Influences of paratendinous innervation and non-neuronal substance P in tendinopathy

– studies on human tendon tissue and an experimental model of Achilles tendinopathy

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"The noblest pleasure is the joy of understanding."

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Leonardo Da Vinci

To my family…

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ABREVIATIONS

α_1-AR	alpha-1 adrenergic receptor
ACh	acetylcholine
BSA	bovine serum albumin
CD31	cluster of differentiation marker 31
CGRP	calicitonin-gene related peptide
CNS	central nervous system
CRPS	chronic regional pain syndrome
DEPC	diethylpyrocarbonate
DIG	digoxigenin
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
H&E	hematoxylin and eosin
HRT	hormone replacement therapy
ICC	intraclass correlation
-LI	- like immunoreactivity (TH-LI etc.)
M2R	muscarinic acetylcholine receptor M2
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTJ	myotendinous junction
NaCl	sodium chloride / saline
NK-1R	neurokinin receptor 1
NPY	neuropeptide Y
NPY1R	neuropeptide Y receptor Y1
OCT	optimal cutting temperature
OTJ	osteotendinous junction
PBS	phosphate buffered saline
PGP9.5	protein gene product 9.5
PNS	peripheral nervous system
PRP	platelet rich plasma
RA	rheumatoid arthritis
RNA	ribonucleic acid
RT	room temperature
SP	substance P
SSC	saline-sodium citrate
ssDNA	single-stranded deoxyribonucleic acid
STE	sodium chloride-tris-EDTA
TACR1	tachykinin receptor 1
TH	tyrosine hydroxylase
TRITC	tetramethylrhodamine isothiocyanate
US	ultrasound or ultrasonography
VEGF	vascular endothelial growth factor

ABSTRACT

Pain of the musculoskeletal system is one of the most common reasons for people seeking medical attention, and is also one of the major factors that prevent patients from working. Chronic tendon pain, *tendinopathy*, affects millions of workers world-wide, and the Achilles tendon is an important structure often afflicted by this condition. The pathogenesis of tendinopathy is poorly understood, but it is thought to be of multifactoral aetiology. It is known that tendon pain is often accompanied not only by impaired function but also by structural tissue changes, like vascular proliferation, irregular collagen organisation, and hypercellularity, whereby the condition is called *tendinosis*. In light of the poor knowledge of tendinosis pathophysiology and recent findings of a non-neuronal signalling system in tendon tissue, the contributory role of neuropeptides such as substance P (SP) has gained increased interest. SP, known for afferent pain signalling in the nervous system, also has multiple efferent functions and has been described to be expressed by non-neuronal cells.

As pain is the most prominent symptom of tendinopathy, the focus of the studies in this thesis was the innervation patterns of the tissue ventral to the Achilles tendon (i.e. the tissue targeted in many contemporary treatment methods) as well as the distribution of SP and its preferred receptor, the neurokinin-1 receptor (NK-1R), in the tendon tissue itself. It was hereby hypothesised that the source of SP affecting the Achilles tendon might be the main cells of the tendon tissue (the tenocytes) as well as paratendinous nerves, and that SP might be involved in tendinosis-development.

The studies were conducted, via morphological staining methods including immunohistochemistry and in situ hybridisation, on tendon biopsies from patients suffering from Achilles tendinosis and on those from healthy volunteers. The hypothesis of the thesis was furthermore tested using an experimental animal model (rabbit) of Achilles tendinopathy, which was first validated. The model was based on a previously established overuse protocol of repetitive exercise.

In the human biopsies of the tissue ventral to the Achilles tendon, there was a marked occurrence of sympathetic innervation, but also sensory, SP-containing, nerve fibres. NK-1R was expressed on blood vessels and nerve fascicles of the paratendinous tissue, but also on the tenocytes of the tendon tissue proper itself, and notably more so in patients suffering from tendinosis. Furthermore, the human tenocytes displayed not only NK-1R mRNA but also mRNA for SP. The animal model was shown to produce objectively verified tendinosis-like changes, such as hypercellularity and increased vascularity, in the rabbit Achilles tendons, after a minimum of three weeks of the exercise protocol. The contralateral leg of the animals in the model was found to be an unreliable control, as bilateral changes occured. The model furthermore demonstrated that exogenously administered SP triggers an inflammatory response in the paratendinous tissue and accelerates the intratendinous tendinosis-like changes such that they now occur after only one week of the protocol. Injections of saline as a control showed similar results as SP concerning hypercellularity, but did not lead to vascular changes or pronounced paratendinous inflammation.

In summary, this thesis concludes that interactions between the peripheral sympathetic and sensory nervous systems may occur in Achilles tendinosis at the level of the ventral paratendinous tissue, a region thought to be of great importance in chronic tendon pain since many successful treatments are directed toward it. Furthermore, the distribution of NK-1R:s in the Achilles tendon described in these studies gives a basis for SP, whether produced by nerves mainly outside the tendon or by tenocytes within the tendon, to affect blood vessels, nerve structures, and/or tendon cells, especially in tendinosis patients. In light of this and of previously known SP-effects, such as stimulation of angiogenesis, pain signalling, and cell proliferation, the proposed involvement of SP in tendinosis development seems likely. Indeed, the animal model of Achilles tendon overuse confirms that SP does induce vascular proliferation and hypercellularity in tendon tissue, thus strengthening theories of SP playing a role in tendinosis pathology.

TENDINOPATHY vs TENDINOSIS

Definitions used in this thesis:

Tendinopathy	a disorder characterised by a swollen, painful tendon, with impaired function
Tendinosis	a disorder characterised by a swollen painful tendon, with impaired function, which in addition has been verified to have structural tissue changes via MRI, US or histological examination

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers:

I **Nerve-related characteristics of ventral paratendinous tissue in chronic Achilles tendinosis**
Andersson G., Danielson P. Alfredson H., and Forsgren S.
Knee Surg Sports Traumatol Arthrosc, 2007; 15 (10): 1272-1279

