providing a certain degree of tendon gliding (Salzman and Bonor, 1994, Józsa and Kannus, 1997).

The fibres of the Achilles tendon rotate about 90 degrees when descending to the calcaneal bone, leading the soleus fibres to insert medially whereas the gastrocnemius fibres insert laterally (Root et al., 1977). It has been speculated that this rotation of the tendon portions

results in an internal stress especially in the midportion 2-6 cm proximal to the insertion (Józsa and Kannus, 1997, Teitz et al., 1997).

1.1.4.2 Ultrastructural architecture of tendon

The dense packing of fibrils forms collagen fibers, which in turn progressively aggregate into units forming collagen fiber bundles, namely primary (collagen subfascicles), secondary (collagen fascicles) and tertiary (collagen fascicle bundles) ultimately defining the tendon (Figure 5). The fiber bundles are able to move slightly relative to each other (pseudo-elasticity) but the overall elasticity of tendinous tissue is very low (about 3-8%) partly due to the texture and partly due to the molecular composition (Putz et al., 1995). It has been suggested that proteoglycan bridges between collagen fibrils play a part in transmitting and resisting tensile stresses in tendons, contributing to the strength of the tissue (Cribb and Scott, 1995).

Under polarized light microscopy, the collagen fiber bundles of tendons appear crimped with alternative dark and light transverse bands with a periodicity of approximately 100 µm (Birk et al., 1990). This pattern disappears when the tendon is stretched about 2 %, which corresponds to the toe region of the stress-strain curve (Figure 6) and is thought to be related to the straightening of the fibers (Józsa et al., 1991). Components defined as knots of collagen fibrils termed “fibrillar crimps” (Figure 5) conform with the overall complex ultrastructure of the tendon that provides high buffer capacity in harbouring forces of different directions; longitudinal, transversal, horizontal and rotational, all being an integral component of the musculoskeletal system (Franchi et al., 2007a and 2007b).

The fiber bundles are held together by a fibrous dense irregular connective tissue called the endotenon (Figure 5), in which small blood vessels and lymphatic vessels and to a small extent nerves are harboured, analogous to the situation in the paratenon. These areas allow the collagen fiber bundles to move independently of each other (Maffulli and Almekinders, 2007).To what extent there are nerve fibers in the endotenon of the human Achilles tendon is not known.

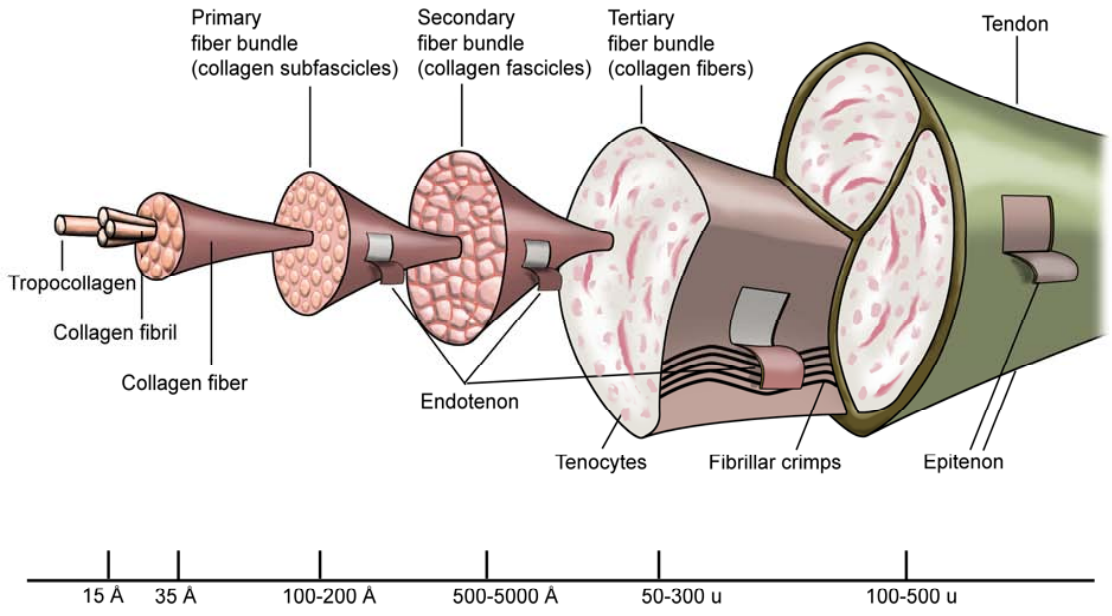


Figure 5. Tendon ultrastructure. Organization of collagen compounds from tropocollagen to the entire tendon.

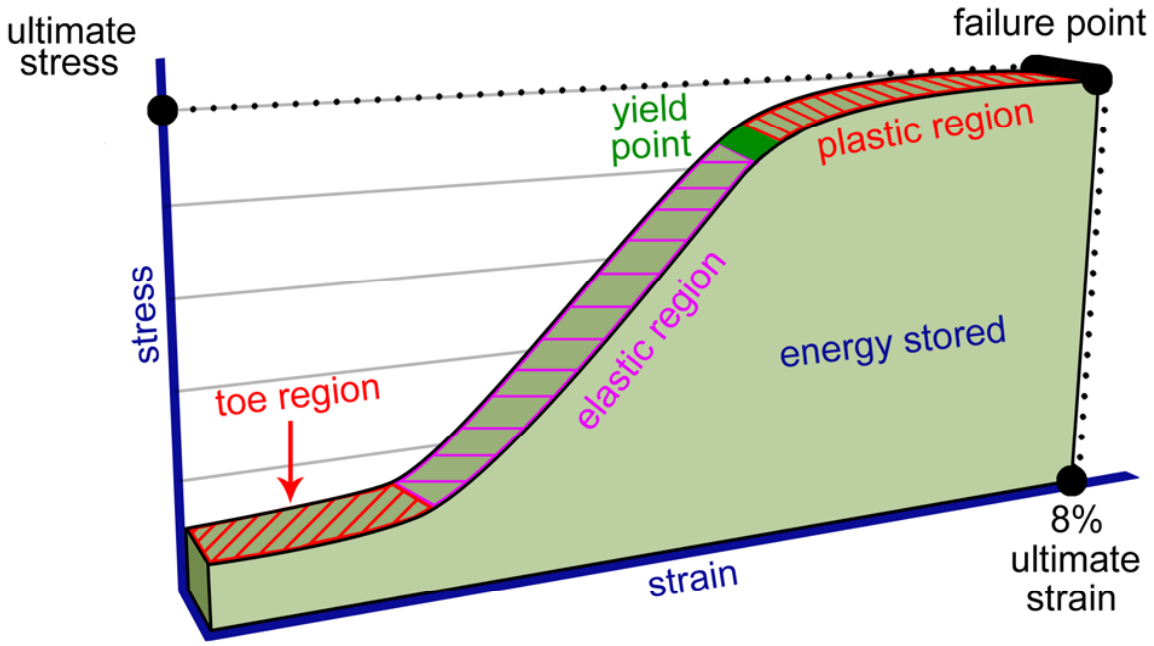


Figure 6. Stress-tension curve. Tendon can be stretched but only to a certain extent. At 4% strain the tendon starts to rupture and at 8% strain it is likely to rupture (Józsa and Kannus, 1997).

1.1.4.3 Molecular composition of tendon

The composition of human tendons have been described in terms of three categories of proteins: (1) collagens, (2) elastin and other extracellular matrix (ECM) proteins with elastic properties such as tenascin-C (Järvinen et al., 2000, Riley et al., 1996) and proteins with multi-adhesive properties (e.g. integrins, fibronectin, laminin), also known as non-collagenous (glyko-) proteins (NCP), and (3) hyaluronan (glycosaminoglycans; GAGs /proteoglycans).

Approximately 70-80% of the dry weight of the tendon tissue is collagen, about 1% is elastin and 1% is the other NCPs. Water, accounts for 65-70% of the total wet weight of the tendon, and is closely associated with the proteoglycans of the ECM (Movin et al., 1997, Kannus, 2000).

The water and the proteoglycans probably provide the lubrication and spacing that are crucial to the gliding function of the tendon (Woo and Tkach, 1989). As the water-binding capacity, foremost provided via the macromolecules (proteoglycans and GAGs), is considerably great and as the hydrophilic gel of the matrix can vary in consistence, the resistence of the tendon against shear and compressive or decompressive forces is high (Jòzsa and Kannus, 1997). Since the tendon has a relatively scarce vasculature, the matrix has a high viscosity not only to provide structural support but also for the purpose of mediating and harbouring nutrients and gases that are indispensable to the tendon.

The paratenon contains mainly two types of collagen, type I and III. The tendon tissue proper mainly consists of type I collagen (95%). There is also type III collagen, mainly in the endotenon (Riley, 2004), as well as V and VI types. Types III and V play a role in regulating fibril diameter (Birk et al., 1990), and collagen type VI, together with decorin (a leucin-rich

proeteoglycan), is important in the function of mediating force between the collagen fibrils longitudinally (Waggett et al., 1998).

The mechanical stability of the tendinous collagen is the most important factor for the mechanical strength of a tendon. The tropocollagen or "collagen molecule" is a subunit of larger collagen aggregates called fibrils, which are held together by electrostatic chemical cross-linking (Kannus, 2000).

The main collagenous component in the myotendinous junction is type I collagen as is the case in the tendon tissue proper. Also small amounts of type III collagen is found at the myotendinous interface. In addition, high concentrations of the ECM adhesive protein fibronectin are present on the muscle cell surfaces of the junction.

1.1.5 Tenocytes and tenoblasts

Tendon tissue is regarded as dense connective tissue and the vast majority of its cells are fibroblasts called tenocytes. 90-95% of the cells in tendons are thus tenocytes, the cells partly being referred to as tenoblasts (cf below), and to 5-10% chondrocytes, located at the insertion, synovial cells, and vascular cells (Kannus, 2000). Many other types of cells such as inflammatory cells, macrophages and cells with myofibroblastic appearances can be found in a pathologically changed tendon (Józsa and Kannus, 1997), but in principle not in healthy tendons (Khan et al., 1999a).

The tenocyes of the Achilles tendon tissue proper are specialized fibroblasts that is situated within the collagen fascicles. In a healthy tendon, they appear as star-shaped cells in cross sections, and they appear as cells lying in rows in parallel with the tendon fibers in longitudinal sections. They synthesize both fibrillar (collagens) and non-fibrillar components of the extracellular matrix, and are able to reabsorb collagen fibrils (Józsa and Kannus, 1997).

Tenocytes are slender, spindle shaped, elongated cells with a sparse cytoplasm (Chuen et al., 2004) and are described to have two different cell processes, one being flat extending laterally and delineating the collagen fiber bundles (McNeilly et al., 1996), the other running longitudinally within the tendon. In this three-dimensional network, intercellular communications take place within the rows of tenocytes as well as between them through gap-junctions. The gap-junction proteins, connexin 32 and 43, are thought to be of importance in co-ordinated response of the tendon cells to mechanical loading, connexin 32 mainly being found between cells lying in a row, and connexin 43 linking cells from different rows together (McNeilly et al., 1996). Gap junction communication with connexin 32 stimulates and that with connexin 43 inhibits collagen synthesis when the tendon cells are subject to loading (Waggett et al., 2006).

There are yet no specific markers for tenocytes or tenoblasts to differ them from other fibrocytes or fibroblasts (Riley, 2005). In the literature, there is also to some extent a confusion about how to define the tenocytes and tenoblasts. Nevertheless, certain criteria are defined in a study by Chuen and collaborators on the patellar tendon (Chuen et al 2004). It is described that tenoblasts are those tendon cells that are rounded, and that have an ovoid nucleus, and that tenocytes are the slender and spindle-shaped tendon cells (c.f. above). Furthermore, the tenoblasts are suggested to be an activated form of tenocytes, which are needed in situations when high matrix turnover is demanded, e.g. during healing responses (Davidson et al. 1997) or that are activated in response to tendon injury (Kakar et al., 1998).

In line with the description given above, and in accordance with tendon cells seen in “growing tendons” in young individuals, several researchers describe the tendon cells with an aberrant (bulky, ovoid, widened, rounded) appearance as being tenoblasts, (Ippolito et al., 1980, Józsa and Kannus, 1997). The immature tendon cells of newborns are numerous, and

are known to vary in appearance (being elongated, ovoid or polygonal), but in the maturing process of the tendon, these cells change into the typical appearance of a slender spindle-shaped form (Kannus, 2000).

There is a theory that states that the ovoid tendon cells are derived from connective tissue progenitor cells (Muschler and Midura, 2002).

Recent research has also shown that the organelles may differ between tenoblasts and tenocytes, the tenoblasts, but not the tenocytes, carrying a well developed rough endoplasmatic reticulum, but rather few mitochondria in their cytoplasm (González Santander et al., 1999). This is analogous to the fibroblast cell being a metabolic activated state of the fibrocyte, capable of synthesizing extracellular matrix compounds and collagen (Kannus, 2000).

The proliferation and apoptosis rates of the ovoid tendon cells and their expression of procollagen type I (procol I), and heat shock protein 47 (hsp47), have been shown to be higher than those of the elongated tendon cells, suggesting that these former cells are more active in matrix remodelling (Chuen et al., 2004). The ovoid cells have also been discovered to express matrix metalloproteinase 1 (MMP1), bone morphogenetic protein 12 (BMP12), and 13 (BMP13), and transforming growth factor beta1 (TGFbeta1) in higher levels than the elongated cells. These findings are suggested to display differences between the cellular activities of tenoblasts and tenocytes (Chuen et al., 2004).

Furthermore, studies on mice cell lines indicate, that some tendon cells have properties partly resembling mesenchymal stem cells (MSC), as they could differentiate into e.g. adipocytes or osteoblasts (Salingcarnboriboon et al., 2003). Studies on human fibroblast cell lines support the existence of stem cell-like characteristics for fibroblasts (Rieske et al., 2005).

1.1.6 Blood supply

The blood supply to the Achilles tendon has been investigated in several studies (e.g. Carr and Norris, 1989, O`Brien, 1997, 2005, Tuite et al., 1997, Ahmed et al., 1998). Branches of the peroneal and posterior tibial arteries supply the Achilles tendon and three regions where the blood supply is received to the tendon have been identified: (1) the musculotendinous junction, (2) along the length of the tendon, and (3) the tendon-bone junction (Figure 7). Vessels originating from the gastrocnemius and soleus muscles supply the tendon at the musculotendinous junction. The blood vessels to the distal part of the tendon, at the region of the enthesis, originate from an arterial plexus at the posterior part of the calcaneal bone. This supply starts at the margin of the insertion and extends up the endotenon for about 2 cm proximally **(**Lagergren and Lindholm, 1959, Karcz et al., 1996, Ahmed et al., 1998, Zantop et al., 2003).

Tendon tissue predominantly contains extracellular tissue with a low metabolic rate. Hence, this tissue is supposed to have a rather low requirement of blood supply, compared to other tissues. Qualitative and quantitative histological analyses have actually shown that the tendon tissue proper (the central parts of tendons) of the Achilles tendon has a rather poor blood supply throughout its length, as determined by the small number of blood vessels per cross-sectional area. This may suggest that poor vascularity may prevent adequate tissue repair following trauma, leading to further weakening of the tendon (Ahmed et al., 1998).

The degree of vascularity within tendon tissue has, nevertheless, been shown to vary in the human Achilles tendon, the distal and proximal part having similar intravascular volume while a lower vascularization volume occurs for the middle part, defined as 2-6 cm from the insertion into the calcaneal bone (Józsa and Kannus, 1997, Stein et al., 2000, Zantop et al.,

2003). The inner part of the enthesis is, however, normally thought to be avascular (Åström et al., 1994, Benjamin and McGonagle, 2001).

Varying suggestions concerning the blood sypply within the tendon tissue proper of the Achilles tendon are, however, reported. Studies using microdialysis technique (Langberg et al., 1998) and Laser Doppler flowmetry (Åström, 2000) have thus shown an even distribution in blood flow in the tendon, but no conclusion about how this may affect tendon pathology has yet been established (Theobald et al., 2005, Langberg et al., 1998). Langberg and collaborators showed a fourfold increase in peritendinous blood flow 5 cm proximal to the insertion, compared to at 2 cm proximally, when exercising, supporting the concept of giving patients exercise to promote circulation to help the healing in the tendon during rehabilitation (Langberg et al., 1998). However, in a recent study in 20 cadaveric lower human limbs, it was again shown that the mid-section of the Achilles tendon was markedly more hypovascular than the rest of the tendon (Chen et al., 2009). Consensus on this issue apparently is awaiting further research.

The small arterioles, venules and capillaries of the intratendinous networks are the microvascular units of the tendon. New imaging techniques can identify these areas as high signal foci, morphologically representing blood vessel areas of the connective tissue septa, called the endotenon (see above) (Hess et al., 1989, Józsa et al., 1991, Mantel et al., 1996).