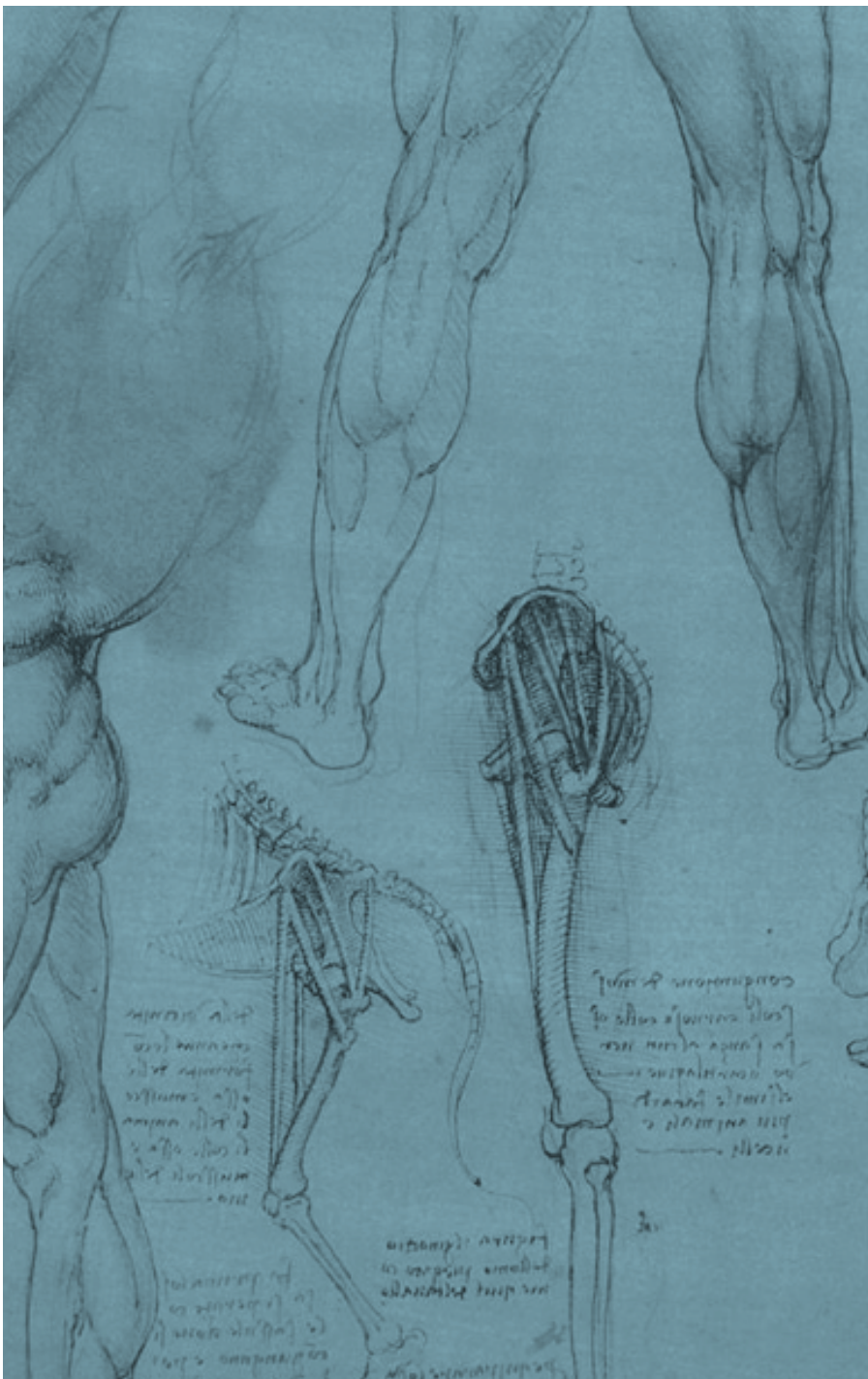
II. **Prescence of substance P and the neurokinin-1 receptor in tenocytes of the human Achilles tendon**
Andersson G., Danielson P., Alfredson H., and Forsgren S.
Regul Pept, 2008; 150 (1-3): 81-87

III. **Tenocyte hypercellularity and vascular proliferation in a rabbit model of tendinopathy: contralateral effects suggest the involvement of central neuronal mechanisms**
Andersson G., Forsgren S., Scott A., Gaida JE., Elgestad Stjernfeldt J., Lorentzon R., Alfredson H., Backman C., and Danielson P.
Br J Sports Med; Online First, published on July 6, 2010, as 10.1136/bjsm.2009.068122

IV. **Substance P induces tendinosis-like changes in a rabbit model of Achilles tendon overuse**
Andersson G., Backman L., Scott A., Lorentzon R., Forsgren S., and Danielson P.
(Manuscript)

INTRODUCTION

Part I
Chapters



"The journey of a thousand miles begins with one step."

Lao Tzu

1 Historical aspects

MYTHOLOGY AND ETYMOLOGY OF THE ACHILLES TENDON

The tendon of focus in this thesis is the Achilles tendon, which is named after Achilles, son of Peleus, of Greek mythology. Achilles was according to Homer the greatest warrior of the Greek army and is considered the centrepiece of Homer's opus the Iliad, dating back to the eight century BC (Vidal-Naquet 2000).

Concerning the etymology of the Achilles tendon, one needs to go back to the dutch anatomist Philip Verheyden, who in 1693 devised the new latin term, Achilles tendo, to the heel tendon. This was coined in reference to the mythological account by the roman poet Statius, who told about the invulnerability of Achilles due to his mother dipping him in the river Styx, and in doing so, she held Achilles by the heel which was therefore not touched by the water (Mozley 1928). Achilles met his death during the Trojan War as a result of the Trojan prince Paris shooting him with an arrow in this vulnerable spot – the heel and Achilles tendon (Homer et al. 1880).

The term Achilles Heel was however not used until the 19th century, as a metaphor for vulnerability, by the English essayist Samuel Taylor Coleridge (Coleridge 1810).

From the point of view of anatomists one should, however, consider – if the myths are to be explained – that the Achilles tendon was probably not the mayor cause of the death of Achilles, but more likely the piercing of the posterior tibial artery, with subsequent complications (Arnott 1846).

The normal tendon

2

In This Chapter

TENDON ANATOMY

Tendons are the connecting structures between striated muscles and bone. In principle, each muscle consists of two connecting structures; one at the muscle origin located proximally, concerning the extremities, and one at the distal end, at the insertion. The point of transition of tension from the intracellular contractile proteins of the muscle, to the extracellular connective tissue proteins of the tendon, is called the myotendinous junction (MTJ) (Ippolito et al. 1986). The other end of the tendon is connected to the bone itself and is called the osteotendinous junction (OTJ). Transmission of force from the muscle via the tendon is required in order to make joint movement possible (Ippolito et al. 1986).

The basic characteristic of a healthy tendon is that of a structure with great resistance to mechanical loads. Macroscopically, tendons vary greatly in shape and in the way they form the osteotendinous junctions. Some are short and broad – ideal in withstanding powerful forces – others are long and thin when arising from muscles involved in subtle, delicate movements (Józsa et al. 1997).

GENERAL TENDON HISTOLOGY

The tendon tissue proper – the core of the tendon – is mainly constituted of tendon cells (tenocytes, cf. 'Tendon cells'), and their products: collagen and proteoglycan-rich extra cellular matrix (ECM). The tenocytes are flat, spindle shaped, cells that lie in-between the collagen fibrils in the tendon. In the normal tendon the tenocytes are quite sparse in numbers (Khan et al. 1999). Besides this, blood vessels and occasional nerve fibres course in the loose connective spaces of the tissue (endotenon; cf. below).

The tendon tissue proper is organised in different levels, ranging from the whole tendon, via tertiary bundles and secondary bundles (fascicles), to the primary bundles (subfascicles). The diameters of the tertiary and secondary bundles are in direct relation to the size of the tendon itself, ranging from 1 mm to 3 mm and 150 µm to 1000 µm, respectively. This can, for instance, be seen in the Achilles tendon, which holds some of the largest bundles found in human tendons (Józsa et al. 1997).

Collagen fibres, the basic tendon unit, form the bundles. These fibres consist of cross-linked tropocollagen molecules that form insoluble col-

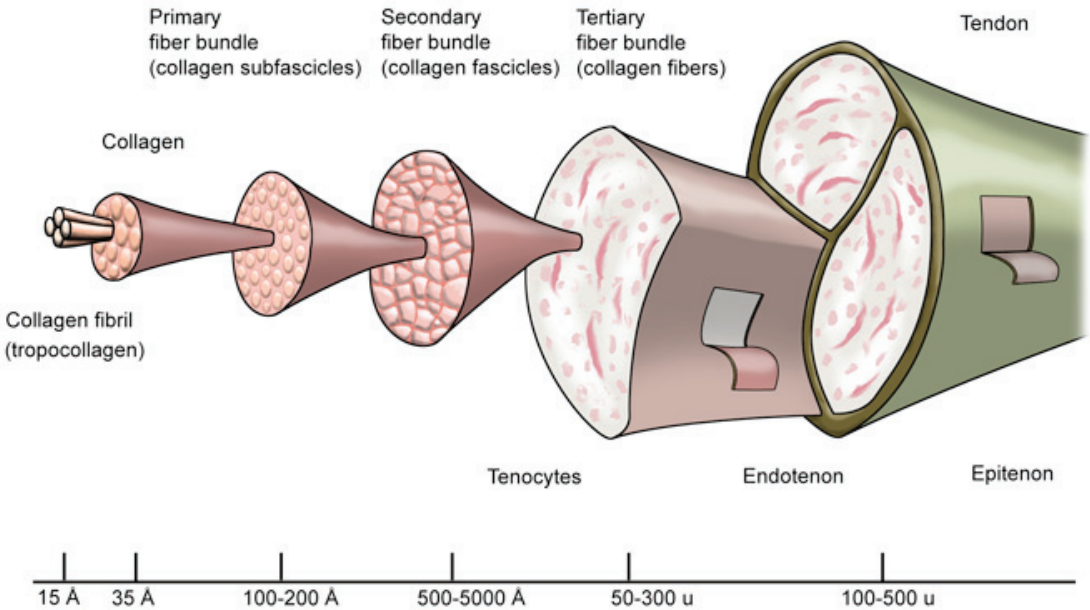


Figure 1. Ultrastructure of tendon. Organisation of collagen from collagen fibre to the entire tendon.

lagen. These molecules in turn form a polypeptide chain with a triple-helix formation, namely the collagen fibril. Multiple collagen fibrils form a single collagen fibre (Elliott 1965) (Figure 1).

The major collagen constituent of the tendon tissue proper is collagen type I. Type I collagen, together with elastin (a glycoprotein), ground substance and anorganic components, form the extra cellular matrix (ECM) (Hess et al. 1989; Jozsa et al. 1989). The collagen gives the tendon its tensile strength, while the elastin, although not clearly understood concerning its function, is considered to contribute to the recovery of the wavy configuration of the collagen fibres, following a stretch of the tendon (Butler et al. 1978). Other glycoproteins include fibronectin, which mediate cell interactions with the ECM (Riley 2005), and is highly active following tendon injury in helping cell adhesion, migration and differentiation (Riley 2005).

The ground substance of the tendon – primarily consisting of proteoglycans with bound glycosaminoglycans (GAG:s) – is hydrophilic in nature, which gives the tendon much of its shear and compression resistance due to a high water content (Józsa et al. 1997; Riley 2005).

A thin layer of loose connective tissue that supplies the tendon with nervous, vascular and lymphatic structures surrounds the tendon tissue proper. This cladding of the tendon is called the epitenon. The epitenon surrounds the tendon itself and divides the tendon into the tertiary bundles by forming sheets between groups of secondary bundles. It also envelops each individual tendon fibre binding them together, and organises it into all of the above-mentioned levels of arrangement in the tendon (subfascicle, fascicle etc.) (Elliott 1965; Hess et al. 1989; Jozsa et al. 1991; Reynolds et al. 1991) (Figure 1).

These separating septa of the epitenon, which go into the tendon tissue, are called endotenon (Elliott 1965; Józsa et al. 1997).

Superficially to the epitenon, one finds a loose areolar connective tissue consisting of primarily type I and type III collagen fibrils, and some elastic fibrils as wells as synovial cells (Kvist et al. 1985; Williams 1986). This tissue is referred to as the paratenon, a structure of gliding membranes continuous with the fascial envelope of the muscles proximally to the tendon (Williams 1986). The synovial cells of the paratenon supply lubrication, and together with the other paratenon-components they not only make the paratenon function as an elastic sleeve, but also allow the tendon to move freely from surrounding tissues (Hess et al. 1989).

The combination of the epitenon and the paratenon is sometimes called the peritendinous sheath – not to be mistaken for the tendon (or synovial) sheath that surrounds parts of some of the tendons in the hand and feet where, due to angulated courses of the tendon, a significant friction demands extra efficient lubrication. A more convenient term to avoid further confusion is peritendon as denominated by Jozsa & Kannus (Józsa et al. 1997). Concerning the term "loose paratendinous connective tissue", which is frequently used in this thesis, see chapter 3.

TENDON CELLS

Tendons consist of a number of different cells: Primarily tenoblasts and tenocytes, i.e. subpopulations of fibroblasts (Riley 2008), which cover about 90-95% of the total cell count; the remaining 5-10% being the chondrocytes at the osteotendinous junction, the synovial cells in the paratenon or tendon sheath, and vascular cells such as endothelial cells and smooth muscle cells (Józsa et al. 1997).

Tendons of the newborn have a higher cell-to-matrix ratio than those of the adult. Initially, the tendon-forming cells, called tenoblasts, are arranged in long parallel chains and have different shapes ranging from elongated to rounded and even polygonal. As the tendon matures, the tendon cells decrease in number and take on a spindle shaped appearance. The cells are now called tenocytes (Ippolito et al. 1980).

The younger cells – the tenoblasts – exhibit a high number of organelles in the cytoplasm and stay in close contact with the fibres of the tendon. These characteristics of the tenoblasts correlate to the idea that the tendon has a high metabolic activity in the young (Ippolito et al. 1986). It is, however, a common misconception that the tendon in adults would be metabolically inactive, when in fact, the tendon constantly maintains and repairs itself (Dudhia et al. 2007).

The older cells – the tenocytes – are elongated and of larger size than the tenoblasts. As the tendon matures the cell-to-matrix ratio decreases, and the general tendon cell count is less than that in the young tendon. The tenocytes have longer cellular processes in order to keep in contact with the surrounding matrix as the lower number of cells-per-matrix needs to be compensated for (Józsa et al. 1997).

TENDON METABOLISM

The tendon is, as stated above, a metabolically active structure, in contrast to what was previously thought. In comparison with skeletal muscle, however, tendons have a much lower oxygen requirement (Vailas et al. 1978).

The most important aspect of tendon metabolism is that of a relatively slow reparation and adaptation to change. As will be discussed in the following sections, the healing of tendons after rupture can take up to a year (Sharma et al. 2006). Though there is a balance between degradation and fresh collagen synthesis at all times in the normal tendons of the young (O'Brien 1997), the reparative capabilities of the tendon diminishes as it gets older (Józsa et al. 1997). One should therefore not be surprised by the fact that injuries and overuse of tendons require long periods to recover.

The Achilles tendon

3

ANATOMY

In This Chapter

The Achilles tendon – also known as the calcaneal tendon or the heel tendon – is one of the longest and strongest tendons in the human body (Lemm et al. 1992; DeMaio et al. 1995). It arises from the triceps surae muscle of the calf, which is comprised of the gastrocnemius muscle and the soleus muscle (Figure 2). The origin of the gastrocnemius muscle is found above the knee joint, on the distal parts of the femur, while that of the soleus muscle is located on the lower leg, on the tibia and fibula. Found between the gastrocnemius and soleus is the plantaris muscle, the tendon of which partly runs between the triceps surae muscles, and is not incorporated in the Achilles tendon but runs adjacent to it (Cummins et al. 1946; Józsa et al. 1997; Doherty et al. 2006).