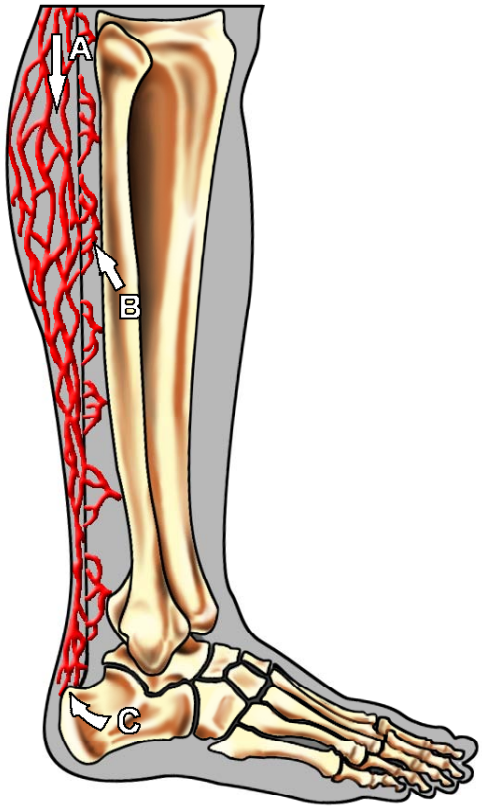


Figure 7. Blood supply of the Achilles tendon comes from three regions: The musculotendinous junction (A), along the length of the tendon (B), and the bone-tendon junction (C).

1.1.7 Innervation and signal substances

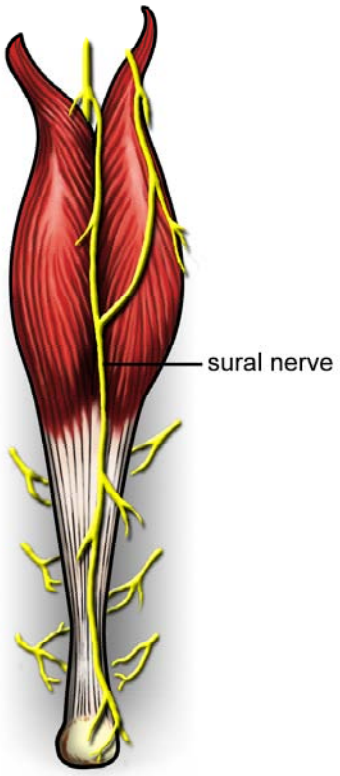
1.1.7.1 Nerves, sensory endings, neuropeptides, neurotransmitters and their receptors

There is no detailed study of the innervation of the Achilles tendon from its myotendinous junction down to the enthesis. Anatomically, the nerves have been described to derive from the attaching muscles and from small nerve fascicles coming from cutanous nerves, especially the sural nerve (Figure 8) (Stillwell, 1957b). Animal studies have shown that many of the nerve fibres terminate in sensory nerve endings in the connective tissue surrounding the tendon, the paratenon (Ackermann et al., 2002). However, a few nerves enter the tendon tissue proper following the vascular channels in the endotenon. They also anastomose obliquely and transversely inside the tendon, ultimately terminating into nerve endings.

Four types of the sensory nerve endings have been decribed in the locomotor system, including to some extent in tendons. These include Type I or Ruffini corpuscles (pressure and stretching sensors), II or Vater-Pacini corpuscles (pressure sensors, reacting to acceleration and deceleration of movement), type III or Golgi tendon organs (tension receptors) and typ IV or free nerve endings (pain receptors, also called nociceptors) (Józsa et al., 1993, Józsa and Kannus, 1997, Kirkendall and Garrett, 1997, O´Brien, 1997). Both Golgi tendon organs and free nerve endings have been found in relation to Achilles tendons, foremost in the myotendinous junction and insertion areas (Józsa and Kannus, 1997, Grey et al., 2007).

Until recently, the tendon tissue proper of larger tendons has been considered not only to be relatively hypovascular but also hyponeural. The innervation that has been identified in the Achilles tendon has been stated to be mainly unmyelinated and afferent (Stillwell, 1957a, Józsa and Kannus, 1997). In 1994, SP-innervation was found to be scarcely present in the cat Achilles tendon (Marshall et al., 1994). In the last decade, several neurotransmitters and neuropeptides have been discovered for the human patellar tendon. Studies on the human patellar tendon have thus shown presence of sensory (SP- and CGRP-containing) nerve fibers, sometimes forming larger nerve bundles (Aune et al., 1996, Lian et al., 2006), in the vicinity of blood vessels, and in relation to arteries and some of the small vessels in the loose paratendinous connective tissue (Danielson et al., 2006a). Furthermore, sympathetic nerve endings have been found in the tendon tissue proper of the patellar tendon, the majority of those being clearly related to blood vessels (Lian et al., 2006). Recent research has also shown that there is a presence of a sympathetic innervation in the paratendinous connective tissue of the patellar tendon and to a small extent in the endotenon of this tendon. The sympathetic innervation is especially marked in the paratendinous connective tissue of the patellar tendon (Danielson et al., 2007b, Danielson et al., 2008).

AChE-containing nerves have been found to be occasionally present in the regions of small blood vessels inside the human Achilles tendon (Alfredson et al, 2001a). Besides this information, nothing is known concerning cholinergic innervation patterns of the Achilles tendon. Apart from the findings of a substantial sympathetic/sensory innervation in the ventral paratendinous connective tissue (Andersson et al., 2007), there is also a lack of information on the sympathetic and sensory innervations innervation within tendon tissue proper of the Achilles tendon of man. There is no information at all concerning the NPY-ergic innervation of the human Achilles tendon. These facts are one of the bases for the studies in the present thesis.



Figue 8. The nerve supply of the Achilles tendon. Innervation of the Achilles tendon occurs via the suralis nerve and cutaneous branches, mainly coming from the saphenus and tibialis nerves. The latter branches are shown principally in the figure.

1.1.7.2 Signal substances traditionally associated with neurons but also being produced by non-neuronal cells

A lot of effort has been made during the last years into investigating the possible production of substances, traditionally found in neuronal cells, in non-neuronal cells. These investigations are of importance as a backround for the present thesis.

The neurotransmitter acetylcholine (ACh) has thus been found in a variety of immune cells, in the epithelium of airways and epidermis, and in smooth muscle cells and endothelial cells (Wessler and Kirkpatrick, 2001**,** Horiuchi et al., 2003, Kawashima and Fujii, 2003). ACh is also known to be produced in skin fibroblasts (Grando, 2006) and urothelial cells (Yoshida et al., 2008). Cancer cells, such as those in small-cell lung carcinoma (Song et al. 2007), do also synthesise and secrete ACh. ACh production also occurs in a variety of lower organisms (Horiuchi et al., 2003, Wessler and Kirkpatrik, 2008).

Catecholamines are produced in e.g. the suprarenal gland and in various endocrine cells (i.e. Zouboulis, 2004). Recent studies in the laboratory at Anatomy suggest that there is local production of catecholamines in cells of synovial tissues (unpublished observations). Neuropeptide Y (NPY) is produced in non-neuronal cells such as neuroblastoma cells (Dozio et al., 2008).

Of particular importance for the present thesis are the reports of local production of nerve signal substances in tendons. Thus, studies using immunohistochemistery and in situ hybridization have given evidence of an occurrence of production of both ACh (Danielson et al., 2006b, 2007a) and catecholamines (Danielson, 2007b, 2007c) in the tenocytes of the human patellar tendon. Of interest is the fact that these evidences were much more evident in

tendinosis patellar tendons than in normal such tendons. Furthermore, results from studies on the vesicular glutamate transporter VGluT2 suggest that glutamate is produced and released by tenocytes in Achilles and patellar tendons, and much more so in tendinosis tendons than normal tendons (Scott et al 2008). mRNA for substance P (SP) has also been shown for Achilles tendon tenocytes, particularly in tendinosis tendons (Andersson., 2008).

In the recent studies on patellar tendinosis, it was shown that the tenocytes not only showed expressions of the enzymes that are related to ACh and catecholamine production but that they also showed expressions of both ACh receptors (muscarinic receptors) (Danielson et al., 2006b, 2007a) and adrenoceptors (Danielson 2007b). In comparison, fibroblasts, the equivalent of tenocytes (tenoblasts), have been shown to express ACh receptors in mammalians (Sekhon et al., 2002). Of further importance is the fact that avian tenocytes have been shown to express mRNA for α_1-adrenoreceptors, as seen via use of the RT-PCR technique (Wall et al., 2004).

Of interest, with tendinosis in mind, is the fact that 1) both norepinephrine and ACh can have proliferative effects and effects on collagen deposition (Oben et al., 2003a, b, Sekhon et al., 2002) and 2) proliferation effects concerning both tenocytes and blood vessels, as well as changes in the continuity of collagen, occur in tendinosis (e.g. Khan et al 1999a). NPY has vasoregulatory as well as angiogenic and proliferative effects (Hansel et al., 2001, Abe et al., 2007, Grundemar & Håkansson 1993).

The observations favouring an occurrence of nerve signal substances and presence of associated receptors in the tendinosis patellar tendon suggest that locally delivered nerve signal substances may play roles in the pathology of, or in the attempted repair response of, tendinosis.

There is no evidence as to whether there is a local production of ACh, catecholamines or NPY in the Achilles tendon. It is also not known if there are cholinergic or adrenergic receptors, nor NPY-ergic receptors, in the tenocytes of the Achilles tendon. That is one background for performing the studies in the present thesis.

1.1.7.3 ACh, catecholamines and NPY: Enzymes for their production and receptors to which they bind

When studying the possible existence of the signal substances described above concerning the Achilles tendon, it is of importance to clarify the enzymes for their production, and the receptors to which they bind. Aspects on acetylcholine, catacholamines and NPY are therefore given.

ACh is mainly synthesised by ChAT (choline acetyltransferase). However, also the enzyme carnitine acetyltransferase (CarAT) can participate in its synthesis (Tusek, 1982). The vesicular acetylcholine transporter (VAChT) shuffles ACh from the cytoplasmic site of synthesis into the storage vesicles in the nerve terminals (Tucek 1982, Eiden 1998). Another transporter is also involved in ACh metabolism, namely the so called choline transporter (CHT1), providing the uptake of choline for ACh synthesis in neurons (Okuda et al., 2000). The enzyme that degrades ACh is acetylcholinesterase (AChE).

It is well-known that there are five different molecular and pharmacological muscarinic acetylcholine receptors (mAChRs): M_1, M_2, M_3, M_4 and M_5. They all display similar pharmacological properties, including activation by acetylcholine (ACh) and muscarine, and inhibition by atropine. Nevertheless, they do also demonstrate varying pharmacology and properties regarding effector mechanisms (Caulfield and Birdsall, 1998). The muscarinic AChRs that are expressed on smooth muscle cells are mainly of the M_2 as well as the M_3 subtypes (for a review, see Caulfield and Birdsall, 1998).

As described above, also catecholamines are interpreted to be produced by tenocytes. In the previous studies depicting catecholaminergic features in tenocytes for the patellar tendon, stainings were made for the rate limiting enzyme in catecholamine production, namely tyrosine hydroxylase (TH) (Kaufman, 1995).

In blood vessels, adrenergic α_1-ARs mediate constriction (Leech and Faber, 1996) and α_{2A}-ARs mediate relaxation (Chotani et al., 2004).

NPY, which belongs to the family of peptides containing peptide YY and pancreatic polypeptide, is a 36-aa neurotransmitter/neuromodulator that was isolated from the porcine brain (Tatemoto et al 1982). This neuropeptide activates the Y receptors, which are G-protein-coupled receptors, highest affinity being shown for Y(1), Y(2) and Y(5) receptors (c.f. Lerch et al 2005, Lindner et al 2008). NPY is markedly involved in blood vessel regulation (Grundemar and Håkanson, 1993, Linder et al., 1996). Given the known effects of NPY, it would be of interest to know if there are Y receptors in the human Achilles tendon and to what extent they occur in tendinosis. Targeting Y receptors has been suggested for several conditions such as obesity, metabolic disorders, hypertension and heart failure (Pedrazzini et al., 2003, Pons et al., 2004, Körner et al., 2008). A main feature in most of these conditions is the occurrence of large numbers of Y1 receptors in the affected tissue (Abe et al., 2007, Körner and Reubi, 2007).

There is no information in the literature concerning the presence or absence of cholinergic, adrenergic or NPY-ergic receptors in relation to blood vessel walls, or in other structures, for the Achilles tendon. That is the fact for man as well as animals.

1.1.8 Tendon metabolism

At the molecular level, all three pathways of energy metabolism are represented in the tendon; the Krebs cycle, anaerobic glycolysis and the pentose phosphate shunt. The ability to use the Krebs cycle and the pentose phosphate shunt decreases with age, whereas the anaerobic glycolysis does not (Józsa and Kannus 1997).

A few decades ago, tendon tissue was suggested to be a rather metabolic inactive structure and to have a low metabolic turnover. More recent studies have, however, revealed that tendon tissue is a tissue with an active energy metabolism, containg cells (tenocytes/tenoblasts) that produce molecules. These cells are related to both structural effects, producing collagens and other matrix proteins (Józsa and Kannus 1997), and signalling effects (Danielson P, 2007), expressing enzymes and receptors normally appearing in cells in other tissues, especially in neurons. The biosynthetic function varies over time, but is generally high during growth and decreases with aging. Tendon disorders (Kannus, 2000) and various loading conditions (Kjaer et al, 2005) may change the levels of this function over time.

It has been shown that the ECM of tendons has the ability to adapt to loading, e.g. through exercise (Kjear et al, 2006). When loading the tendon, there is an increase in collagen synthesis and proteolytic metalloproteinase activity. These changes modify the mechanical properties and the viscoelastic characteristics of the tissue, decrease its stress-susceptibility and probably make it more load-resistant (Riley 2004, Kjaer et al., 2006).

1.1.9 Biomechanical aspects

1.1.9.1 General aspects

As late as in the 1960s, tendons were considered to be relatively inert and inelastic structures, although it is now well accepted that tendons have the ability to store and recoil energy (Kjaer et al., 2006).

When the calf muscles contract they act on the Achilles tendon, forcing the foot into plantar flexion. This contraction enables standing on the toes, walking, running, and jumping. The Achilles tendon is subject to a person's entire body weight during each step and depending upon speed, stride, terrain and additional weight being carried or pushed, each Achilles tendon may be subjected to substantial forces.

In the rat, the mechanical properties of tendons have been shown to change, e.g. leading to increased stiffness, during loading (Monti et al., 2003). A strain level above approximately 4 % starts damaging the tendon fibers and at 8 % the tendon ruptures (ultimate strain) (Józsa and Kannus, 1997). Heavy forces are involved in the Achilles tendon function. Forces that are 12,5 times the body weight during running and 3,6 times the body weight during slow walking have been described to act on the tendons (Komi, 1990, Komi et al., 1992) (c.f. Fig 6).

Elastic energy is, to various degrees, stored in tendons. The capability of this is very important and the Achilles tendon has been shown to be specialized in this respect. It has been shown that the shorter time between the switch from dorsi- to plantarflexion is, the greater is the elongation of the tendon. Furthermore, the work that is loaded onto the tendon increases with higher switch frequency (Kubo et al., 2000). Plantarflexion immediately preceded by dorsiflexion of the foot (as in walking, running and jumping), has been shown to leave a task of storing and releasing elastic energy to a larger extent to the tendon, compared to plantar flexing the foot solely, presumably due to nearly isometric work of the muscle fibers in the calf muscles around the time of the switch (Kawakami et al., 2002). This demonstrates the importance of coordinated structural elements in the muscle and tendon to withstand the very rapid force shifts that are present in these tissues.

1.1.9.2 Exercise, immobilization and age

Studies by Kjaer and collaborators have recently shown an increase in matrix turnover, blood flow, oxygen demand, and levels of synthesis of collagen synthesis and matrix metalloproteinases with mechanical loading (Kjaer et al., 2005). Several studies in animals have shown the tendons to become larger, stronger and more resistant to injury and to receive increased tensile strength, elastic stiffness and total weight by exercise (Józsa and Kannus, 1997, Kannus et al., 2000, Buchanan and Marsh, 2001). Younger animals seem to adapt by increasing the size and weight of their tendons and mature animals to adapt more by structural changes inside the tendon (Kannus et al., 2000).

Immobilization results in reductions in the mechanical properties of the tendon (Kirkendall and Garrett 1997, O´Brien 1997). Research also indicates that tendons subjected to injury and immobilization require mechanical loading to recover **(**Kjaer, 2004**,** Ingber, 2005), the adequate loading though still remaining unknown.

In vivo and in vitro data on tendons during aging are to some degree contradictory, but taken together one could argue that most of the studies confirm that the aging processes lead to alterations in the biomechanical properties in the muscle-tendon complex, such as a loss of elasticity in tendon and a decline in muscle force (Narici and Maganaris, 2006).

1.2 Achilles tendinosis

1.2.1 Terminology, definition, classification

1.2.1.1 Terminology of tendon disorders

The decriptions of tendon diseases and their pathology are historically rather heterogenous.

Until the late 1990s the most commonly used nomenclatures included *tendinitis (tendonitis), peritendinitis (peritendonitis, paratendonitis, paratendinitis), overuse injury of the tendon, tenopathy, and tendinopathy* (Åström and Rausing, 1995, Järvinen et al., 1997, Khan et al., 1999a). In addition, partial and total ruptures can be diagnosed. In the insertion area, including the region of the retrocalcaneal bursa, descriptive diagnoses such as *bursitis, distal achillodynia, enthesitis, insertion tendinopathy, insertion tendinitis, insertitis and retrocalcanear bursitis* are referred to. Often these conditions are temporally distinguished and referred to as acute or chronic.

A very commonly used and term is tendinopathy This is a generic description of the clinical condition in tendons arising from overuse characterized by a clinical combination of pain and swelling of the tendon accompanied by impaired ability to perform strenuous activity (Järvinen et al., 2001, Sharma and Maffulli, 2006). However, the term tendinopathy, does not give any information about the underlying pathology of the tendon disorder (Maffulli et al., 1998, Khan et al., 1999a).The widespread use of this term underlines the fact that the knowledge of the pathogentic processes in painful tendons is to a large extent still lacking.

Tendinitis, peritendinitis, and paratendonitis (Kvist et al., 1987) describe conditions with an inflammatory component (Puddu et al., 1976), and in clinical practice, these have even been the misnomers for conditions in tendons when no inflammatory reactions can be found (Khan and Cook, 2003a). During the past decades researchers have, through histological (e.g., Khan et al., 1996, Movin, et al., 1997, Järvinen et al., 1997, Teitz et al., 1997, Maffulli et al., 1998, Riley, 2004) and intratendinous microdialysis (Alfredson et al, 1999, Alfredson et al, 2001b) studies shown that the chronic (more than 3 months of symptoms and signs) painful tendon conditions are not inflammatory at the moment in time when tissue is harvested or the microdialysis is performed, respectively.

Not all tendinopathies are overuse chronic conditions, as one third of the patients with Achilles tendinopathy have not participated in vigorous physical activities (Rolf and Movin, 1997). Several studies have instead suggested a process with partly degenerative tendon tissue changes. This started discussions suggesting another term, “tendinosis”, for chronically painful tendon conditions (e.g. Khan et al., 1999a, 2002, Alfredson and Lorentzon, 2000a, b). Even this term has been under debate. Degenerating and/or mechanically damaging, or other, processes are leaving the tendon to loose its features and the tendon tissue becomes yellowish, looses its glistening appearance, and changes with respect to biomechanical properties (Kvist, 1994).

1.2.1.2 Definition of tendinosis

Patients having tenderness, swelling and impaired tendon function are generally diagnosed to have tendinopathy. If objective evaluation of the tendon, using ultrasound, MRI or biopsies, show structural tendon changes in the affected part of the tendon, this is generally defined as tendinosis (Alfredson, 2005a).

There are also other definitions. One definition implies that tendinosis should be regarded only as a histopathological diagnosis (Maffulli et al., 1998, Peers and Lysens, 2005).

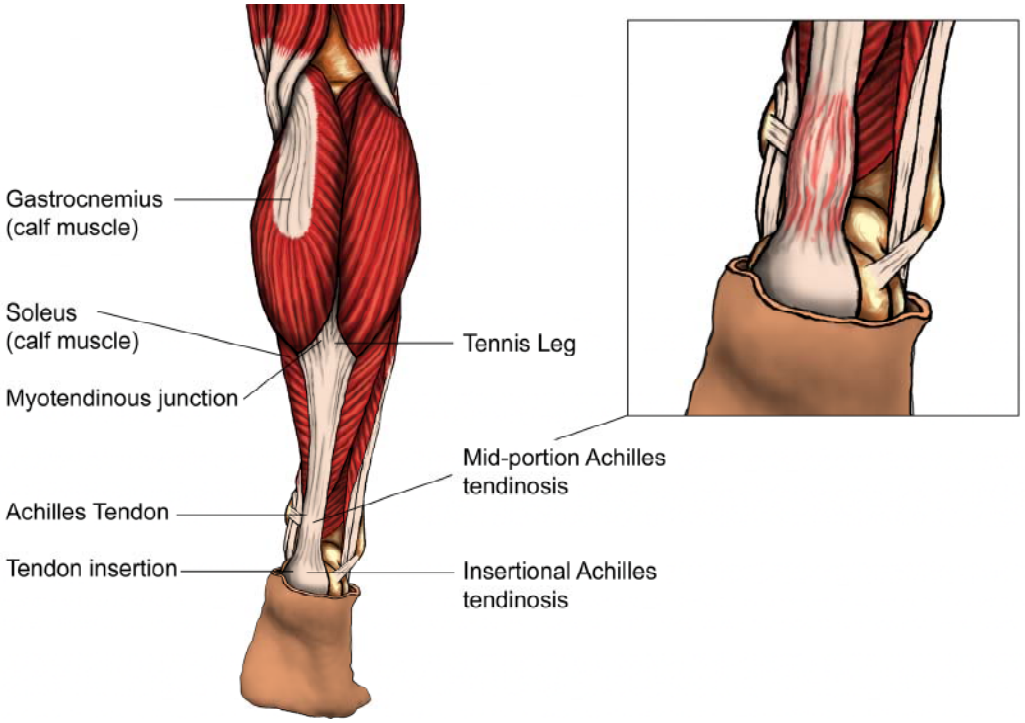


Fig. 9. The lower limb, right leg. Overview of the anatomy. Location of tendon pathology (inset). Note the hyperaemia (symbolically shown) in the midportion Achilles tendnosis.

1.2.1.3 Classification and grading of tendinosis

Chronic painful Achilles tendon conditions can also be assessed topically, three main areas along the tendon being identified; the proximal part (muscle-tendon junction), midportion (Midportion Achilles tendinosis) (Movin, 1998) and distal part (Insertional Achilles tendinosis) (Fahlström, 2001) (Fig. 9). At the proximal part, “Tennis leg” rupture can occur (Johnson, 2000).

The severity of Achilles tendinosis has been described to evolve in four stages. From no pain during exercise (Stage 1), to a stage when it hurts too much to exercise or run (Stage 4). These are expressed in the VISA-A questionnaire (Victorian Institute of Sport Assessment-Achilles) (Robinson et al., 2001). This questionnaire is a helpful tool in evaluating the symptoms, and to assess adequate starting therapeutic interventions, but also to depict work ability. A validity investigation of this questionnaire has been performed by (Grävare Silbernagel, 2006), where it is stated that it can be used in research as well as in the clinic.

Histopathologic classifications, or rather grading systems, have also been suggested. Åström and Rausing, in 1995, graded the histopathologic appearances from 0 (normal) to 3 (maximally abnormal) when comparing normal Achilles tendons with Achilles tendons with severe tendinosis (Åström and Rausing, 1995). The Bonar scale (Cook et al., 2004a), uses a fourpoint scale to semiquantitatively assess histopathological changes in tendinosis.

1.2.2 Histopathological tendon tissue changes

The histopathological findings in Achilles tendinosis have been well described by several authors (Åström and Rausing, 1995, Józsa and Kannus, 1997, Järvinen et al, 1997, Khan et al., 1999a). In tendinosis tendons the collagen is disorganized, and there is an increased mucoid ground substance, mostly GAGs (glycose aminoglycanes) (Movin et al., 1997) that is deposited between the collagen fascicles. The tenocytes become bulky, plump and ovoid, and have more rounded nuclei. Some tenocytes show a fibroblastic or myofibroblastic appearance and there is a varying degree of hypercellularity. Ingrowth of small vessels are seen, but no inflammatory cells as a sign of inflammation (Khan et al., 1996, 1999a).

1.2.3 Diagnostics, symptoms and signs

1.2.3.1 Patient history, physical examination

Physical examination should include thorough inspection to search for muscle atrophy, swelling, asymmetry, and erythema of the tendon, range-of-motion testing, palpation for tenderness, and tiptoeing that simulates tendon loading in order to clarify if this reproduces pain (Wilson and Best, 2005). The Achilles tendon is easy to inspect and palpate with the patients standing on their knees on the examination bench, allowing their feet to hang over the side. The continuity of the muscle-tendon complex can be assessed through the calf muscle squeeze test (Grävare Silbernagel, 2006). If the muscle tendon unit is intact the foot will plantarflex during the test. Clinically it is important to avoid missing an Achilles tendon rupture as the treatment is totally different compared to that of tendinosis. A total rupture leaves an inability of tiptoeing and the calf muscle squeeze test is thus negative in affected patients (Grävare Silbernagel, 2006).

1.2.3.2 Diagnosis and imaging

Classification can be taken further by different imaging techniques such as magnetic resonance imaging (MRI) and ultrasonography (US).

US techniques for examining the musculoskeletal system became widely accepted and spread in the beginning of the 1980`s (Moss and Mowat, 1983, Laine 1984). In its simplest forms and concerning Achilles tendinosis, US shows changes in the tendon consisting of localized widening, an irregular fibre structure of collagen and hypoechoic areas (Archambault et al., 1998, Öhberg et al., 2001b), whereas MRI shows a localized widening and increased signal intensity (e.g. Shalabi et al., 2002, 2004). US, at its best using high-resolution probes (Grechenig et al., 2002) and/or colour power Doppler flowmetry, can measure the velocity (colour Doppler velocity; CDV) and direction of the blood flow in the tendon tissue proper and in the paratendinous connective tissue. Both these methods contribute to reveal hypoechoic areas, irregular tendon structure, localized tendon widening,

increased blood flow due to hypervascularity (neovascularisation), and paratenon thickening (Öhberg et al., 2001a, Öhberg and Alfredson, 2002, Leung and Grifith, 2008). MRI and US are regarded as the methods of choice in the investigation of the Achilles tendon, both being described to be justified in tendon diagnostics in general and to have a good correlation to surgical and histological findings (Neuhold et al., 1992, Lehtinen et al., 1994., Paavola et al., 1998, Åström et al., 1996, Movin et al, 1998, Jacobson, 1999, Goodwin, 2000, Rasmussen, 2000). Both methods do also show a relatively good correlation with clinical assessment (Archambault et al., 1998, Khan et al., 2003b, Movin et al., 1998)

Clinically, the severity of pain and functional impairment has been shown to be correlated to increased mean intratendinous MR signal in the painful chronic midportion Achilles tendopathy (Gärdin et al., 2006), and clinical outcome to be positively associated with graded MRI, i.e. the better clinical outcome, the lesser are the grades of MR signal abnormality (Khan et al., 1999b, 2003b).

1.2.3.3 Symptoms and signs

While acute overloading often leads to ruptures and tears in the soft tissue of the musculoskeletal system, the clinical symptoms of Achilles tendinosis do instead include gradually increasing load-related localized pain, morning stiffness, tenderness and swelling in the morphologically changed zones (Alfredson and Lorentzon, 2000a, b, Kader et al., 2002, Wilson and Best, 2005). In initial stages, pain disappears during warm up allowing the affected indviduals to proceed with, their physical activity, but thereafter the pain gradually progresses, and ultimately the pain totally inhibits loading (Rolf, 1995). Many patients have had pain for many months, or pain that comes and goes during long periods, when they seek for help. Initially, pain often starts subsequently to heavy physical activity, but as injury progresses some patients start feeling pain during physical activity. Sometimes daily activities such as walking are eliciting pain, and in some severe cases patients even report pain at night. The tenderness is located in the midportion of the Achilles tendon, 2-6 cm proximal to the tendon insertion. Often there is a thickening of the tendon in the more chronic stages (Grävare Silbernagel, 2006).

The symptoms do not always correlate positively to the actual function of the muscle-tendon unit. In a study on 37 patients suffering from Achilles tendinopathy in the midportion of the tendon, with symptoms for >2 months, symptoms and function were evaluated at the initiation of the study and after 1 year, using the Swedish version of the Victorian Institute of Sports Assessment-Achilles questionnaire (VISA-A-S) for defining the symptoms, and a validated test battery for evaluation of the lower leg muscle-tendon function. A rehabilitation programme, under the supervision of a physiotherapist, was utilized for 6 months. Only 25% (4/16) of the patients who had full symptomatic recovery had achieved full recovery of muscle-tendon function as measured by the test battery (Silbernagel et al., 2007). This shows the importance of further research and development of validated treatment follow-up studies.

1.2.4 Epidemiology

Midportion Achilles tendinopathy has been reported to account for 55-65% of all the Achilles tendon injuries (Kvist , 1991, 1994, Järvinen, 1992, Järvinen et al., 2005).
Achilles tendinopathy is mostly seen in middle-aged people, 30-60 years old (Kvist, 1994 Paavola et al., 2000 and 2002, Alfredson et al., 2003c). 30% have bilateral injuries (Öhberg and Alfredson, 2004a, Grävare Silbernagel, 2006). The incidence has increased during the past decades as a result of greater participation in recreational and competitive sporting activities. In a study on 3336 competetive and recreactionallly active patients, 698 patients were found to have Achilles tendon complaints, of whom, 66 % had Achilles tendinopathy (Kvist, 1991). Jörgensen and collaborators reported that Achilles tendinopathy accounted for 10.5% of all overuse injuries in badminton players (Jörgensen and Winge, 1990), and and in several studies it has been reported that the incidence of Achilles tendinopathy among runners is 6-18% (Clement et al., 1984, Soma and Mandelbaum, 1994, Józsa and Kannus., 1997, Lysholm and Wiklander, 1987).

Treatment studies show that men is accounting for 45-86% of cases with Achilles tendinosis, with the lower percentages in the more recent studies (Nelen et al., 1989, Alfredson et al 1998, Paavola et al., 2000, Mafi et al., 2001, Öhberg and Alfredson, 2002). In a recent study it was stated that Achilles tendinopathy is equally common in men and women (Grävare Silbernagel, 2006).

It is nowadays stated that the condition is spread among people with a rather sedentary lifestyle (Alfredson and Lorentzon, 2000a). In a study of 58 patients with tendinoses, 31% of these did not participate in active sports or in any vigorous physical activity (Rolf and Movin, 1997).

The musculotendinous junction has been described to be the weakest point in the muscle-tendon complex, the junction having a pronounced force absorbing function. This area is at risk for strain injuries, especially through acute high force loading (Józsa and Kannus, 1997). Acute injuries with ruptures, specifically in the distal medial head of the gastrocnemius, often referred to as "tennis leg", is more common than chronic lesions.

1.2.5 Etiology, pathogenesis

It is very important to establish the underlying pathology of Achilles tendinopathy/tendinosis as a basis for effective validated high level of evidence treatment methods. Although overuse is described to be commonly involed in the condition (Leadbetter, 1992, Józsa and Kannus, 1997), the etiology of Achilles tendinopathy is, still not fully understood. Many basic risk factors have despite this been suggested, presumably to a great extent valid even for Achilles tendinosis as it has been stated that as much as 90% of cases with Achilles tendinopathy may be tendinosis (Åström and Rausing, 1995).

It is out of the scope of this thesis to describe all ris-factors in detail. To summarize, they can be devided into intrinsic risk factors (e.g. anatomic misalignment and high body weight) and extrinsic risk factors (e.g. training errors, sedentary lifestyle). Involvement of biochemical factors, exercise in excess of healing capacity, lack of rest, ECM matrix changes, existence of insufficient vascular beds, occurrence of hypoxia/anoxia, and an overexpression of NO-synthase have been discussed. For further information, literature is recommended (e.g. Kvist, 1991, Józsa and Kannus, 1997, Riley, 2005, Holmes and Lin, 2006, Jonsson, 2009, Grävare Silbernagel, 2006).

When a lesion as in chronic Achilles tendinopathy already has arisen, it is considered to be associated with hyperaemia from an uncertain origin. The findings of hyperaemia in Achilles

tendinosis patients are confirmed in several studies (e.g. Öhberg et al., 2001b, Knobloch et al., 2006). Thus, studies on Achilles tendons using ultrasonografic Doppler teqnique have shown signs of increased vascularity inside and outside the ventral part of the area with tendon tumification changes (measured as increased blood flow and interpreted as neovascularisation) in midportion Achilles tendinosis but not in any of the pain-free control tendons.

In women, oral contraceptives or hormone replacement therapy is a risk factor for Achilles tendinosis (Holmes and Lin, 2006), although the mechanisms for this is still unclear.

To summarize, the etiology of chronic Achilles tendinosis, it is in principle discussed in terms of three main theories: A mechanical, a vascular, and a neural theory. None of these theories solely fully explain the intriguing questions of chronic painful midportion tendinosis. Maybe an interface theory combining these is the way to go in the future.

1.3 Tendon healing in general

The tendon is believed to undergo three phases during the process of healing in response to advanced tendon disease/injury. This was first shown in animal studies (Parry et al., 1978, Reddy et al., 1999). Approximately the same phases are believed to occur in humans (Sharma and Maffulli, 2006). In the acute inflammatory phase, that renders 3-7 days after injury, the infiltrating inflammatory cells remove damaged tissue. Initially vasoactive and chemotactic factors are also being released. Increased vascular permeability, initiation of angiogenesis, stimulation of tenocyte proliferation, and recruitment of more inflammatory cells occurs (Murphy et al., 1994). This cascade of events is thought initiate tenocytes to migrate to the wound and start synthesizing type III collagen (Oakes, 2003). After a few days, a remodeling phase starts and type III collagen synthesis peaks during this stage. This stage lasts for a few weeks and during which glycosaminoglycan and water content remain high (Oakes, 2003). A third stage involving further modelling commences after approximately 6 weeks, were the healing tissue is resized and reshaped. A decrease in cellularity and in collagen and glycosaminoglycan synthesis occurs. The first step in this stage is consolidation, which continues up to 10 weeks (Tillman and Chasan, 1996). In this period, the repair tissue changes from cellular to fibrous and the tenocyte metabolism is high. The collagen fibres become aligned in the direction of stress (Hooley and Cohen, 1979) and a higher proportion of type I collagen is synthesized (Abrahamsson, 1991). After approximately 10 weeks, the maturation stage occurs, with gradual change of fibrous tissue to scar-like tendon tissue over the course of one year (Hooley and Cohen, 1979). In the latter half of this stage, the tenocyte metabolism and tendon vascularity decline (Amiel et al, 1987).