The most proximal part of the Achilles tendon arises from the two heads of the gastrocnemius muscle (originating from the medial and lateral condyles of the femur, respectively) and forms a flat and broad connective tissue. The tendon, which spans from 11-26 cm in total (Curvin et al. 1984), becomes more narrow and rounded distally. After about half of its length, the tendon receives support from the soleus muscle fibres, which attach on the anterior/ventral surface of the tendon. The distal part of the tendon attaches to the calcaneal bone in the shape of a delta via a fibrocartilaginous expansion (Reynolds et al. 1991).

It is interesting to note how the fibres travel in the Achilles tendon as they descend towards the calcaneal insertion. Fibres that at the origin are found on the posterior side are twisted towards the lateral side; lateral fibres are twisted towards the anterior/ventral part, and so on. The tendon itself may in some cases spiral up to 90° laterally (Cummins et al. 1946) (Figure 3a). This rotation has been speculated to affect the tensile strength of the tendon as well as to assist in supination of the foot in initiation of the gait (Morimoto et al. 1968; Williams et al. 1989). The rotation of the tendon has been found to correlate with the way the tendon is constituted by the gastrocnemius and soleus originating fibres, as the degree of fibre content supplied from the respective muscle (for variants of this, see Figure 3b), and the level where the fusion takes place, are determinants for how much the tendon rotates (Cummins et al. 1946).

In close relation to the distal part of the Achilles tendon, one finds

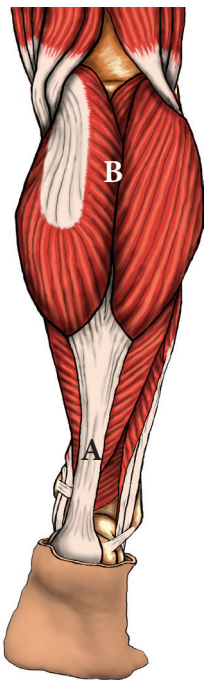


Figure 2. Anatomy of the Achilles tendon (A) and the triceps surae muscle (B).

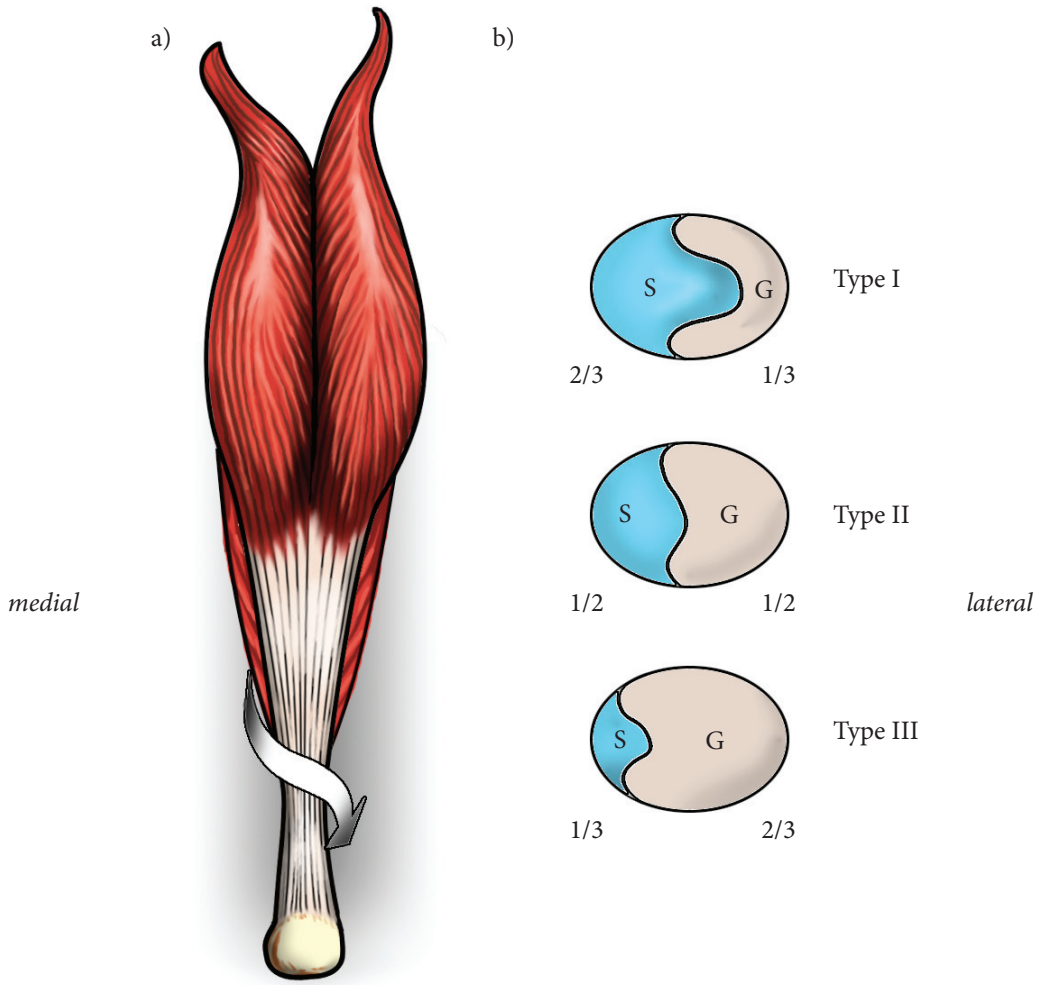


Figure 3: **a)** Posterior view of the right leg. Arrow illustrating the Achilles tendon rotation.
b) Transverse section of the Achilles tendon (right leg, superior view). The degree of fibre content of the Achilles tendon supplied by the gastrocnemius (G) and soleus (S) muscles, respectively, varies in different people (Cummins et al. 1946). The different types (I-III) range from gastrocnemius-soleus ratio 1:2 to 2:1.

the retrocalcaneal and subcutaneous calcaneal bursae. These synovial fluid containing structures help to decrease friction between the tendon and the calcaneal bone (Reinherz et al. 1991).

In this thesis the term "loose paratendinous connective tissue" is frequently used as a denomination for the loose connective tissue in principle also the fat (by some called Kager's fat pad, cf. below) that is found ventral to the Achilles tendon (Figures 4). However, it should be noted that the histological distinction between the paratenon and the other paratendinous tissues is at times hard to discern in the specimens examined in this

study, especially in the small biopsies.

Ventral to the Achilles tendon, one finds the "Kager's fat pad" (or "Kager's triangle") – a mass of tissue comprised of primarily adipose cells intertwined by small bundles of elastic fibres and type I collagen, as shown in animal studies (Shaw et al. 2007). It is visualised as a radiolucent triangle in lateral radiographs of the ankle. Its boundaries are the Achilles tendon, the calcaneus, and the posterior border of the flexor hallucis longus muscle (Ly et al. 2004) (Figure 4).