Unfortunately, the repair process after tendon rupture often results in a morphologically different and biomechanically inferior structure compared to the normal tendon. In animal studies, impaired tendon healing has been reported to have negative effects. The biomechanical properties are changed, e.g. tensile strength and energy absorption are reduced (Kader et al., 2002). The tenocytes in regenerated tissue are described to have greater amounts of rough endoplasmatic reticulum and contractile proteins (actin and myosin), and furthermore, the tenocytes are more abundant and less uniformly distributed (Postacchini F et al., 1978). There is still little knowledge of how the healing process in detail is proceeding, specifically regarding the collagen restitution process.

Adequate tissue perfusion and oxygenation is regarded as an absolute prerequisite for a successful repair of a tissue, since essential wound healing mechanisms such as collagen deposition are oxygen-dependent reactions (Beckert et al., 2007).

The results from a study on the rat suggested early nerve regeneration to be a prerequisite for healing as an orchestrated, temporal appearance of nerve fibers expressing peptides with different actions in early healing of ruptured Achilles tendon was found (Lin et al., 2001). NOS (nitric oxide synthase) has been shown to be overexpressed in the healing process of the Achilles tendon. NOS activity is expressed in a temporal fashion in healing fibroblasts, whilst in normal tendons, very little NOS activity is found (Murrell, 2007). Studies in human tendon cells, show that nitric oxide can enhance collagen synthesis in vitro (Xia et al., 2006).

Furthermore, nerve signal substances have been described to participate in tendon healing in animals (Messner et al., 1999, Ackermann et al., 2001, 2002). Detailed information on nerve signal substances within the tendon tissue proper in the human Achilles tendon is therefore needed. Thus, it might be asked as to whether such signal substances are expected to play roles in remodelling in Achilles tendinosis.

1.4 Tendon and pain

Pain symptoms in individuals with tendinosis tell us very little about the pathology behind it. How pain is mediated in healthy tendons and tendons with tendinosis still remains a puzzling issue. Nevertheless, the existence of a sensory innervation inside and around tendons (the patellar tendon) (Danielsson et al., 2006a), in the ventral peritendinous tissue of Achilles tendons (Andersson et al., 2007), give aspects on this issue. Neuroanatomical changes, like the occurrence of nerve sprouting and disorganized small nerve fibers in patellar tendinosis (Sanchis-Alfonso et al., 2001) are also interesting. Furthermore, neurovascular ingrowth has been suggested to be a possible source of pain in chronic midportion Achilles tendinosis (Alfredson et al., 2003c).

Regarding tendon pain, a “biochemical model” has been discussed during recent years. The presence of biochemical mediators that may influence nociceptors, inside or around the tendon, have thus been suggested to be of importance (Khan, 2000, Danielson 2007, 2009). There may thus e.g. be an involvement of the nociceptor neuropeptides SP and CGRP. The SP-preferred neurokinin 1 receptor (NK-1 R) has actually been detected in the Achilles tendon, particularly in tendinosis (Forsgren et al., 2005, Andersson et al., 2008).

Of interest is the fact that glutamate and the related receptor NMDAR1 (N-methyl D-aspartate1 receptor) have been found in tendons. In the Achilles tendon, Alfredson and collaborators showed higher levels of glutamate in tendinosis than in normals in a microdialysis study (Alfredson et al 1999), and moreover a presence of NMDAR1 in relation to nerves has been revealed (Alfredson et al., 2001a).

In addition to these findings, mechanical loading is found to elicit increased interstitial concentrations of the nociceptive agent bradykinin, which is known to cause vasodilation in resistance vessels and to be involved in regulation of tissue blood flow, in the paratendinous connective tissue of human tendon (Langberg et al., 2002). It also is of interest, to notice that ACh seems to play a modulating role in peripheral nociception (Wess et al., 2003) and is even capable of inducing pain (Vogelsang et al., 1995). In the muscarinic cholinergic receptor family, the M_2 receptor is of special interest as it may be involved in mediating nociception (Haberberger and Bodenbenner, 2000), and to have an antinociceptive potential (Bernadini et al., 2002).

There is no information as to whether there is local production of ACh, being possibly related to the “biochemical pain model” for Achilles tendinosis. Nor is it known if M_2 receptors are present in Achilles tendon tissue. It is also of importance to clarify the patterns

for catecholaminergic signal substances. It is known that the efferent sympathetic nervous system can enhance pain (Baron et al., 1999).

A new method to treat tendinosis pain: Polidocanol injection therapy
A newly developed technique using sclerosing Polidocanol injections targeting the area with the increased blood flow has been developed concerning Achilles tendinosis. The injections reduce the blood flow and after a series of injections, a majority of patients experience a relief of the pain. Follow-up studies have thereto shown no signs of Doppler detectable blood flow, interpreted as an absence of neovessels (Lind et al., 2006).
This technique has also been utilized with success in patellar tendinosis (Alfredson and Öhberg, 2005a, Hoksrud et al., 2006) and lateral epichondylitis (Zeisig et al., 2006).

1.5 Final comments: What became the focus in the studies of this thesis

As seen above, many aspects are of great interest concerning the structure and function of the normal Achilles tendon and concerning the events that occur when it is diseased. Focus was in the present thesis devoted to the nerve signal substances ACh, catecholamines and NPY, and to their receptors, as well as to the innervation patterns of the normal and diseased tendon (Achilles tendinosis). There is a lack of knowledge in these respects. This is a drawback as effects of locally produced signal substances and the innervation are likely to be of great importance in tendinosis.

My personal aim for making the studies that developed into this thesis was hereby to try to contribute to the process of revealing the “Achilles heel of the chronic Achilles tendon pain”, the scarcity or even total lack of information on the above aspects being a “weak point” (“an Achilles heel”). In the extension of the findings that are made, I hope we could discover more of the mechanisms that occur in Achilles tendinosis and to get new ideas of future treatment regimes for the condition.

2. AIMS

The overall aim of this thesis was to learn more about the characteristics of the normal and midportion tendinosis Achilles tendon, specifically regarding the innervation patterns and the intercellular communication systems.

The specific aims were

1) to **investigate the innervation patterns** of the normal and tendinosis Achilles tendon regarding

a) **general innervation** (PGP 9.5) [I].

b) **sensory innervation** (SP/CGRP) [I].

c) presence or not of **cholinergic markers** (VAChT/ChAT), and the presence or not of muscarinic M_2 receptors [II].

d) **sympathetic innervation/NPY-ergic innervation** (TH/ NPY) [III, IV], and the presence or not of α_1 adrenoreceptors [III] and Y1 and Y2 receptors [IV]

2) to examine for the possible occurrence of **a non-neural local intercellular communication system** as shown by evidence of production of signal substances and **presence of their receptors** in normal and tendinosis Achilles tendons. This was done by investigating the possible occurrence in tenocytes of

a) the ACh synthesizing enzyme ChAT, ChAT mRNA, VAChT and M_2 receptors [II].

b) the catecholamine-synthesizing enzyme TH, TH mRNA and of α_1 adrenoreceptors [III].

c) NPY [III, IV], Y1 and Y2 receptors [IV]

3) to study the pattern of morphologic derangements of Achilles mid-portion tendinosis tendons in relation to the aspects described above.

3. MATERIAL AND METHODS

3.1 Subjects

3.1.1 Subjects in total

The subjects participating in the studies of this thesis were either symptomatic patients with midportion Achilles tendinosis (Table 1), or asymptomatic individuals with normal Achilles tendons (controls; normals) (Table 2). In total, samples from 49 individuals, 27 females and 22 males (Table 3) were analyzed. All participants choose on a voluntary basis to be included in the research program. They were otherwise healthy, free from medication and were non-smokers.

3.1.2 Achilles tendinosis patients

This group consisted of in total 42 patients surgically treated for chronic painful midportion Achilles tendinosis (Table 1), diagnosed through clinical examination combined with ultrasonography or MRI. Their level of physical activity varied from low recreational to elite sport levels. The duration of symptoms varied from 12-36 months (mean: 19 months), and there was activity related pain in a tender thickening of the Achilles tendon midportion (located 2–6 cm cranial to the tendon insertion into the calcaneus bone). The tender thickening of the tendon corresponded to a region were tissue changes were revealed by ultrasonography (localized widening, irregular structure and focal hypo-echoic regions) or MRI (localized widening, increased signal intensity).

Sixteen patients in the tendinosis group (8 females, 8 males) in Study IV, the subgroup, had been subjected to sclerosing Polidocanol injection treatment (Table 3) for 3-5 times before tissue samples were harvested (for details of this treatment see Öhberg and Alfredson 2002, Alfredson and Öhberg, 2005a, b). The patients had prior to the injection treatment undergone eccentric training with poor clinical results.

3.1.3 Controls

In this group, 10 individuals (Table 2) were included as normal controls. All had pain-free and normal Achilles tendons at clinical examination. Ultrasonography showed normal tendons (normal tendon thickness and no structural changes or hypoechoic areas). The level of physical activity varied from low to moderate recreational activities (jogging, work-out, walking, cycling).

Code	Age	Study	Comment
ATK1	46 years	I, II, III, IV	
ATK2	49	I, II, III, IV	
ATK3	51	I	
ATK4	56	I, II, III, IV	
ATK5	46	I, II, III, IV	
ATK6	48	I, II, III, IV	
ATK7	42	I, II, III, IV	
ATK8	50	I, II, III, IV	
ATK9	55	I, II, III, IV	
ATK10	49	I, II, III, IV	
ATK11	34	I, II, III, IV	
ATK12	49	I, II, III,	
ATK13	41	I, II, III, IV	
ATK14	51	II, III, IV	
ATK15	54	IV	Scl
ATK16	39	IV	Scl
ATK17	49	IV	Scl
ATK18	57	IV	Scl
ATK19	56	IV	Scl
ATK20	57	IV	Scl
ATK21	36	IV	Scl
ATK22	58	IV	Scl
ATM1	44	II, III, IV	
ATM2	42	II, III, IV	
ATM3	51	I, II, III, IV	
ATM4	35	I, II, III, IV	
ATM5	38	I, II, III, IV	
ATM6	54	I, IV	
ATM7	36	I, IV	
ATM8	42	I, II, III, IV	
ATM9	46	I, II, III, IV	
ATM10	47	I, II, III, IV	Scl
ATM11	42	IV	Scl
ATM12	41	IV	Scl
ATM13	36	IV	Scl
ATM14	61	IV	Scl
ATM15	26	IV	Scl
ATM16	40	IV	Scl
ATM17	48	IV	Scl

Table 1: Patients with symptomatic Achilles tendons from whom biopsies were harvested; painful midportion Achilles tendinosis. ATK: female, ATM: male. Scl: Biopsies harvested from patients previously treated with 3-5 injections of sclerosing Polidocanol.

Code	Age	Study
AK1	42 years	I, IV
AK2	22	I, II, III, IV
AK3	44	I, II, III, IV
AK4	46	I, II, III, IV
AK5	46	I, II, III, IV
AM1	32	II, III, IV
AM2	34	I, II, III
AM3	45	I, II, III
AM4	40	I, II, III, IV
AM5	60	I, IV

Table 2: Subjects with asymptomatic Achilles tendons from whom biopsies were harvested; controls. AK: female, AM: male

	Study I	***Study II***	***Study III***	***Study IV (all)***	***Study IV (subgroup)***
Subjects	30	29	29	45	
M/F	12/18	12/17	12/17	20/25	(cf below)
Age (range)	45 (22-60)	41.8 (22-56)	41.8 (22-56)	45.4 (22-61)	
Tendinosis	21	21	21	37	16
M/F	8/13	8/13	8/13	17/20	8/8
Age (range)	45 (34-56)	45 (34-56)	45 (34-56)	46.2 (26-61)	46.8 (26-61)
Controls	9	8	8	8	
M/F	4/5	4/4	4/4	3/5	
Age (range)	41.2 (22-60)	38.5 (22-46)	38.5 (22-46)	41.5 (22-60)	

Table 3. Overview of subjects for all studies. M/F: Male/Female, Age: mean age. Study IV (Subgroup): Patients previously treated with 3-5 injections of sclerosing Polidocanol. It was not possible to use specimens from all 49 individuals in all studies.

3.1.4 Inclusion and exclusion criteria

Achilles tendinosis

Inclusion criteria

- Pain in the Achilles tendon for more than 3 months
- Clinical symptoms: tender thickening in the Achilles tendon midportion, and ultrasound or MRI verified tendinosis changes corresponding to the region with clinical findings

Exclusion criteria

- Acute or chronic inflammatory diseases
- Diseases or injuries causing radiating pain in the lower limb
- Smokers

Controls

Inclusion criteria

- No ongoing or previous pain inthe Achilles tendon
- Normal findings on ultrasonography
- Good health – on no medication
- No diseases or injuries affecting the lower extremities
- Non smokers

3.2 Ethics

All studies included in this thesis were approved by the Committee of Ethics at the Faculty of Medicine, Umeå University, and by the Regional Ethical Review Board in Umeå. All experiments, contacts with participating subjects, study design and implementation were conducted according to the principles in the Declaration of Helsinki 2000, 5th revision.

3.3 Sampling and tissue processing

All surgical procedures were performed under strict sterile conditions. A local anesthetic (4-5 ml Prilocaine hydrochloride, 10 mg/ml; Södertälje, Sweden) was injected into the skin and subcutaneous tissue in the controls, whilst the tendinosis patients were submitted to general anaesthesia during the surgical treatment.

The biopsies from the controls were carefully taken from the midportion of the Achilles tendon (2-6 cm from the tendon insertion), and corresponding to the level were the tissue samples were harvested in the tendinosis subjects (cf below). The biopsies from the tendinosis tendons were taken during surgical treatment. In study I-IV the biopsies were taken from the central and dorsal parts of the tendon (measuring approximately 2 mm in width and 1-5 mm in length). In the subgroup in study IV, the biopsies were taken from the ventral part of the tendon.

The tissue samples from both tendinosis and normal tendons were processed in two different ways. They were either chemically fixed before freezing, or were directly frozen after the sampling process, i.e. were processed chemically unfixed. Usually, two different samples were taken from the same individual. Both chemically fixed and unfixed samples were thus available from a majority of the individuals studied. In order to fixate the specimens, they were treated by immersion overnight at 4°C in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, and were then thoroughly washed in Tyrode's solution, containing 10% sucrose. They were then mounted and frozen in propane, chilled with liquid nitrogen, and thereafter stored at –80°C until sectioning. The specimens

that were not chemically fixed were frozen directly after transport to the laboratory. The chemically fixed and the unfixed specimens were mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, Illinois). The specimens were further processed for immunohistochemistry

Concerning tissue samples for processing with in situ hybridization (ISH), both unfixed and fixed biopsies were utilized, and postfixation according to the ISH protocol was performed (cf below 3.6).

3.4 Sectioning and mounting

After having been frozen and stored at –80°C, the samples were cut in a cryostat. Consecutive series of sections, that had a thickness of 7-10 microns, were produced. The sections were mounted on slides pre-coated with crome-alun gelatin, and were then left to dry, whereupon they were ready to be processed either for immunohistochemistry (immunofluorescence by TRITC/FITC or enzymatic antigen detection (PAP)) or to be stained with hematoxylin-eosin in order to display the tissue morphology. For in situ hybridization, see below (3.6).

3.5 Immunohistochemistry (IH)

3.5.1 Immunofluorescence (TRITC, FITC) methods

These methods were used for demonstration of α_1-AR (III), αSMA (II), CD 31 (II), CGRP (I), ChAT (II), M_2R (II), NPY (III, IV), PGP9.5 (I), SP (I), TH (III), VAChT (II), Vim (II), Y1R (IV), and Y2R (IV). All antibodies were polyclonals, except for the antibodies used to detect CD 31, αSMA and Vim, which were monoclonals.
Sections to be processed for α_1-AR, ChAT, NPY, TH, and VAChT, were first pretreated with acid potassium permanganate for 2 min, a procedure that was used to enhance specific immunofluorescence reactions (Hansson and Forsgren, 1995), followed by rinsing three times for 5 min each in phosphate-buffered saline (PBS). Then followed incubation for 20 min in a 1% solution of detergent Triton X-100 (Kebo Lab, Stockholm) in 0.01 M PBS, pH 7.2, containing 0.1 % sodium azide as preservative, and again rinsing three times for 5 min each in PBS.