The large number of different structures surrounding the tendon easily gives rise to misunderstandings concerning the anatomical topography of the tendon, with the different sheaths and structures being mixed up. In an attempt to clarify this, Franklyn-Miller and colleagues (Franklyn-Miller et al. 2009) injected silicon in the "paratenon space" of cadavers, and in dissection of these verified that the paratenon indeed is continuous with the fascial envelope of the triceps surae muscles, as previously reported by Williams (Williams 1986) (cf. Chapter 2). Distally, the paratenon blends with the calcaneal periosteum (Williams 1986). A recent study has shown that the paratenon on the dorsal side of the tendon – the paratenon in itself being a continuation of the muscle fascia – confluences with the crural fascia at about 4 cm from the calcaneal insertion (Carmont et al. 2010).

The paratenon of the Achilles tendon is described as consisting of gliding membranes on the dorsal, lateral and medial sides of the tendon (Kvist et al. 1987), but the ventral aspects of the paratenon is usually summarised as being an fatty areolar tissue with rich vascularisation (Kvist et al. 1987; Schepsis et al. 1994). Others imply that, like the situation in the lateral, medial and dorsal aspects, the paratenon is constituted of a membranous structure also on the ventral side, and that it is not continuous with Kager's fat pad (Pierre-Jerome et al. 2010), as visualised by ultrasound.

In summary, the details of the ventral aspects of the tendon is unclear in the literature, and further anatomical and radiological studies are desired to avoid continued misunderstandings of the anatomy. However, one study by Soila and colleagues, using

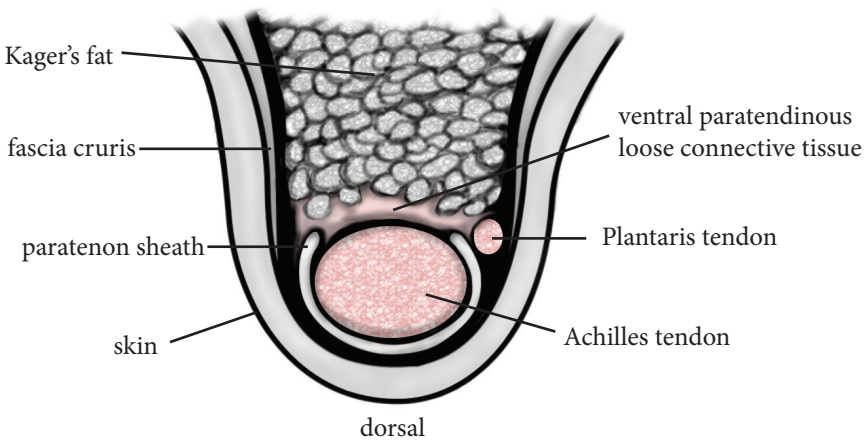


Figure 4: Transverse section of the Achilles tendon (midportion) region. Relation between the Achilles tendon and adjacent structures is shown. Note paratenon sheat (medial, dorsal, lateral) and paratendinous loose connective tissue (ventral) surrounding the Achilles tendon. (Left leg, superior view)

high-resolution MRI, further support the idea of the paratenon fusing distally with the crural fascia, and that it is only found on the dorsal, lateral and medial aspects of the Achilles tendon (Soila et al. 1999). Ventral of the tendon only Kager's fat pad was found (Soila et al. 1999), protecting and stabilising the blood vessels entering the Achilles tendon (Theobald et al. 2006). In the light of these findings, it is this author's opinion that whenever the structure surrounding the tendon is discussed, the term paratenon should preferably be confined to the sliding membranes dorsal, lateral and medial of the tendon, while the adipocyte- and loose connective tissue-rich structure on the ventral aspect (Kvist et al. 1987) should be called "the paratendinous loose connective tissue" (as is done in this thesis) (Figure 4).

BLOOD SUPPLY

All tendons share a general set-up of vascular supply, with the three origins of the tendon blood vessels being: (1) vessels of the related muscle, (2) vessels coming from the bone and periosteum at the osteotendinous junction, and (3) vessels from tissues surrounding the tendon (paratenon, mesotenon and synovial sheath) (Figure 5) (Carr et al. 1989). This gives rise to a intra- and peritendinous network of blood vessels capable of supplying the entire tendon.

Concerning the Achilles tendon, the vessels originating in the muscle, arriving from the perimysium of the triceps surae, continue between the tendon fascicles, maintaining

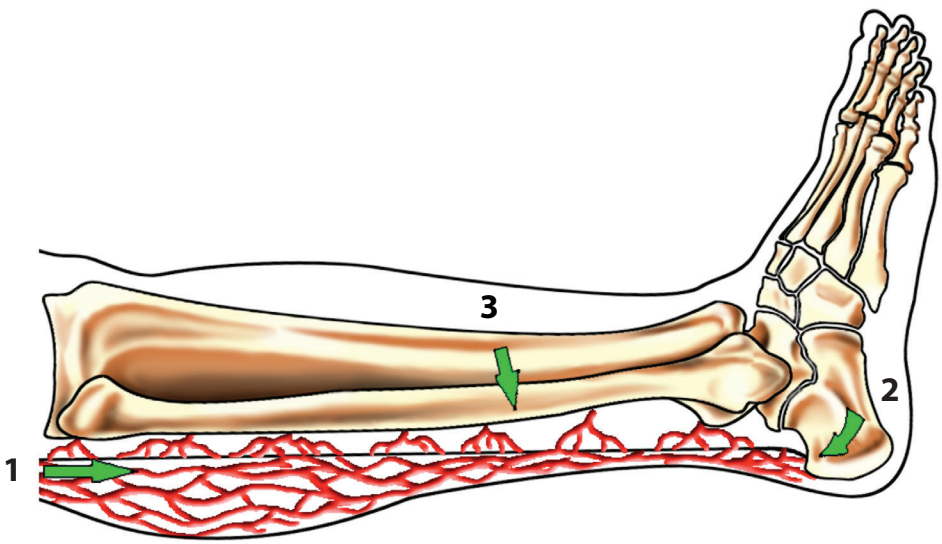


Figure 5. Vascular supply of the Achilles tendon, 1-3 denotes the three origins of the blood supply (cf. numbers in text).

their original size. However, some studies (Peacock 1959; Carr et al. 1989) have shown that these vessels only supply the upper third part of the tendon. In consequence, there is an area with poor vascular distribution found 2-6 cm proximal to the calcaneal insertion (Lagergren et al. 1959; Carr et al. 1989). On the other hand, Doppler examinations of the Achilles tendon have shown an even blood flow distribution throughout the Achilles midportion, but with a lower flow at the calcaneal insertion (Astrom et al. 1994). One should however differ between actual blood flow as measured with Doppler, and the anatomical distribution of vessels. The blood supply is, however, not only related to vessels from the muscle.

The main blood supply of the Achilles tendon is actually considered by some to be that of the paratendinous network of blood vessels which originates from the anterior and posterior tibial arteries, as well as the peroneal arteries (Lagergren et al. 1959). As the Achilles tendon has no synovial sheath, the paratendinous vascular network is comprised of branches from these arteries, the branches transversely penetrating the paratenon (Reynolds et al. 1991) and the loose paratendinous tissue found ventral to the tendon (Theobald et al. 2006). Most of these paratendinous vessels can be found on the ventral side of the tendon, and less are seen on the dorsal side of the tendon (Zantop et al. 2003). In fact, when the paratenon, the origin for the majority of the blood supply for the Achilles tendon (Peacock 1959; Williams 1986), is removed, the only vessels visualised are those in the myo- and osteotendinous junctions (Peacock 1959; Carr et al. 1989) possibly supporting the importance of the paratendinous blood supply. One should however note, that surgery including paratenon stripping (cf. chapter 5) does not cause necrosis of the tendon. The paratendinous vessels pass through the paratenon, then penetrate the epitenon transversely or obliquely, and via the endotenon septa, the vessels connect to the intratendinous vascular network (Józsa et al. 1997).