The sections were thereafter incubated for 15 min at room temperature in 5 % normal swine serum (code: X0901; Dakopatts, Glostrup, Denmark) in PBS supplemented with 0.1 % bovine serum albumin (BSA) for α_1-AR, CGRP, NPY, M_2R, PGP9.5, SP, TH, and VAChT (Sigma antibody) immunostaining or in 5 % normal donkey serum (code: 017-000-121; Jackson ImmunoReserach, PA) in PBS supplemented with 0.1 % BSA for ChAT, VAChT (Santa Cruz antibody), Y1R and Y2R immunostainings. Then the sections were incubated with the primary antibody, diluted in PBS with BSA, or in only PBS for ChAT and VAChT (Santa Cruz antibody), in a humid environment. Incubation was performed overnight at 4°C or for 60 min at 37°C. After incubation with specific antiserum, and three 5 min washes in PBS, another incubation in normal swine serum (or normal donkey serum) followed as described above, after which the sections were incubated with secondary antibody corresponding to tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine antirabbit IgG (code: R0156;

Dakopatts, Glostrup, Denmark), diluted 1:40 in PBS with BSA, for 30 min at 37°C for α_1-AR, M_2R, NPY, TH, VAChT (Sigma) staining or with FITC-conjugated AffiniPure donkey antigoat IgG (Jackson ImmunoResearch, PA, 705-095-147), diluted 1:100 in PBS, for 30 min at 37°C, for ChAT, VAChT (Santa Cruz), Y1R and Y2R demonstration. After a last 3 x 5 min wash in PBS, the sections were finally mounted in Vectashield microscopy mounting medium.

Concerning immunostaining for the monoclonal antibodies (αSMA, CD31 and Vim) the following exceptions from the above scheme was used: The normal serum used was normal rabbit serum and the secondary antibody was TRITC-conjugated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark, Z0259), diluted 1:40.

3.5.2 Double-staining

For double-labeling, the sections were incubated with antibodies raised in rabbits and mice. Rabbit antibodies raised against M_2Rs and mouse monoclonal antibodies raised against CD31 or alpha smooth muscle actin (Table 4) were used as primary antibodies. Incubation for each was made for 60 min at 37°C.

As secondary antisera, the same TRITC-conjugated swine antirabbit IgG as described above was used, as well as FITC-conjugated rabbit antimouse IgG or donkey antimouse IgG (Dakopatts, Glostrup, Denmark) secondary antbodies. Incubation with each secondary antiserum lasted for 30 min, and was performed at 37°C. When FITC-conjugated donkey antimouse IgG was utilized, normal donkey serum was used instead of normal rabbit serum.

3.5.3 Peroxidase antiperoxidase (PAP) teqnique

Concerning demonstration of CD31(II), CGRP(I), M_2R(II), NPY(III), Neurofilament (II), PGP 9.5 (I), SP (I), and TH (III) (Table 2), PAP staining was used.

In order to reveal epitopes hidden by the formaldehyde fixation, microwave antigen retrieval was used as a first step for NPY (III) and TH (III) detection. The slides were hereby initially placed in plastic Koplin jars filled with 0.01 mol/l citrate buffer, pH 6.0. Thereafter the jars were placed in a microwave oven (55°C) and were then boiled at 650 W for 5 min x 3. After each cycle, the slides were transferred to new jars with fresh citrate buffer (0.01 mol/l, pH 6.0) and cooled down to room temperature. After 20 min cooling, the sections were rinsed in PBS buffer for 5 min x 3.

After a possible antigen retrieval as described bove (performed as an alternative for TH and NPY detection in III), all sections to be processed with the PAP technique were initially pretreated with acid potassium permanganate for two min, after which followed rinsing in PBS for 5 min x 3. Thereafter the slides were incubated in a 1 % Triton X-100 solution (Kebo Lab) in 0.01 M PBS, pH 7.4, for 20 min, and then washed in PBS 5 min x 3, after which endogenous peroxidase activity was blocked by 30 min incubation in 1 % H_2O_2. After subsequent washing in PBS 5 min x 3, the sections were incubated with 5 % normal swine serum (code: X0901; Dakopatts, Glostrup, Denmark) in PBS supplemented with 0.1 % BSA for 15 min at room temperature. Incubation with primary antibody, diluted in PBS with BSA, was thereafter performed for 60 min at 37°C. After an additional washing in PBS 5 min x 3 and another incubation with normal swine serum as described above, the secondary antibody was applied (swine anti-rabbit, diluted 1:100 in PBS with BSA; code: Z196; Dakopatts, Glostrup, Denmark). The sections were thereafter further rinsed in PBS 5 min x 3, then

incubated for 30 min in room temperature with peroxidase-labeled IgG immunoglobulin (1:100; PAP-rabbit; code: Z0113, Dakopatts, Denmark), prepared from horseradish peroxidase and polyclonal rabbit anti-horseradish peroxidase and to which the secondary antibody used is known to bind. Thereafter followed, again rinsing in PBS for 5 min x 3, and development in diaminobenzidine (DAB) solution for 5 min. Before dehydration, the sections were dipped for 20 sec in Mayers hematoxylin for delineating general tissue morphology. After that the sections were dehydrated and finally mounted in DPX microscopy mounting medium.

Concerning the staining for CD31, the pattern of staining was somewhat different: Normal rabbit serum was used, and the secondary antibody corresponded to rabbit anti-mouse (1:50; Dakopatts, Glostrup, Denmark; Z0259) and PAP-mouse (1:100; P0850) was utilized.

3.5.4 En Vision® detection

To visualize VAChT (II), the EnVision® detection was found to give the most clear-cut delineated immunoreactions.

As in the PAP staining technique, microwave antigen retrieval was used (c.f. above), and after this the slides were left cooling for 20 min. The sections were then rinsed in PBS buffer for 5 min x 3, thereafter pretreated with acid potassium permanganate for two min, and again rinsed in PBS for 5 min x 3. The slides were subsequently incubated in a 1 % Triton X-100 solution in 0.01 M PBS, pH 7.4, for 20 min after which followed rinsing in PBS 5 min x 3. To block endogenous peroxidase activity, the sections were incubated in 1 % H_2O_2 for 30 min. The sections were washed in PBS 5 min x 3, and then incubated with normal 5 % goat serum in PBS supplemented with 0.1 % BSA for 15 min. Incubation with primary antibody (antibody against VAChT from Sigma) was performed for 60 min at room temperature. After again washing in PBS 5 min x 3, another incubation with normal goat serum as described above was performed, and then the secondary antibody complex was applied (Dako EnVision®+, goat antirabbit IgG conjugated to a peroxidase-tagged polymer, code K4002; undiluted; DakoCytomation, Glostrup, Denmark) for incubation at room temperature for 30 min. The sections were thereafter again rinsed in PBS 5 x 3, and then developed in diaminobenzidine (DAB) solution for 5 min. Prior to dehydration the sections were dipped for 20 sec in Mayers hematoxylin in order to delineate general tissue morphology. The sections were dehydrated and finally mounted in Pertex microscopy mounting medium for examination.

3.5.5 Hematoxylin-eosin staining

Hematoxylin-eosin staining of parallel sections to those processed for IH and ISH were performed to delineate tissue morphology in all studies (I-IV). This staining method involves application of the basic dye hematoxylin, which colors basophilic structures such as nucleic acids, ribosomes and cell nucleus blue-purple, and eosin, which colors eosinophilic structures such as intracellular proteins in the cytoplasm the or extracellular proteins bright pink.

3.5.6 Primary antibodies

Polyclonal and monoclonal antibodies were used as primary antibodies in the immunohistochemical stainings (Table 4). The study in which the respective antibody was utilized is presented in Table 4.

Antigen	Antibody Code	Source	Raised in	Raised against (antigen)	Dilution	Tissue	Method for staining	Study
αSMA	M0851	Dakopatts, Glostrup, Denmark	Mouse	N-terminal decapeptide of human alpha-smooth muscle actin	1:2000	Unfixed	TRITC	II
α_1-AR	PC160	Oncogene, Boston, MA, USA	Rabbit	Synthetic peptide (KFSREKKAAKT) corresp to aa 339-349 of the human α_1-AR	1:100	Fixed	TRITC	III
CD31	M0823	Dakopatts, Glostrup, Denmark	Mouse	CD31 of membrane preparation of human spleen	1:100, 1:40	Unfixed	TRITC, PAP	II
CGRP	RPN-1842	Amersham International, Buckinghamshire, UK	Rabbit	Synthetic rat α-CGRP conjugated to BSA	1:100	Fixed	TRITC	I
CGRP	PEPA27	Serotec, Oxford, UK	Rabbit	Synthetic rat α-CGRP conjugated to BSA	1:100	Fixed	TRITC, PAP	I
ChAT	AB144P	Chemicon, Temecula CA	Goat	Human placental ChAT enzyme	1:25 - 1:50	Fixed	FITC	II
M_2R	M9558	Sigma, St Louis, MO	Rabbit	Purified GST fusion protein of a part of the i3 intracellular loop of human M_2R corresp to aa residues 227-356	1:100	Unfixed	TRITC, PAP	II
M_2R	AB5166	Chemicon, Temecula, CA	Rabbit	Purified GST fusion protein of a part of the i3 intracellular loop of human M_2R corresp to aa residues 225-356	1:100	Unfixed	TRITC, PAP	II
Neurofilament	N4142	Sigma, St Louis, MO	Rabbit	Purified neurofilament 200 from bovine spinal cord	1:100	Fixed	PAP	II
NPY	PC223L	Oncogene, Boston, MA, USA	Rabbit	Synthetic peptide corresp to porcine NPY	1:500 - 1:1000	Fixed	TRITC, PAP	IV, III
PGP 9.5	7863-0504	Biogenesis, Poole, UK	Rabbit	Native brain PGP9.5	1:1000	Fixed	TRITC PAP	I
PGP 9.5	PH164.XS	Bindingsite, Birmingham, UK	Sheep	24-aminoacid sequence of PGP9.5	1:1000	Fixed	PAP	I
SP	i675/002	UCB, Brussels, Belgium	Rabbit	Synthetic SP conjugated to bovine thyroglobulin	1:100 - 1:200	Fixed	TRITC	I
SP	8450-0004	Biogenesis, Poole, UK	Rabbit	BSA-conjugated SP	1:100 - 1:200	Fixed	TRITC, PAP	I
TH	P40101	Pel-Freez, Rogers, Arkansas, USA	Rabbit	Denatured tyrosine hydroxylase	1:50 – 1:100	Fixed	TRITC, PAP	III
Vim	MON3005	Sanbio, Uden, the Netherlands	Mouse	Porcine Vimentin	1:1000	Unfixed	TRITC	II
VAChT	v5387	Sigma, St Louis, MO	Rabbit	Synthetic peptide corresp to the C-terminal of rat VAChT, aa residues 512–530, conjugated to keyhole limpet hemocyanin	1:100	Fixed	TRITC, EnVision	II
VAChT	sc-7716	Santa Cruz, CA	Goat	C-terminus of VAChT of human origin	1:25 - 1:100	Fixed	FITC	II
Y1R	sc21992	Santa Cruz, CA	Goat	Peptide mapping near the C-terminus of the Y1R of human origin	1:100	Fixed	FITC	IV
Y2R	sc14736	Santa Cruz, CA	Goat	Peptide mapping near the C-terminus of the Y2R of human origin	1:100	Fixed	FITC	IV

Table 4: Properties of primary antibodies. aa; amino acids. Antibodies raised in mouse were monoclonals; those raised in rabbit, goat and sheep were polyclonal antibodies.

3.5.7 Control stainings

To confirm the validity of the methods iterated test stainings on reference tissues were performed in parallel with staining of sections from normal and tendinosis tendon tissue samples. These corresponded to specimens of unfixed and chemically fixed human colonic tissue (II, III), rat spinal cord (II), rat superior cervical ganglion, rat adrenal medulla, and rat fetal heart (III). Eventually it turned out that the above mentioned protocols for stainings were the most appropriate to delineate specific immunoreactions

Preabsorption stainings were furthermore used in all the studies (Table 5). Sections were here submitted to incubation with antisera that had previously been preabsorbed with their corresponding antigen overnight at 4°C. Replacement of the primary antibodies with normal serum (I-III) or with PBS together with 0.1 % BSA (III) were used as further control stainings. As a control for the evaluation of double-staining, either one of the primary antibodies was omitted whilst both secondary antibodies were applied (II).

Preabsorption Antigen	Preabsorption antigen code	Source of antigen	Antibody to be evaluated	Concentration used[1]	Papers
CGRP	C2806	Sigma, St Louis, MO	PEPA27	10-20	I
ChAT	AG220	Chemicon, Temecula CA	AB144P	20	II
M_2R	AB5166-peptide	Chemicon, Temecula, CA	AB5166	50-100	II
NPY	sc115P	NeoMPS, Strasbourg, France	PC223L	50	III, IV
VAChT	sc-7716 P	Santa Cruz, CA	sc-7716	150	II
SP	S3144	Sigma, St Louis, MO	8450-0004	10-20	I
SP	S6883	Sigma, St Louis, MO	8450-0004	50	I
Y1R	sc21992P	Santa Cruz, CA	sc21992P	20	IV
Y2R	sc14736P	Santa Cruz, CA	sc14736	20	IV

Table 5: Corresponding antigens and antibodies used for preabsorption controls.
[1]μg antigen/mL antiserum

3.6 In situ hybridization (ISH)

In two studies (II, III), in situ hybridization (ISH), a type of hybridization histochemistry method that uses a labeled complementary DNA strand (i.e., a probe), to localize a specific mRNA sequence in a section, was utilized.

In study II, a GreenStar* digoxigenin (DIG)-hyperlabeled oligonucleotide ssDNA probe for the detection of human ChAT mRNA (GD1001-CS, custom made; GeneDetect, New Zealand) and in study III, a digoxigenin (DIG)-hyperlabeled oligonucleotide probe (ssDNA) for detection of human TH mRNA was used (for details, see Table 4). In both studies, these probes were utilized on sections from biopsies from two of the tendinosis tendons and one of the control tendons.

In situ hybridization was performed according to the protocol from Panoskaltsis-Mortari and Bucy 1995. An alkaline phosphatase (AP)-labeled anti-DIG antibody (raised in sheep; catalog no.: 11 093 274 910, Roche), with a few modifications, was used for detection.

A cryostat equipped with a knife washed in 70% ethanol in diethylpyrocarbonate (DEPC)-H_2O were utilized for a series of cryosections (10 μm thick) that were cut and then mounted onto Super Frost Plus slides (no. 041300, Menzel-Gläser). Sections were air-dried at room temperature for 30 min and thereafter fixed for 60 min at room temperature in a paraformaldehyde solution (4% paraformaldehyde in 0.1 M PBS), that was first passed through a 0.45-μm sterile filter.

Out of 3 M NaCl and 0.3 M sodium citrate, pH 7.0 (+20°C), a 20× SSC (saline sodium citrate) solution was prepared. This solution was autoclaved, and diluted 10× with DEPC-H_2O to obtain a 2× SSC solution, in which the slides then were washed for 2x10 min. Subsequently the sections were incubated in 0.2 M HCl for 8 min at room temperature to inhibit endogenous alkaline phosphatase acitivity, and then acetylated by incubation for 15 min at room temperature in a mixture of 195 mL DEPC-H_2O, 2.7 mL l tiethanolamine, 0.355 mL HCl, and 0.5 ml acetic anhydride being added after the slides had been placed in their slide holder. The slides were then again rinsed in 2× SSC. The ssDNA probe (50 ng in Study II and 100 ng i n Study III) was added to 15 μL hybridization solution (500 μL formamide, 200 μL 20× SSC, 50 μL 20× Denhardt's solution, 50 μL heatdenatured herring sperm DNA (10 mg/ml), 25 μL bakers yeast RNA (10 mg/ml), and 175 μL dextran sulfate (50%) in a total volume of 1.0 mL) in a 1.5-mL Eppendorf tube, denaturated for 5 min in 80°C, and then placed on ice.

To each section, the probe-containing hybridization solution was applied, and the sections were then sealed for incubation at 56°C overnight by a cover-slip and nail polish. The sections were thereafter washed at room temperature in 2× SSC for 2×10 min, and in STE-buffer (500 mM NaCL, 20 mM TRIS-HCl pH 7.5, 1 mM EDTA) for 5 min. They were then placed for incubation in 100 μL RNase A (40 μg/ mL in STE) for 30 min at 37°C, washed for 20 min at 56°C in 2× SSC, 50% formamide (25 ml 100%, 25 mL 2× SSC buffer), and rinsed at room temperature in firstly in 1× SSC for 2×5 min, and then in 0.5× SSC for another 2×5 min. Thereafter the slides were washed for 5 min in buffer 1 (100 mM TRIS-HCl pH 7.5, 150 mM NaCl), followed by incubation in buffer 1 containing 4% normal horse serum (NHS) for 60 min at room temperature in a humid chamber, and after that in 100 μL AP-labeled anti-DIG antibody (diluted 1:500 in buffer 1 with 4% NHS) for 60 min at room temperature in a humid chamber. The sldes were then washed in buffer 1 for 2×10 min, and in buffer 2 (100 mM TRIS-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 2×5 min. Subsequently the enzyme (AP) substrate solution (20 μL NBT/BCIP [Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate; toluidine salt (gives a dark blue indigo staining as an oxidation product after been reacting with the AP-substrate)] in 1 mL buffer 2 with 10 μL levamisole) was sterile-filtered through a 22 μm filter and added to the sections, and the slides were incubated upside down in the dark at 4°C overnight. The color reaction was therafter stopped by placing the slides in buffer 3 (10 mM TRIS-HCl pH 8.0, 1 mM EDTA). Counter-staining of the slides in methylgreen (0.5%-0.5 g/100 mL) was then performed, using the following procedure: the slides were dipped for 30 s in 75% ethanol, for 30 s in 95% ethanol, and for 4-5 s in 0.5% methylgreen, and thereafter washed x3 in pure (99.5%) ethanol. The sections were lastly mounted in Pertex mounting medium.

As controls the corresponding sense DIG-hyperlabeled ssDNA probe (negative control) and the antisense probe β-actin probe (GD5000-OP) from GeneDetect, New Zealand (positive control) was used.

Probe	Code	Source	Dilution	Fixation
Antisense probe[1], recognizing human TH mRNA	GD1811-OP	GeneDetect, New Zealand	100 ng in 15 µL hybridization solution	Postfixation according to ISH protocol
Antisense probe[2], recognizing human ChAT mRNA	GD1001-CS (custom made)	GeneDetect®, New Zealand	100 ng in 15 µL hybridization solution	Postfixation according to ISH protocol

Table 6: GreenStar* DIG-hyperlabeled oligonucleotide probe. Sequence:
[1]AACCGCGGGGACATGATGGCCT
[2]CCATAGCAGCAGAACATCTCCGTGGTTGTGGGCACCTGGCTAGTGGAGAG

3.7 Evaluation by microscopy

All sections were evaluated by two (D.B., and S.F. (I, IV)) or three (D.B., S.F., and P.D. (II, III)) indepentdent microscopists. All sections immunoassayed for a specific antigen were examined consecutively and iteratively by each microscopist. This made it possible to perform a semi-quantitative assessment of the degrees of intensity and the frequency of the immunostainings.

Either a Leitz Orthoplan photomicroscope (I) equipped with epifluorscence optics, or a Zeiss Axioscope with epifluorescence optics and an Olympus DP70 digital camera, was used (II- IV).

3.8 Statistics

In Study IV, statistics were used in the evaluation process. The statistic material of was rather limited, with small non-paired samples sizes (37 tendinoses tendons and 8 controls), with non-continuous variables. For these reasons, the Mann-Whitney test was used to compare the groups. The statistical software applied was SPSS 11.0 (SPSS, Chicago, Ill, USA) for Macintosh. $P < 0.05$ was considered significant. The Spearman rank correlation test was used for age-related correlation analyses.

4. RESULTS

4.1 Morphological characteristics

4.1.1 Overall morphology of the tissue

Htx-eosin staining was used to display the general morphology.

Achilles tendon specimens from controls consisted of tendon tissue proper and paratendinous connective tissue. The border between the two tissue types was readily defined microscopically (III: Fig.1). The tendon tissue proper consisted of large groups of collagen fibrils lying in parallel alignmen, inbetween which narrow channels with loose connective tissue, containing blood vessels and nerve structures, were running (I: Fig. 2).
The paratendinous connective tissue mainly consisted of loose connective tissue (with collagen fibrils showing wavy courses) (III: Figs. 1a, b), and blood vessels (large arterioles/venules and occasionally even arteries/veins and nerve structures.
Vascularity was more prominent in the paratendinous connective tissue than inside the tendon.

During surgery for Achilles tendinosis, the tissue from which samples were taken, did not to show the typical tendon tissue features (glistening white, regular parallel appearance of makroscopic “fiber bundles”, (c.f. Khan et al., 1999a), but was amorphous and grey to the naked eye. Macroscopically, it was also often observed that the outer part (the paratendinous connective tissue) had coalesced with the tendon tissue proper in these specimens.

There was a great variability in the morphology among the specimens of the tendinosis group and within the same specimen of this group. Some regions in the tendinosis specimens showed a rather normal histologic tendon appearance with collagen fibrils of parallel alignment (II: Fig. 1) and fairly slender tenocytes, while others exhibited irregular collagen fibre structure and aberrant tenocytes (cf below) (III: Figs. 1c, d). In some specimens severe derangement was noted.

Vascularity was in general more prominent in the tendon tissue proper in the tendinosis group (hypervascularity) than in the tendon tissue proper of the control specimens. In both types of specimens, the blood vessels were more abundant in the paratendinous connective tissue than in the tendon tissue proper. Perivascular cells, frequently forming groups of cells, occurred in association with the finest blood vessels in several tendinosis tendon specimens.

In Study IV, the tissue samples were taken from both the ventral and the dorsal side of the tendon. No obvious differences in morphology of the tendon tissue proper were noticed between these biopsies.

4.1.2 Morphology and frequency of the tenocytes (II, IV)

Most of the tendon tissue samples of controls harboured typical slender, spindle-shaped, fairly straight tenocytes. The tendon tissue proper of the specimens was relatively hypovascular and hypocellular (III; Fig. 1). Variability in tenocyte appearance was, however, identified in some controls, and very occasionally, hypercellularity occurred in limited parts of the specimens.

In the tendinosis specimens, there was a much higher degree of variability in tenocyte appearance and hypercellularity was common. Most tendinosis tissue samples exhibited a large number of tenocytes, whereas in a few specimens the tenocyte number almost resembled that found in control tendons. Many of the tenocytes in the tendinosis specimens had a

disfigured shape; from “bulky” and “wavy” to rounded, widened appearances. The amount of aberrant cells varied between different tendinosis specimens, but also within the same specimen. In most tissue samples, the number of “abnormal” tenocytes was higher than was the number of the tenocytes with normal (slender, spindle-shaped, straight) appearances.

4.2 Summary of results in relation to aims and methods

4.2.1 Study I

The innervation pattern of the human Achilles tendon: studies of the normal and tendinosis tendon using markers for general and sensory innervation

Aims

- to describe the general and sensory innervation patterns in the midportion of the painless normal Achilles tendon and the chronic painful midportion Achilles tendinosis via markers for general (protein gene-product, PGP9.5) and sensory (substance P, SP and calcitonin gene-related petide, CGRP) innervation.
- to investigate if there are differences between the normal Achilles tendon and the Achilles tendinosis tendon.

Subjects and methods

The investigation included tissue samples from 30 individuals, 21 patients suffering from chronic Achilles tendinosis, and 9 individuals, who had painless, and clinically normal Achilles tendons. For further details, see Material and methods.

Immunohistochemistry with the immunofluorescence technique and the PAP technique was used.

Main results

In Achilles tendon specimens of controls, PGP9.5-like immunoreactions (LI) were seen in nerve fascicles and nerve fibers of the tendon tissue proper. These were located inside the channels of loose connective tissue, the endotenon, but foremost in the paratendinous loose connective tissue.

The large nerve fascicles occurred in the vicinity of blood vessels (I: Figs. 1, 4) and the smaller nerve fibers were found in association with the media–adventitia junction of arterioles (I: Fig. 3). On the whole, the nerve structures appeared to be more abundant in the paratendinous loose connective tissue compared to the tendon tissue proper.

In the tendinosis Achilles tendons, the same innervations patterns as in the controls were seen. However, in some specimens, PGP9.5-immunoreactive varicose fibers were found to be intimately associated with fine blood vessels (I: Fig. 8).

In control Achilles tendon specimens, nerve fibers exhibiting CGRP-LI and SP-LI could, to a small extent, be seen in association with the blood vessels of the tendon tissue proper. SP-LI (I: Fig. 2b) and CGRP-LI (I: Fig. 4) were also detected in nerve fascicles in the paratendinous connective tissue. Immunoreactions for CGRP appeared to be more marked than those for SP. In Achilles tendinosis specimens, nerve fibers displaying CGRP-LI (Figs. 6b, 7) and SP-LI were also seen in nerve fascicles and in perivascular locations.

On the whole, CGRP and SP immunoreactions were only observed in association with a subpopulation of the blood vessels, and thereto seldom deep in the tendon. Overall, the SP/CGRP innervation was very sparse.

Sensory corpuscles (mechanoreceptors) were occasionally observed in the paratendinous loose connective tissue (I: Fig. 9). They appeared as lamellated (Pacinian) corpuscles (c.f. Halata et al. 1999, Albuerne et al. 2000).

4.2.2 Study II

Presence of a non-neuronal cholinergic system and occurrence of up- and down-regulation in expression of M_2 muscarinic acetylcholine receptors: new aspects of importance regarding Achilles tendon tendinosis (tendinopathy)

Aims

- to investigate the innervation patterns of the normal and tendinosis Achilles tendons regarding cholinergic innervation by studying the immunohistochemical expression patterns of VAChT, ChAT and M_2R in nerve structures, blood vessel walls and tenocytes.
- to investigate if local production of acetylcholine (ACh) can be evidenced at both the protein and mRNA levels as a prerequisite for an existence of a non-neuronal cholinergic system in the Achilles tendon.
- to clarify differences between normal Achilles tendon and midportion Achilles tendinosis

Subjects and methods

Achilles tendon tissue samples were taken from the midportion in 29 individuals, 21 individuals of these were suffering from chronic painful midportion Achilles tendinosis.

Another group defined as the normal control group consisted of 8 individuals. For further details c.f. Material and methods.

Immunohistochemistry using antibodies against VAChT, ChAT, and M_2Rs were performed. Antibodies against Vimentin, CD31, αSMA were used as tissue markers. Double staining for M_2R/CD31 and M_2R/αSMA, and in situ hybridization to reveal ChAT mRNA, was utilized.

Main results

ChAT or VAChT immunoreactivities were not detected in nerve fascicles nor in perivascular nerve fibers. That was the case in control Achilles tendon specimens as well as in tendinosis specimens, including in the innervaion in the paratendinous connective tissue of both types of specimens. VAChT-LI was occasionally seen in the cells of the blood vessel walls.

The tenocytes of control tendons exhibited only weak or no immunoreactions for ChAT. In tenocytes in tendinosis tendons, ChAT immunoreactions were much more clearly detected. The reactions were especially marked in specimens harbouring a high degree of hypercellularity, and an abundance of disfigured tenocytes (II: Fig. 12). Nevertheless, the degrees of ChAT-LI showed substantial variations between different tenocytes within the specimens and also between different tendinosis specimens. Not all disfigured tenocytes showed ChAT-LI in the tendinosis tissue samples, and those with a more slender appearance in these samples were never seen to be ChAT immunoreactive.

Tenocytes in tendinosis tendons clearly exhibited AP reactions after incubation for ChAT mRNA (II: Figs. 16a, 17). Variability within the specimens as well as between the tissue

samples was the rule also in this repect (II: Figs. 16, 17). The most prominent reactions were displayed in tenocytes that not showed the normal straight and slender appearance. Tenocytes in tissue samples from control Achilles tendons did only in a few cases exhibit faint AP reactions for ChAT mRNA.

Concerning VAChT in tendinosis specimens, specific immunoreactions could most clearly be seen in aberrant tenocytes in tendinosis specimens, particularly in samples with high cell density, (II: Fig. 14a, b). However, as was the case for ChAT, there was a wide variation in reactions between different tenocytes.

M_2R immunoreactions, found in blood vessel walls in normal (II: Figs. 2a, 3a) and tendinosis tendons (II: Figs. 2b, 3b, 4a, b), appeared as punctuate labelings. In the in the walls of the large vessels, the reactions were mainly confined to the innermost located cells (II: Figs. 4a,b). In tendinosis specimens with hypercellularity and hypervascularity, a high degree of immunoreactivity was noticeable (II: Figs. 2b, 3b, c, 4a), whilst in tendinosis specimens with a markedly aberrant morphology, only occasional immunoreaction (II: Figs. 5a–c) was noted, or the specimens were not immunostained at all. As revealed by double-staining, the M_2R immunoreactivity in vessel walls was to a large extent co-localized with the endothelial marker CD31 (II: Fig. 7a–c).

M_2R immunoreactivity was to some degree also detected in nerve fascicles and perivascular nerve fibers in both control and tendinosis tendons.

In normal tendons, a moderate to high degree of M_2 immunoreactivity was detected in tenocytes, whilst in tendinosis specimens with hypercellularity and hypervascularity a high degree of immunoreactivity (II: Figs. 2b, 3b, c, 4a) was regurlarly detected in these cells. In tendinosis specimens, in which the tenocytes showed a markedly rounded/widened appearance, immunoreaction was only occasionally noted (II: Figs. 5a–c, Fig. 6).

In the double stainings, that were used to further reveal the distribution of M_2R, neither αSMA nor CD31 immunoreactions were detected in tenocytes, but these cells were regularly exhibiting pronounced vimentin immunoreactions (II: Fig. 10).

4.2.3 Study III

Immunohistochemical and *in situ* hybridization observations favor a local catecholamine production in the human Achilles tendon

Aims

- to reveal the general pattern of sympathetic innervation and the possible occurrence of adrenergic receptors in the Achilles tendon.
- to reveal if a local catecholamine production in tenocytes can be evidenced both at protein and in mRNA levels.
- to describe differences between the normal Achilles tendon and midportion Achilles tendnosis

Subjects and methods

Tissue samples from 29 individuals were studied. Biopsies were taken from 21 Achilles tendinosis patients and biopsies from 8 individuals with pain-free and clinically normal Achilles tendons. For further details, see Material and methods.

All specimens were subjected to immunohistochemical processing. TH and NPY was displayed via TRITC and PAP stainings, and α_1- adrenoreceptors were visualized via TRITC.

In situ hydridization was performed to display the presence of TH mRNA.

Main results

TH- (III: Fig. 2a) and NPY- LI were sometimes detected in the nerve fascicles of the paratendinous loose connective tissue in control and tendinosis Achilles tendons, but only exceptionally in the tendon tissue proper. Fine immunoreactive nerve fibers also lay more or less in association with the media-adventitia junction of the vessles (III: Fig. 3a). In most specimens though, the walls of the blood vessels, did not show neither TH nor NPY immunoreactivity (III: Figs. 2b, c, 3b).

On the whole, no convincing differences could be seen between the specimens from controls and tendinosis patients with respect to the magnitudes of NPY and TH immunoreactivities in blood vessel walls.

Tenocytes showed intracellular punctuate TH immunoreactions (III: Figs. 4c) in both control and tendinosis tendon specimens (III: Figs. 4a, b). A variability within and between specimens, was, however, noted in this respect. Thus, immunolabeled tenocytes could be seen to be intermingled with non-immunolabeled cells (III: Fig. 4a). The TH immunoreactions were in general more marked in tendinosis specimens compared to control tendon tissue samples. The most distinct reactions appeared in cells that were widened/cylindrical or rounded (Fig. 4b) or very wavy in appearance, and foremost in specimens showing hypercellularity.

The tenocytes in tendinosis tendons also displayed reactions for TH mRNA after having been processed by ISH. Overall, the abnormal tenocytes, displaying rounded (III: Fig. 6a), wavy and widened (III: Fig. 6b) and bizarre and rounded (III: Fig. 6c) appearances were showing the most marked reactions.

α_1- AR immunoreactions could be detected in nerve fascicles, and in blood vessel walls (Fig. 5b). α_1-AR-LI was also detected in tenocytes, particularly in tendinosis specimens (III: Fig. 5a). The tenocytes showing the most prominent α_1-AR-LI were those that not showed the typical normal features of slender, straight tenocytes. In some tendinosis specimens, it was noted that the disfigured, in these cases mostly widened, tenocytes showing α_1-AR-LI, were lined up in rows. At high magnification, the immunoreactions in the tenocytes showed a granular appearance (III: Figs. 5a, 5a inset).

4.2.4 Study IV

Precence of the neuropeptide Y1 receptor in tenocytes and bood vessel walls in the Achilles tendon

Aim

- to clarify the NPYergic innervation pattern by investigating the possible occurrence of NPY and the NPY receptors Y1 and Y2 in normal and tendinosis Achilles tendons of man.
- to investigate if NPY is related to local production and function in the Achilles tendon
- to map differences between normal the Achilles tendon and midportion Achilles tendnosis

Subjects and methods

Achilles tendon specimens from 45 individuals, one group consisting of 37 patients with painful midportion Achilles tendinosis were examined. 16 individuals of this group had previously been treated with sclerosing Polidocanol injections 3-5 times in order to reduce pain. The other main group consisted of 8 individuals with painless, normal Achilles tendons.

Immunohistochemistry was utilizeded, using antibodies against NPY (TRITC) and Y1R and Y2R (FITC), and immunofluorescence. A semi-quantitative evaluation was performed

concerning levels of Y1R immunoreactions on the tenocytes. A 4-graded (1-4) scale was hereby used, were grade 1 corresponded to very weak reactions and grade 4 to very strong reactions.

Main results

NPY immunoreactions could be detected in nerve fascicles and perivascularly (IV: Fig. 1a), particularly in the paratendinous loose connective tissue. Nevertheless, there was, on the hole, a very scanty NPY innervation in both normal and tendinosis tendons.

NPY immunoreactions were not detected in tenocytes in any of the specimens from either controls or tendinosis patients.

Y1R immunoreactions could not be detected in the nerve fascicles. On the other hand, the smooth muscle cells of the blood vessel walls exhibited marked immunoreactions for the Y1R. In the endothelial layer, there were no immunoreactions; valid for both large (IV: Figs. 2a, 3b, d) and smaller (IV: Figs. 3a, 3d) blood vessels as well, and for both normal and tendinosis tendons. No convincing differences were revealed with respect to the immunoreactivity intensity concerning the blood vessles between specimens from control tendons compared to those from tendinosis tendons.