Intratendinously, the blood vessels (as well as nerves and lympathic vessels) follow the endotenon in-between the tertiary fibre bundles, usually in the constellation of one artery per two veins. These vessels run in the longitudinal direction of the tendon and communicate with each other via anastomoses (Edwards 1946). The sizes vary, the larger ones at times being called “the main arteries of the intratendinous vascular bed” (Józsa et al. 1997).

INNERVATION

The Achilles tendon receives its innervation from different origins, including nerves that innervate the triceps surae muscle and cutaneous branches of the sural nerve (Stilwell 1957). However, the innervation is quite sparse inside the tendon tissue proper, with just a few small nerve fibres following the endotenon septa (Józsa et al. 1997), as compared to smaller tendons, such as the finger flexors, for which the level of innervation is quite high. The main part of the innervation of the Achilles tendon is found in the paratenon, and penetrates the epitenon to reach the tendon tissue proper (Stilwell 1957; Andres et al. 1985; Ippolito et al. 1986).

The nerve endings found inside human tendons in general consist of Ruffini corpuscles, Vater-Pacini corpuscles, Golgi tendon organs, and free nerve endings (Józsa et al. 1997). All these are important for the signalling to the central nervous system (CNS) concerning the pressure changes and the tensile stress of the tendon, which

helps regulate balance and changes in movement status (Jozsa et al. 1993). The free nerve endings are primarily found in the tissues surrounding the tendon tissue proper and are involved in transmission of pain (Zimny et al. 1989; Katonis et al. 1991; Jozsa et al. 1993). Looking specifically at the Achilles tendon, the prescence of Golgi tendon organs and free nerve endings has been shown both in the tendon tissue proper and the paratenon (Andres et al. 1985). However, generally there are few studies on the innervation patterns of the human Achilles tendon. Recent studies, including a paper of this thesis (paper I), have further delineated the innervation patterns concerning the sympathetic and sensory innervation of the Achilles tendon (Bjur et al. 2005).

In studies on rats, the adipose tissue ventral to the Achilles tendon (Kager's fat pad, cf. 'Anatomy') has shown to be supplied by sensory nerve fibres, primarily in the proximal portion (Ackermann et al. 2003; Shaw et al. 2007).

COMPARATIVE ANATOMY - RABBIT vs MAN

As two of the papers of this thesis (III-IV) are based on animal (rabbit) studies, it is important to clarify the differences between the anatomy and histology of the human and rabbit Achilles tendon.

Both species have the same components of the triceps surae muscle; namely medial and lateral gastrocnemius muscle heads as well as a soleus muscle part. The lateral rotation of the Achilles tendon, as described above, also occurs in the rabbit (Doherty et al. 2006).

The most distinctive, and macroscopically notable, difference is that of a more distal fusion of the tendon fibres originating from the two gastrocnemius heads in rabbit. In humans, these meld together after reaching 23% of their course (beginning proximally) as compared to the rabbit, in which they do not fuse until after 93% of their course – about 5 mm from the distal end (Doherty et al. 2006). In our histological samples from rabbits, the two fascicles could be clearly visualised, and the endotenon clearly divided them. In human histological samples, the Achilles tendon has a more homogenous appearance, and any separation between the tertiary bundles is very hard to notice. The importance of the soleus muscle in the contribution to the rabbit Achilles tendon is unclear, but it appears to have a negligible contribution to the tendon (Doherty et al. 2006) as compared with the human Achilles tendon where it constitutes up to two thirds of the fibre content (cf. 'Anatomy' and Figure 3) (Cummins et al. 1946).

Another, very important, difference between the human and rabbit Achilles tendon, is the relationship to other tendon structures. In humans, the tendon of the plantaris muscle accompanies the Achilles tendon, but usually does not become part of the Achilles tendon itself. In the rabbit, however, there is a flexor digitorum superficialis muscle (not found in human), which travels in close relation to the Achilles tendon fibres from the medial gastrocnemius muscle and is inserted just posteriorly to the Achilles tendon at the calcaneus (Popesko et al. 1990). At the level of the calcaneal insertion, it shares the paratendinous tissue with the Achilles tendon making it hard to discern from the gastrocnemius tendon-parts in a transverse section (Doherty et al. 2006).

What effects these differences may have on the possibility of creating a tendinopathy model, as well as when comparing the results in regard to biomechanical stress, are not known, and can only be speculated on.

Tendinopathy and tendinosis

4

In This Chapter

DEFINITION OF TENDINOPATHY AND TENDINOSIS, AND OTHER TERMINOLOGY

The tendons of the loco-motor system of man are prone to develop many different pathological conditions. The Achilles tendon is in focus of this thesis, but there are many aspects to consider when talking about the ways in which a tendon can be affected.

A term widely used is tendinopathy, which can be literally translated as "the disease of a tendon". This term is however often more specifically used for conditions of tendon pain; the symptom to which the majority that suffers from tendinopathy can attest (Khan et al. 2000; Alfredson 2005; Riley 2008). A painful condition in the tendon is one of the basic characteristic of all tendinopathies, but for the full definition of tendinopathy in modern medicine, a swelling as well a loss of function, are required (Khan et al. 1999). This use of the term, which is a clinical definition, gained popularity under the last decade, and is probably the most accepted way of talking about a tendon disorder. However, this says nothing about the underlying cause (Riley 2004).

Further nomenclature that has been popular to use is tendinitis (or tendonitis) and peritendinitis (alt. peritendonitis, paratendinitis, paratendonitis) or variations thereof. This use of words instantly adds a level of pretence as to the understanding of the cause of the condition at hand. The suffix "-itis"is used to describe an inflammatory process, and would in this case attribute the swelling and pain symptoms as being caused by inflammation. There may be an acute form of inflamed paratenon/peritenon, but this condition should not be confused with the long-lasting, painful tendinopathies, upon which this thesis is based. Several histopathological, biochemical, and molecular studies have actually shown that there is a lack of a prostaglandin mediated inflammatory process inside the tendon of the 'chronically' painful tendon (Khan et al. 1999; Alfredson et al. 2001; Riley 2005). The classical definition of inflammation includes not only pain (dolor) and swelling (tumor), but also heat (calor) and redness (rubor) as recorded by the Roman encyclopaedist Celsus in the 1st century A.D. The latter two characteristics are lacking in tendinopathy. Consequently, it cannot be justified to us the suffix "–itis" for this condition.

As recent studies have started to elucidate the complexity of the disorders affecting tendons, a more restrictive nomenclature has come into use by clinicians and researchers alike. Aside from the above-mentioned term tendinopathy, terms like achillodynia, coined by Åström (Astrom 1997), which describes a symptomatically painful Achilles tendon, is sometimes used.