Y2R immunorections were not detectable in perivascular nerve fibers, nor in cells of the blood vessel walls (IV: Fig 3c) nor in the nerve fascicles. This was true for the paratendinous loose connectivte tissue as well as for the tendon tissue proper, and for both controls and tendinosis tendon specimens.

Y1R immunoreactions were, on the other hand, seen in tenocytes of both control (IV: Fig. 5a) and tendinosis (IV: Figs. 5b, c) tendons. The intensities of the immunoreactions were particularly marked in the tendinosis specimens (IV: Figs. 5b, c, 6). In aberrant tenocytes, foremost those with a rounded/widened appearance, marked immunoreactions appeared to be displayed on the plasma membrane, i.e. the exteriors of the cells (IV: Fig. 5c).

A semiquantitative analysis was made. This showed that the Y1R immunoreactions for the tenocytes in the tendinosis group were stronger than those in the non-tendinosis group ($p<0.01$). When comparing scores of specimens from males with those from females in the tendinosis group no statistical difference could be established. In the Spearman rank correlation test no correlations between age and immunoreactions intensity values were found. A comparison of the intensities of Y1R immunoreactions in tenocytes of the tendinosis patients that had been treated with Polidocanol injections with those tendinosis patients that not had been given this treatment showed no statistically significant difference between the two subgroups (IV: Fig. 6; treated, and IV: Figs. 4a, 5b,c; non-treated).

Y2R immunorections were not detected in the tenocytes (Fig 4b), neither in controls nor in tendinosis specimens.

4.3 Brief summary of the results

The present studies display features concerning the innervation patterns and the expression of signal substances and their receptors in normal and midportion tendinosis Achilles tendons (Table 7). The investigations show that there on the whole is a difference in the magnitude of the innervation deep inside the tendon compared to that in the paratendinous loose connective tissue outside the tendon. The deeper parts of the tendon tissue proper are found to exhibit a relatively scarce innervation, whilst the innervation is more pronounced in the paratendinous loose connective tissue. Of particular interest are the findings suggesting production of nerve signal substances in Achilles tendinosis specimens, and the findings of marked receptor immunoreactivities in these tendons.

Compound	Nerve fascicles and peri-vascular nerve fibers		Vessel walls	Tenocytes	
	Paratentendinous loose connective tissue	*Tendon tissue proper*		*Controls*	*Achilles tendinoses*
PGP9.5	++	+	-	-	-
SP/CGRP	+	(+)	-	not examined	not examined
ChAT/VAChT	-	-	-/(+)	+	+++
TH	+	(+)	-	+	+++
NPY	(+)	(+)	-	-	-
M_2R	+	+ - ++	+ - ++	+	++ - +++
α_1R	+	+	+	++	++
Y1R	++	++	++	++	+++
Y2R	-	-	-	-	-

Table 7. The table shows the magnitudes of innervations at the level of the paratendinous loose connective tissue and the tendon tissue proper concerning the substances/enzymes investigated (PGP9.5, SP/CGRP, ChAT/VAChT, TH, NPY). ++: moderate, +: low, (+): sparse, -: no innervation. Furthermore, the degrees of expressions for the receptors in nerve structures and vessel walls are summarized (M_2R, α_1R, Y1R, Y2R). ++: moderate, +: low levels of immunoreactions, -: no immunoreactions.The magnitudes of expressions seen in the tenocyte populations are also depicted. +++: pronounced, ++: moderate, +: low, (+): sparse, -: no immunoreactions. The immunoreactions were in these cases seen in a subpopulation of the tenocytes.

5. DISCUSSION

5.1 Overall comments

Chronic painful midportion Achilles tendinosis is a common tendinopathy that often, but not exclusively, is seen in response of overuse (Maffulli et al., 1998). Despite being the strongest tendon in the human body, the Achilles tendon is one of the tendons that is more prone to injury than others (Rees et al., 2009), presumably to a certain extent due to its functional duties absorbing substantial forces when walking, running and jumping (Józsa and Kannus, 1997). In mid-portion Achilles tendinosis, as well as in Achilles tendon ruptures, many risk factors have been speculated upon (c.f. Introduction), but overall it has been established that degenerative tissue changes are deeply involved in tendinosis. The prerequisites that are needed prior to these changes do still to this date remain an enigma. Furthermore, another fact is, that the occurrence of degenerative processes does for many patients not manifest clinically, i.e. they do not have any symptoms such as pain or swelling. In a study where 891 tendon ruptures were examined, 397 of these being confined to Achilles tendons, two-thirds of the patients had no symptoms prior to rupture, but 97% had degenerative changes, compared to controls, of whom 34% had such changes (Kannus and Józsa, 1991). The reason of this lack of clinical symptoms, foremost pain, despite degenerative changes, is still mainly unknown.

5.2 Overall scope the thesis

In the present thesis, the patterns concerning local productions of nerve signal substances and their receptors were evaluated. The results provide evidence for the occurrence of a local production, in tenocytes, of nerve signal substances and presence of corresponding receptors, normally found in neurons. The findings presented in this thesis do also delineate the appearances concerning signal substances in relation to the morphological changes that occur in tendinosis. Furthermore, the innervation patterns of the human Achilles tendon are shown.

5.3 Locally produced nerve signal substances and their receptors

5.3.1 Expression patterns; presumable functions

The results of this thesis show that the tenocytes in Achilles tendinosis tendons 1) display expressions for ChAT, VAChT and TH, suggesting that they synthesize ACh and catecholamines, 2) show expressions for the M_2 receptors and α_1-receptors, favouring that the cells are under influence by ACh and catecholamines. The tenocytes of these tendons also exhibited marked reactions for the Y1 receptor.

The observations may imply that both ACh and catecholamines may be produced by the tenocytes in tendinosis tendons. The fact that NPY-expression was not detected in the cells

must not mean that they do not produce NPY. The production levels may be very low, or alternately the release rate is very high.

A central finding was that the immunoexpression levels concerning ChAT, VAChT, M_2R, TH, α_1AR and Y1R were clearly more pronounced in the tenocytes of tendinosis tendons than in those of the control tendons. These observations show that the expression levels had increased in parallel with tendinosis manifestation. The observations furthermore suggest that more pronounced signal substance effects on the tenocytes are likely to take place when tendinosis has evolved. There is thus a marked morphological basis for occurrence of autocrine/paracrine events in the tendon, involving the cholinergic, catecholaminergic and NPY-ergic systems. Furthermore, it is evident that the "biochemical concept" (Khan et al., 2000, Wang et al., 2006), as being a concept for the symptoms and signs that occur in tendinosis, should be further considered when interpreting the processis of Achilles tendinosis.

It is obvious that ACh (Phillips et al., 2000, Jacobi et al., 2002), catecholamines (Leech and Faber, 1996, Zhang and Faber, 2001, Anesini and Borda, 2002), and NPY (Grundemar & Håkansson 1993, Hansel et al., 2001, Abe et al., 2007) have functions in common, that relates to proliferative, angiogenic, and blood vessel regulating effects. Interestingly, hypercellularity, hypervascularity and angiogenesis occur in tendinosis (e.g. Åström and Rausing, 1995, Movin et al., 1997, Khan et al., 1999a, Maffulli et al., 2004). It is thus possible that effects via M_2R, α_1AR and Y1R are important in the tendinosis process as being related to these phenomena.

A receptor that was very markedly expressed in the tenocytes, particularly in tendinosis tendons, was the Y1R. Interestingly, the Y1R has marked effects in several conditions. Apart from blood vessel regulating and proliferative effects (Pons et al., 2008), these include effects on tumor cell proliferation (Ruscica, et al., 2007) and effects on the immune system (Wheway et al., 2007).

It appears as if there is a continuous process in terms of signal substance production and expression of receptors for these substances. The more evident the tendinosis features was, the more evident were the signal substance/receptor immunoreactions. However, it was also evident that there was a heterenogeneity in tendon tissue concerning expressions or not of signal substances and their receptors. Some cells in the tendons were thus non-reactive.

5.3.2 There are especially expressions in tendon cells with tenoblast appearances

It was evident that especially tendon cells with rounded/oval appearances were immunolabelled. These tendon cells are generally considered to represent the tenoblast cell type of tendon cells (c.f. Introduction), i.e. cells with a high metabolic rate and being an activated form of tenocytes. As the tenoblasts are activated in response to injury (Kakar et al. 1998), it is logic that these particular cells exhibit marked signal substance reactions.

When discussing the characteristics of tenoblasts, it is of relevance to comment on those of fibroblasts, i.e. the counterparts to tenoblasts/tenocytes in other tissues. Active fibroblasts can be recognized by their abundant rough endoplamic reticulum. Inactive fibroblasts, which are also called fibrocytes, are smaller and spindle shaped and have a less pronounced rough endoplasmic reticulum. The fibroblasts are often crowded in the tissue and are often locally aligned in parallell in clusters (McNeilly et al., 1996, Ralphs et al., 1998). The latter morphological features were often seen for the rounded/oval tendon cells in the present studies.

The main function of fibroblasts is to maintain the structural integrity of connective tissue by continuously secreting precursors of the extracellular matrix. Fibroblasts make collagens, glycosaminoglycans, reticular and elastic fibers, and glycoproteins. Tissue damage stimulates fibrocytes, inducing mitosis of these.The apparent counterpart in the tendons (the tenoblasts) in tendinosis do apparently also show a high metabolic rate concerning signal substance production.

It was, however, also a fact that some tendon cells with rounded/oval appearances exhibited a low level of expression of M_2R. One possibility is that the receptors in these cells have been degraded in response to a very high and long-lasting metabolic activity.

Results of recent studies suggest that also other signal substances than those examined here are produced by tenocytes. That includes glutamate (c.f. Introduction), neurotrophins (Bagge et al., 2009) and VEGF (Petersen et al., 2003).

5.4 Innervation patterns

The present findings show that there is a scarce innervation inside the tendon, and that most of the innervation is located to the paratendinous connective tissue outside the tendon and here being located close to blood vessels. This was seen in the stainings performed for the general nerve marker PGP9.5. The existence of a scarce innervation inside the tendon, conform to observations made for the patellar tendon (Danielson, 2007).

The findings show that there indeed is a morphological correlate for the occurrence of nerve-related effects at the level of the paratendinous connective tissue. There was an existence of sensory and sympathetic components in this innervation. SP is a neuropeptide that has been demonstrated in the innervation of the paratendinous region in both rat Achilles tendons (Messner et al., 1999) and cat popliteus tendons (Marshall et al., 1994).

The paratendinous connective tissue located dorsally to the tendon tissue proper was the tissue examined in the present thesis. As judged from recent studies performed in the research group, it is obvious that there is actually a more marked nerve supply in the paratendinous tissue located ventrally to the Achilles tendon (c.f**.** Andersson et al., 2007).

The observations of innervation in association with blood vessels at the the level of the paratendinous loose connective tissue are of interest from a clinical point of view. Ultrasound and color Doppler-guided sclerosing of blood vessels via injections at this level do thus drastically decrease tendinosis pain (Öhberg and Alfredson, 2002, Alfredson and Öhberg, 2005b).

Interestingly, fine nerve fibers exhibiting PGP9.5- as well as to some extent SP- and CGRP-LI could sometimes be detected in association with small blood vessels in tendinosis tendons. This can be related to an effect of the nerves in relation to angiogenesis. It is thus previously shown that neuropeptides can have effects in relation to angiogenesis (Fan et al., 1993). This aspect will be further discussed below.

The sympathetic innervation pattern (III) was found to follow the general innervation pattern. However, the sympathetic innervation was rather scanty. The magnitude of the NPYergic innervation was very scanty. The cholinergic markers, a transporter for ACh (VAChT) and the enzyme that is responsible for ACh synthesis (ChAT), could not be detected in nerve structures at all (II). In accordance with this finding, nerve fibers displaying AChE activity have only been very occasionally detected in tendinosis Achilles tendons (Alfredson et al., 2001a). This implies that there in principle is a very low or almost non-existing cholinergic innervation in relation to the Achilles tendon. In comparison, immunoreactions for ChAT were also not seen with certainty in nerve structures in the patellar tendon (Danielson et al., 2006b).

5.5 The locally produced signal substances and the innervations in relation to tendon pain

It would be very interesting to know if and to what extent the locally produced signal substances are related to tendinous pain.

There were no nerve fibers in close relation to the tenocytes and the great part of the innervation is located in the paratendinous connective tissue. Nevertheless, it might be that neuropeptides/neurotransmitters to some extent can reach receptors at rather distant sites within the tissue. In any case, it is known that stimulation of adrenergic as well as ACh receptors can modulate pain (Vogelsang et al., 1995, Baron et al., 1999).

The source of the pain in tendinopathy is in any case likely to be derived from sensitised nerve endings within the tendon and particularly from nerve endings in the paratendinous connective tissues. Here it should be recalled that the number of nerve fibers within tendon tissue proper of the Achilles tendon was very low. Ingrowth of nerves, in parallell with that of blood vessles, has been suggested to occur for tendinosis tendons (Alfredson et al., 2003c). Maybe this nerve ingrowth to some extent is related to pain-effects.

A well known phenomenon is that there may be interactions between sensory and sympathetic nerve fibers. These may partly be mediated via receptors on the sensory nerve fibers (Wong, 1993). It can therefore not be excluded that sympathetic/sensory influences can be involved in the pain mechanisms in the paratendinous connective tissue in Achilles tendinosis. The morphologic correlate for this is more evident in the paratendinous connective tissue located ventrally (Andersson et al., 2007), than dorsally (here examined) in relation to the tendon proper.

It is of interest to note that the nociceptive role of SP is known to be additive with that of glutamate in mice (Murray et al., 1991) and that glutamate has been found to have excitotoxic effects in the CNS (Camacho and Massieu, 2006). Glutamate and one its receptors, the NMDAR1 (Alfredson et al., 1999, 2001a, 2001b), have been found in the Achilles tendon, and in a very recent study, coexistence of up-regulated NMDA receptor 1 and glutamate on nerves, vessels and transformed tenocytes has been discovered in patellar tendinopathy (Schizas et al., 2009), suggesting a regulatory role in intensified pain signalling.

5.6 Relation to histopathology, exercise, the collagen and apoptosis

A general feature when examining all the specimens in these studies was the fact that there were marked inter-individal morphologic variations between the specimens of both groups, as well as within the specimens as such. The variability in morphology in tendinosis specimens in our studies is in principle in accordance with that found in previous investigations (Åström 1995 and Rausing, Józsa and Kannus 1997, Khan, 1999a, Shalabi, 2004).

Metabolic activity, circulatory responses and collagen turnover are all strikingly increased in tendons after exercise, all leading to remodelling and defending of tendon tissue against quality loss. The time pattern of this adaptation may limit athletic training, and help us to understand why overuse injuries occur in work, sport and recreational activities (Kjaer et al., 2005). It is presumable that effects of locally produced signal substances are highly involved in these adaptations. The adaptations include effects on collagen structure. It is actually well-known that there is a tendency for discontinuity of the collagen in tendinosis (Khan et al 1999a). Here it should be remembered that both ACh (Oben et al., 2003b) and norepinephrine

(Oben et al., 2003a) can induce collagen in myofibroblastic cells. Furthermore, that the use of an ACh agonist has effects on collagen production (Jacobi et al., 2002).

In summarizing tendon biomechanical behaviour in vivo, Magnusson and collaborators suggested that "human tendons are metabolically active, especially during exercise, and exhibit visco-elastic properties that enable them to interact with the contractile element and mediate the outcome of muscle contraction and whole body performance". These authors also concluded that the tendon properties are worsened by chronic disuse and ageing, but that training can partly mitigate these changes (Magnusson et al., 2008). It would be very interesting to know if and to what extent the levels of signal substance productions in the tenocytes/tenoblasts are influenced by training/exercise.

It is well-known that tenocytes in tendinosis tendons may be subject to apoptosis (Yuan et al., 2003). Signal substances may hereby have an effect in modifying this process. It is thus known that interference with adrenergic effects can influence apoptotic/degenerative events, beta-adrenoceptor antibodies inducing apoptosis in rat cardiocytes (Staudt et al,. 2003).

5.7 The vasculature

The blood vessel were consistently equipped with M_2, α_1 and Y1 receptors. These observations suggest that they are under marked influences via effects on these receptors. It is a well-known fact that the sympathetic nervous system and also NPY are involved in cardiovascular regulation (Pons et al., 2008). NPY can hereby potentiate the effects of alpha-adrenergic agonists (Linder et al., 1996). The observations of M_2 immunoreactions in the endothelial parts of the blood vessel walls suggest that vasodilatory mechanisms occur in the Achilles tendon via effects of ACh.

Previous studies have indicated that neovascularization occurs at certain stages in tendinosis (Öhberg et al 2001b, Alfredson et al 2003c, Cook et al. 2004b) and that neuropeptides on the whole can have effects in relation to angiogenesis (e.g., Fan et al. 1993). In comparison, ingrowth of blood vessels is suggested to be associated with ingrowth of nerves in vertebral discs (Brown et al., 1997). The aspects of angiogenesis for tendinosis tendons are further discussed below.

In experimental studies in which Achilles tendon disorder corresponding to tendinosis was induced in the rat, increased numbers of nerve filaments and increased immunoreactivity to CGRP and SP occurred in parallel with the occurrence of hypervascularization at 7–11 weeks after treatment (Messner et al. 1999). The vasculature has also been postulated to have a qualitative significance, in that tendon grafts promote a revascularisation allowing normal function of the tissue, while a vascular response in chronic tendinopathy apparently does not lead to repair or resolution of the condition (Fenwick et al., 2002).

5.8 Does the up-regulation of signal substance production/receptor levels have a healing effect?

It might be asked as to wether the up-regulation of signal substance production/receptor levels is a drawback for tendon tissue or if it has a positive outcome. The occurrence of proliferative, angiogenic, vascular and collagen-modifying functions should here be recalled. An increase in tenocyte numbers is likely to be of positive character in view of the collagen production function that the tenocytes have. The angiogenesis process may also be important for the tissue to cope with an increasing demand. Angiogenesis processes initiated by effects of

neuropeptides may actually participate in healing processes (Brain 1997). NPY is one of the neuropeptides which have angiogenic effects (Abe et al., 2007).

It is a fact that neuropeptides like SP are reported to have healing and growth promoting effects in tendons (Steyaert et al., 2006). SP is actually considered to exhibit healing effects also for other tissues (Katz et al., 2003). In an experimental study, artificially ruptured rat Achilles tendons were found to elicit extensive nerve ingrowth into the rupture site in the early phase of healing, followed by almost complete fiber disappearance and that this may prove that the observed temporal occurrence of different neuropeptides in this situation reflects a role of the peripheral nervous system in synchronously regulating nociception and healing (Ackermann et al., 2002). The fact that the effects of ACh may include "wound healing" (Grando, 2006) is also noteworthy. It is furthermore interesting to note that locally produced ACh in inflammatory cells in inflammatory conditions is considered to have anti-inflammatory effects (Kawashima and Fujii, 2004).

The increase in signal substance production/function is likely to influence tissue organization and function and to be intimately coupled to the occurrence of an increase in the metabolic activity of the tendinosis tendons. However, it cannot be excluded that the high levels of signal substance production/function may have a negative effect in the long run, eventually leading to tissue deterioration. Concerning another factor shown to be locally produced in tendons, VEGF, it is considered that this growth factor plays a significant role for the pathogenetic processus during degenerative tendon disease (Pufe et al., 2005). VEGF may, on the other hand, be this important growth factor for vessel regeneration (Rosenbaum et al. 2008).There have been a few studies suggesting there is an importance of various angiogenic growth factors in normal tendon healing (e.g. Chang et al., 1997, Duffy et al., 1995, Kuroda et al., 2000).

Healing of tendon has also been studied with regard to the involvement of stem cells. Still though, studies on human tendons are lacking in this respect. In vitro studies and animal studies on rabbit healing tendon parts have shown the tendons to heal better when applying mesenchymal stemcells (MSCs) (Young et al., 1998, Chong et al., 2007).. MSCs have thereto been shown to express high levels of vascular endothelial growth factor

5.9 Existing treatments; Do the findings in the present thesis suggest new treatments?

Although it is not the principal scope of the present thesis to evaluate the outcomes of treatments for overuse tendon injuries, certain aspects shall be commented on.

Non-invasive treatments such as performance of relative rest stretching, analgesics (Wilson and Best, 2005), cryotherapy (Bleakley et al., 2004), deep friction massage in combination with stretching (Cyriax, 1980, Kvist, 1991) altering biomechanics by heel pads (Lowdon et al., 1984), or orthotics (Sergesser et al., 1995), therapeutic ultrasound (Enwemeka, 1989), extracorporeal shockwave therapy (Costa et al., 2005), and low level laser treatment Stergioulas et al., 2008), may have effects. Complete immobilization should be avoided to prevent muscular atrophy and deconditioning. NSAID´s instead of analgetics is not a convincing strategy (Åström and Westlin., 1992)

A very favourable treatment method has been found to be eccentric training. Initially eccentric training was performed without tendon pain (Curwin, 1984). Alfredson and collaborators modified the eccentric training regimen in using painful training, heavier loads and single leg exercises (Alfredson et al., 1998). Eccentric exercise has gradually been proven

beneficial in Achilles tendinosis (Fahlström M et al., 2003, Jonsson et al., 2008) and patellar tendinosis (Roos et al., 2004, Jonsson P and Alfredson, 2005, de Vos et al., 2007, Rompe et al., 2007) and superior short-term and medium-term results have been shown compared to concentric training (Mafi et al., 2001). Eccentric training has been shown to normalize the Achilles tendon structure, decrease its thickness (Öhberg et al., 2004b, Shalabi et al., 2004) and blood flow (Öhberg et al., 2004b, Knobloch et al., 2007). There is still, however, a lack of evidence showing exactly why eccentric training gives this relief in pain and the rearrangement of the tendon structures. Are locally produced signal substances involved?

Earlier studies implying inflammatory genesis suggested injections of corticosteroids to be used, but these drugs should be used with caution. Recent research has made it obvious that these should be avoided, not least due to a high rupture risk (Åström, 1998, Csizy and Hintermann, 2001). Sonography-guided intratendinous injections of hyperosmolar dextrose have been shown to decrease clinical symptoms, tendon thickness and neovascularity in chronic Achilles tendinosis patients (Maxwell et al., 2007).

A recently developed method that has been found to be very successful is, as described in Introduction, a treatment by which sonography-guided Polidocanol injections are given outside the tendon tissue proper. Via this treatment, the vessels entering the tendon from the ventral side are sclerosed. This treatment has shown good clinical results (Alfredson and Öhberg, 2005b, Lind et al., 2006, Willberg et al., 2008). In the early period after sclerosing Polidacanol treatment Doppler ultrasonography have revealed increased intratendinous vascularity postulated to be possibly related to a healing respons (Alfredson and Öhberg, 2006). Nevertheless, not all patients with Achilles tendinosis are cured by Polidacanol injections. In one study in the present thesis (IV), it was found that the immunoexpression levels for Y1 in blood vessel walls (as well as on tenocytes), were as marked for tendons of patients who had been sclerosed as those found for tendinosis patients that not had been sclerosed. It should here be pointed out that these patients had not been cured in response to the sclerosing treatment. Numerous other drugs or substances have been used as injection treatments for chronic tendon disorders. Various interpretations about the healing improvement potential of these substances have been made, but few stand up to scrutiny.

Surgery is recommended to be considered if an athlete has been treated for 3 to 6 months without progress conservatively (Angermann and Hovgaard, 1999). Different procedures for this have been utilized. The specimens examined in the present thesis represent excised material from tendon parts for which tendinosis tissue appearance macroscopically was clearly observed.

The results of the present studies show that there are marked reactions for M_2 receptors, as well as α_1 and Y1 receptors in especially tendinosis tendons. That was the case for blood vessel walls as well as tenocytes. The observations show that there is a morphological correlate for an achievement of effects via interfering with all these receptors. Here it should be recalled that adrenergic agonists/blockers and medications that block or increase effects of ACh and adrenergic effects are since long used in practise medicine. An interesting aspect of today is that medications that increase ACh effects are used with positive outcome for Alzeheimer´s disease (Shah et al., 2008). The compounds used for achieving these effects are AChE blockers. The possibility that interference with cholinergic effects might be worthwhile for different aspects concerningt the locomotor system has recently been reviewed (Forsgren et al., 2009).

Concerning the Y1 receptor, it is suggested that targeting this receptor may be favourable for certain cancers (Körner et al., 2008) and for the establishment of a modification of vascular remodelling in cardiovascular disease (Abe et al., 2007). Another field in which targeting NPY may be favourable is obesity (Pedrazzini et al., 2003).

Future treatment methods for tendinosis may possibly involve mesenchymal stem cells. A few promising results have been reported in animal studies (Young et al., 1998, Smith et al., 2003, Smith and Webbon, 2005, Chong et al., 2007). Interesting studies, in rabbit, also show that tenocytes have a potential, similar to different MSCs, of being used as graft cells in the healing process after an artificial rupture (Kryger et al., 2007). Gene therapy has also shown promising results but no large randomized controlled trials have so far been reported (Hoffmann and Gross, 2009). Even gene therapies have been tested in rat with promising results (Bolt et al., 2008, Hou et al., 2009). In recent years animal studies have found growth factor treatment to be beneficial to promote tendon healing; bFGF (Rickert et al., 2005, Tang et al., 2008), VEGF (Zhang et al., 2003 , Pufe T et al., 2005), TGFβ_1 (Kashiwagi et al., 2004), PDGF (Thomopousolos et al., 2007). Another interesting treatment possibility would probably be glutamate antagonists, as both NMDA receptor and glutamate are found in tendon and are involved in mediating pain in the CNS (Chizh, 2002). NMDA receptor antagonists are already in clinical use in other diseases. That includes the use of the NMDA receptor antagonist memantine for Mb Alzheimer.

5.10 Study design, limitations, and aspects of research approaches

In the present thesis, an observational analytic epidemiological study design, the case-control study, was used. The study design itself has the limitations of being retrospective, and non-randomized to its nature. The cases were collected prospectively, with cases added as they occured.

The reports (I-IV) are descriptive to their charactaristics, describing the morphology in Achilles tendons with or without tendinosis, especially regarding innervations and signal substances and their receptors, but do not concern the exposure circumstances.

To establish causality of Achilles tendinosis, longitudinal studies would be preferable. This is a difficult task. Another approach is to use animal models and then conduct a longitudinal study in which tissue samples are taken after various fixed time intervals. Outcome of such studies are of importance in order to better understand the temporal course of development of Achilles tendinos. Some animal tendinosis models do actually exist (Warden, 2007). The importance of the signal substances here examined has not been evaluated in these. An animal experiment model is currently being used with this aspect in focus in our research group. Yet another approach would be to use cell culturing as an experimental model for tendinosis. Such studies are currently being initiated in our laboratory.

There were appearances that to some extent were of histopathological type also in some of the samples of the controls, but they were never as severe as in any specimen from the tendinosis samples. Notable is that our inclusion criteria for controls included individuals that had normal painless Achilles tendon at clinical examination and normal tendon findings on ultrasonography, while the morphologic changes were discovered subsequently in the evaluation process through microscopy.

The tissue samples were carefully taken with emphasis on minimizing tissue trauma in the controls. It should also be remembered that the samples of the controls were taken rather superficially, whilst in the tendinosis group, the tissue samples were taken during surgical treatment and were taken more deeply in the tendon tissue.

Tissue samples of tendinosis patients were in all studies taken from the dorsal and central parts of the tendon. In a subgroup in study IV (patients previously treated with Polidocanol), tissue samples were harvested from the ventral side of the tendon. This was done because

ultrasound and Doppler examination showed structural tendon changes and high blood flow localised to the ventral side. At this time, new research findings had shown a correlation between the combination of structural tendon changes and locally high blood flow, and pain during tendon loading activity (Öhberg et al., 2001b, Alfredson et al, 2003c).

5.11 Social impact of tendinosis pain

Quite many individuals are impaired in the functions of their daily life and/or in their physical activity or sport activities when suffering from midportion Achilles tendinosis. As is the case for many other diseases, this substantially decreases quality of life and can in certain cases even have economic consequences. Athletes, at least those that are at the elite level, are often doomed to be dependent on sponsorships for their economics. Continuous high performance level over time is in this context of utmost importance. Elite athletes may actually have to give up their sport careers due to Achilles tendinosis.

A sedentary lifestyle is not to prefer, as one of the most threatening health-problems of our time, the metabolic syndrome, could be the outcome. Midportion Achilles tendinosis, being, a not-life-threatening disease, is laying in the “border zone” of government health insurance systems (at least just now in Sweden), making it rather important to find reliable, high quality and effective treatments in the future. It is therefore necessary to further develop the already existing treatments and to develop new treatments.

6 FINAL REMARKS AND CONCLUSIONS

Achilles tendinopathy can be resistant to treatment, and symptoms may persist despite both conservative and surgical intervention. The pathology of overuse tendinopathy is mainly thought to be non-inflammatory, with a degenerative or failed healing tendon response. Neovascularisation evident on Doppler ultrasound correlates well with pain and poor function. The diagnosis of Achilles tendinopathy requires excellent differential diagnosis and an understanding of the role of tendon imaging. Conservative treatment must include exercise, with a bias to eccentric contractions. Paratendinous injections, as well as eccentric training, decrease neovascularity, relieve pain and improve outcome. Although surgery is the last resort in those patients failing conservative management, it is still unclear how the removal of adhesions and excision of affected tendinopathic areas affects healing and vascularity, or resolves pain.

The results in the present thesis depict the innervation patterns in the inner parts of the Achilles tendon as well as outside the (in the paratendinous connective tissue). The results also show that quantitative as well as qualitative structural and biochemical changes occur inside the tendon; a non-neuronal paracrine/autocrine system being altered as a part of these processes in tendinosis. The signal substances ACh, catecholamines and NPY are involved. The observations give evidence for the occurrence of local productions of ACh and catecholamines. There is a marked morphologic correlate for an occurrence of effects of ACh, catecholamines and NPY not only in the vasculature but also on the tenocytes. Of utmost importance is the fact that these observations were the most marked in the tendinosis tendons. Potential autocrine/paracrine effects related to ACh, catecholamines and NPY are likely to be of great importance during the process of tendinosis. What remains to be defined is the importance of the presumable proliferative, angiogenic, collagen-modifying and degenerative/apoptotice effects of these substances in the disease process.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Besvär från hälsenan (Akillessenan) är relativt vanligt förekommande. Det kan handla om att senan delvis eller helt gått av men det kan ännu hellre handla s.k. kronisk hälsenesmärta (Akillestendinos), med svullnad, stelhet och typiska mikroskopiska såväl som röntgenologiska avvikelser jämfört med normala ej smärtande hälsenor. Akillestendinos uppstår successivt och förekommer t.ex. hos löpare som belastar benen under många och långa löpträningspass med en stor snabbt alternerande belastning på hälsenorna som följd. Även personer som är idrottsligt passiva kan få besvären. Tidigare trodde man det rörde sig om ett inflammatoriskt tillstånd, varför behandlingen ofta bestod av vila och inflammationsdämpande mediciner. Numera vet man dock att det i princip inte rör sig om ett inflammatoriskt tillstånd.

Många har spekulerat i och även forskat i hur Akillestendinos uppstår. En del hävdar att det rör sig om en kombination av åldersförändringar (degeneration) och upprepad och/eller felaktig belastning utan tillräcklig vila mellan belastningstillfällena, andra att det rör sig om anatomiska eller molekylära brister eller förändringar. Denna avhandling utgör ett bidrag till denna forskning och inriktar sig främst på att undersöka vilken typ av nerver och deras signalsubstanser (nervtransmittorer) och mottagarmolekyler (receptorer) som kan återfinnas i hälsenor och huruvida antalet, typen och var de förekommer förändras efter utveckling av kronisk hälsenesmärta. Avhandlingen belyser även hur senvävnadens celler (tenocyter) och blodkärlens celler uttrycker dessa ämnen.

Vävnadsprover från kroniskt smärtande (tendinotiska) och normala Akillessenor studeras med mikroskopiska metoder (immunohistokemi och in situ hybridisering). Vidare studeras vävnaden med sedvanliga mikroskopiska metoder.

Avhandlingen visar att innervering främst finns utanför själva senvävnaden (tendon tissue proper), nämligen i den luckra bindväven i det som kallas paratenon. Innerveringen består främst av en sympatisk innervering men även till delar av en sensorisk sådan. Det är på det hela taget väldigt sparsamt med innervering i bindvävssepta i tendon tissue proper.

Det mest intressanta med avhandlingen är att det visas att tenocyterna uppvisar reaktioner för enzymer som deltar i bildandet av nervsignalsubstanserna acetylkolin och katekolamniner. Det är av extra stor relevans att dessa fynd mycket tydligare ses i de kroniskt smärtande (tendinotiska) senorna än i normala smärfria senor. Dessutom ses förekomst av receptorer för dessa substanser i tenocyterna. Även i detta fall ses de tydligaste reaktionerna i de tendinotiska senorna. Tenocyterna uppvisar även ett höggradigt uttryck för receptor för nervsignalsubstansen NPY.

Vidare ses tydliga reaktioner för receptorer för nervsignalsubstanserna i blodkärlsväggar.

Fynden visar att det de facto finns ett underlag för erhållande av smärtsensationer i den luckra paratendinösa vävnaden och att substanserna ifråga kan påverka blodkärlen i vävnaden. Intressant nog talar även fynden för att det sker en lokal produktion av nervsignalsubstanser i senorna, nämligen i tenocyterna och att detta sker betydligt mer i kroniskt smärtande än normala senor. Vidare talar fynden för att substanserna ifråga har effekter på tenocyterna, eftersom de uppvisar receptorer för dessa. Allt detta talar för att s.k. autokrina/parakrina effekter avseende flera signalsubstanser i avsevärd omfattning sker vid tendinos. Dessa skulle kunna tänkas vara relaterade till proliferativa, blodkärlsbildande/blodkärlsreglerande funktioner och effekter avseende senornas kollagen.

Fynden visar på en ny aspekt avseende Akillesenans vävnad, nämligen att nervsignalsubstanser kan tillhandahållas i denna trots att innerveringen är mycket sparsam. Fynden ger alltså ny förståelse för existensen av substanser i senvävnaden som kan ha stor betydelse för tendinos-processen. Förhoppningen är att de nya fynden ska leda till utveckling av nya alternativa behandlingsmetoder.

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