The term tendinosis is a further way of describing the condition in front of the clinician. It is used in the studies in this thesis to describe a tendon with all the characteristics of tendinopathy in combination with verified structural tissue changes as seen by use of ultrasound, MRI or histological evaluation of biopsies (cf. below) (Khan et al. 1999; Alfredson et al. 2003). It could be considered that to the patient suffering from a painful tendon, the terminology may not be of utmost importance, as the main interest is to be relieved of the condition, particularly the pain. However, from a research or clinicial point of view, the terminology is highly relevant as there are tendinopathies without a chronic overuse component (Rolf et al. 1997), and the subsequent pathology and optimal treatment may differ substantially depending on the true characteristics of the condition at hand.

As it in some cases is uncertain in what way some researchers use the terms tendinopathy, tendinosis etc., the name tendinopathy will still be widely used in this thesis to avoid misinterpretation of studies where the pathology has not been clarified. This is especially relevant when it appears to be no histological, or otherwise, verified structural changes of the tendon – which should be a requisite for calling the condition tendinosis. Therefore, the reader should not be confused with the mixed use of these two terms (tendinosis and tendinopathy) as it is only a way of avoiding misquotations of earlier studies that mostly describe the same thing.

Most of the tendon conditions related to tendinopathy/tendinosis have at times been considered to have both an acute and a chronic manifestation. The definition of chronic, as postulated by Kettunen and collaborators, implies that the condition has been ongoing for more than 3 months, with continuous symptoms during activity (Kettunen et al. 2002). However, we cannot really tell for certain whether a patient's tendon problem is truly chronic, in the meaning "forever-lasting", as the natural progression of the condition is unclear at the time of clinical examination. Furthermore, the denomination 'long-lasting', persisting, or variants thereof, could possibly give the patient a brighter outlook on the development of the condition.

TENDINOSIS

As mentioned above in this thesis, the condition tendinosis is one of a long-lasting painful and swollen tendon, which often results in a loss of the desired function in the affected tendon, and with verified structural changes (Alfredson 2005). In order to diagnose a patient with tendinosis, these structural changes including hypercellularity, increased vascularity, and/or irregular collagen structure, need to be confirmed (Khan et al. 1999; Alfredson 2005). The latter two characteristics are commonly visualised using ultrasound + colour-Doppler, which can detect a high (higher than the normal) blood flow in the tendon, and often a flow originating from the anterior/ventral side of the tendon (Ohberg et al. 2001; Ohberg et al. 2002; Leung et al. 2008). Also, a hypoechoic tendon, showing an increased width is seen (Archambault et al. 1998; Ohberg et al. 2001). However, as the Doppler describes the degree of flow in vessels, one cannot

be certain that this relates to more vessels, nor can the ultrasound, or MRI, verify an increase in the number of tenocytes, wherefore it has been discussed whether the tendinosis diagnosis entirely should be regarded as a histopathological diagnosis (Maffulli et al. 1998; Peers et al. 2005). However, it should be pointed out that in general practice, the diagnostics with ultrasound and colour-Doppler is less complicated than an invasive histological confirmation, and is often more readily available than MRI.

Tendons prone to develop tendinosis are the Achilles tendon, the patellar tendon (the condition known as "Jumpers knee"), the supraspinatus tendon, and the adductors of the leg (Khan et al. 1996; Khan et al. 1999; Zeisig et al. 2006; Riley 2008). The condition can also affect the extensor carpi radialis brevis muscle origin at the lateral humeral epicondyle (the condition known as "Tennis elbow") and the flexor muscle origin at the medial humeral epicondyle ("Golfers elbow"). All these tendons/muscle origins are lacking tendon sheets, and instead have a paratenon (Józsa et al. 1997).

TENDON HEALING

Whenever a tendon is subjected to some kind of trauma, it will go through a series of steps in order to repair itself. These steps can be summarised in the following phases: (1) cellular reaction, (2) fibrous protein synthesis, and (3) remodelling (Reddy et al. 1999). These findings, which are largely based on animal studies, are believed to be the same in humans (Sharma et al. 2006).

During the cellular reaction, inflammatory cells infiltrate and remove the damaged tissue, and there is a release of vasoactive and chemotactic factors, which stimulate angiogenesis, tenocyte proliferation, and further recruitment of inflammatory cells (Murphy et al. 1994). The tenocytes are responsible for synthesising type III collagen, which constitute the initiation of the second phase of healing (Sharma et al. 2006).

The type III collagen synthesis (step 2) lasts for a few weeks, during which time the water content and glycosaminoglycan concentrations are high (Sharma et al. 2006).

The final step, which involves the remodelling of the healing tissue, begins with a strengthening of the repair tissue involving a transition from a cellular to a fibrous state. The tenocytes, highly metabolically active at this point, and collagen, get aligned in the direction of stress (Sharma et al. 2006), and the collagen production changes into primarily type I collagen (Abrahamsson 1991). After about 10 weeks, a gradual change of the fibrous tissue into a scar-like tendon tissue takes place. This can take up to a year, and in the end tenocyte metabolism and tendon vascularity decline (Sharma et al. 2006).

5

Tendinopathy of the Achilles tendon

In This Chapter

LOCALISATION OF ACHILLES TENDINOPATHY

The most common tendinopathic conditions pertaining to the Achilles tendon can be found at three different locations along its length; the three being: (1) insertional – at the calcaneal insertion(Carmont et al. 2007), (2) midportion – at 2-6 cm above the insertion, and (3) myotendinous – at the muscle-tendon junction (Movin 1998).

Midportion Achilles tendinopathy is the one that is of major interest in this thesis, and all patients in the papers included were diagnosed with midportion Achilles tendinosis. This type is considered to be involved in 55-65% of all Achilles tendon injuries (Kvist 1991; Kvist 1994; Jarvinen et al. 1997; Jarvinen et al. 2005).

EPIDEMIOLOGY

It is considered that about 7-9% of professional athletes participating in sports that contain a high frequency of running and jumping have Achilles tendinopathy (Lysholm et al. 1987; Almekinders et al. 1998; Cook et al. 2002; Alfredson 2003), and the condition makes up 6-18% of all injuries for recreational runners (Alfredson et al. 2000; Fahlstrom et al. 2002a; Schepsis et al. 2002). The age group in which tendinopathy is often seen is the group of individuals ranging in age from 30 – 60 years (Kvist 1991; Paavola et al. 2000), and some studies have shown that up to 30% suffer from bilateral symptoms (Nelen et al. 1989; Kvist 1991; Paavola et al. 2002b; Ohberg et al. 2004). Ordinary modes of activity that can lead to Achilles tendinopathy include middle- or long distance running, badminton, track and field activities etc. (Kvist 1991; Fahlstrom et al. 2002b), but it has also been shown that individuals with a low level of physical activity are prone to be afflicted (Kvist 1991; Kvist 1994). Achilles tendinopathy has in later years indeed been shown in people with relatively sedentary lifestyles, with some studies reporting up to almost a third of patients being non-athletes (not participating in sports/physical activity on a regular basis) (Rolf et al. 1997; Alfredson et al. 2000). For differences between males and females, see “Issues of Sex and Gender” below.

AETIOLOGY, PATHOGENESIS AND PATHOLOGY - THE ACHILLES HEEL OF THE ACHILLES TENDON

There are many theories and suggestions concerning aetiological factors of importance for Achilles tendinopathy, but importantly, most of them rest on weak grounds with sparse scientific evidence.

Underlying factors that possibly predispose for tendinopathies are considered to be multifactoral and of both intrinsic and extrinsic nature.

One of the most discussed intrinsic factor – i.e. factors related to the patient – is that of repetitive strain on the Achilles tendon (Kader et al. 2002; Paavola et al. 2002a), and it has also been suggested that anatomical malalignment of the lower extremity predisposes for Achilles tendinopathies (Kvist 1991; Kvist 1994; Kaufman et al. 1999).

Other suggested factors include age, with high age leading to diminishing mechanical properties (Tuite et al. 1997; Dudhia et al. 2007), sex (Hart et al. 1998), muscle weakness (Haglund-Akerlind et al. 1993) and lack of flexibility (Clement et al. 1984).

There are also studies linking adiposity to tendon overuse injuries (see (Gaida et al. 2009) for a review) which is of interest as physical activity may be one of the most important interventions concerning the health of obese patients, and as activity related pain can hinder exercise.

Extrinsic factors include poor equipment, such as not optimal shoes, training errors (Clement et al. 1984), and running on uneven surfaces (Hart 1994). Another factor is that of the well-documented complication of fluoroquinolone antibiotics leading to tendon disorders (van der Linden et al. 1999; Khaliq et al. 2003) primarily in those with renal dysfunction.

As a result of the Achilles tendon characteristics described in chapter 3, a region of concentrated stress is likely to occur at the site of fibre fusion of soleus and gastrocnemius derived fibres, due to the rotation of the tendon (Reynolds et al. 1991). This coincides with the region of the Achilles tendon which has been suggested to have the most poor vascular supply (Lagergren et al. 1959). This region, 2-6 cm proximal to the calcaneal insertion, is also the place where the Achilles tendon is the thinnest (a cross-section of 0.4-1.4 cm^2) (Kvist 1994; Magnusson et al. 2003).

These factors, although unclear to what degree they predispose for tendinopathic conditions, together with repetitive overuse of the tendon, is traditionally considered some of the most common pathways for the chronically painful Achilles tendinosis, with repetitive microtrauma exceeding the reparative capabilities of the tendon (Leadbetter 1992).

HISTOPATHOLOGICAL CHANGES

The theory of incomplete healing of the tendon as a basis for tendinosis is widely accepted, and the histopathological changes can all be correlated to such a condition.

The characteristics of tendinosis tendons include degeneration of the ECM (Riley 2005) with disordered arrangement of collagen fibres and increased vascularity (Khan et al. 1999). Furthermore, it is well established that the afflicted tendon exhibits an increase in tendon cells, especially cells with rounded nuclei (Astrom et al. 1995). The vessels, which are considered neovessels, are by some described to be randomly oriented (Wil-

liams 1986; Khan et al. 1999), while others have noted an increase also in the number of vessels aligned in parallel with the tendon fibres (Maffulli et al. 2000).

A hallmark in the tendinosis field has become the lack of inflammatory lesions and granulation tissue, with the exception of when there are partial ruptures in the tendon (Ljungqvist 1967; Denstad et al. 1979). On the other hand, it is considered that inflammation may be an important first step in development of tendinosis, but as seen in some studies on tendon healing in rabbits, the inflammatory infiltrates appear to disappear after 18 days post tenotomy with suturing, of the Achilles tendon (Enwemeka 1989). If this is true for humans as well, the patients seeking medical attention are likely not in a primary inflammatory phase anymore as they usually see the clinician after a long time of pain symptoms and the condition has entered a "chronic" stage (Khan et al. 1999).

When looking at the collagen component of tendinosis tendons, one finds an increase in type III collagen (Jarvinen et al. 1997; Riley 2005) as compared to the normal tendon. The accumulation of GAG:s and lipids as well as calcification of the tendon tissue has also been described in tendinopathy (Riley 2005).

TREATMENT METHODS OF TENDINOPATHIES

A wide range of treatment alternatives is suggested in the treatment of tendinopathies. Unfortunately, there is sparse scientific evidence favouring certain methods. As the understanding of the pathological mechanisms is increasing, older methods are left behind in some countries and the field is opened for new alternatives.

Rest

In the case of acute injuries, or as an initiating step of treatment, rest from the painful activity is usually recommended (Angermann et al. 1999). However, completely avoiding physical activity can have negative effects on both the tendon itself (Kannus et al. 1997) as well as have a negative impact on the general health of the patient. As tendinopathy is considered as an overuse injury (Józsa et al. 1997), continued abuse of the structure is suggested to worsen the condition. However, interestingly, the majority of patients seeking help for Achilles tendinopathy have had a long duration of pain symptoms, rest having had no effect on the painful condition.

Physical therapy – Eccentric training

In recent years, one regimen of importance in relation to tendinosis at several anatomical locations has been painful eccentric muscle training (Alfredson et al. 1998). Although it is unclear what mechanism that is at play – with theories ranging from neurological modulation to structural response to the forces involved – the eccentric training regimen has shown promising long term results, especially in the case of chronic Achilles tendinosis (Fahlstrom et al. 2003). Similar effects on pain-relief and a return to physical activity have been seen concerning eccentric training in the treatment of tendinosis in other tendons, such as the patellar (Purdam et al. 2004; Jonsson et al. 2005), and supraspinatus tendon (Jonsson et al. 2006). In a review of nine studies on eccentric training for chronic Achilles tendinopathy, 60% of patients undergoing this rehabilitation regime showed reduction of pain, as compared to only 33% in the control group (Kingma et al. 2007).

Injection treatment

There are many different types of injection treatments for Achilles tendinosis, the injections being given to intra- or extra-tendinous locations. Injections of cortisone with the purpose to cure inflammation has been used for many years, but is now heavily questioned (Andres et al. 2008). Recently, injections of platelet rich plasma (PRP) inside the tendon has become popular worldwide, but interestingly, in a recent randomised trial PRP had similar effects to saline injections (de Vos et al. 2010). Other injected substances tested are autologous blood, hyperosmolar dextrose and MMP-inhibitors (Edwards et al. 2003; Maxwell et al. 2007; Orchard et al. 2008).

One injection treatment concerning Achilles tendinosis is that of sclerosing Polidocanol injections, directed towards the region with high blood flow at the ventral side of the Achilles tendon, guided by utrasonography and colour Doppler (Alfredson et al. 2005). This regimen has shown promising results (Alfredson et al. 2005; Willberg et al. 2008), and a follow-up study (Lind et al. 2006) showed remaining pain relief, improved tendon structure, and a thinner tendon, with very few complications being reported (Alfredson et al. 2007a). However, despite showing promising results, the mechanism – as with the eccentric muscle training – is still unknown. The sclerosing agent may act not only on the neovessels but also the pain-transmitting nerves (Alfredson et al. 2003; Andres et al. 2008).

Traditional surgery

There is a variety of surgical approaches to treat chronic Achilles tendinopathy, grouped by Tallon and collaborators into four different variants: (1) open tenotomy with removal of abnormal tissue, paratenon not stripped; (2) open tenotomy with removal of abnormal tissue, paratenon stripped; (3) open tenotomy with longitudinal tenotomy, with or without paratenon stripping; (4) and percutaneous longitudinal tenotomy (Tallon et al. 2001).

Surgery is often seen as the last resort, when no positive results have been achieved either by conservative or other treatments (Alfredson et al. 2007a), and is often recommended to be considered only after at least 3-6 months of non-surgical treatment has been tried (Angermann et al. 1999). Surgical resection basically involves removing the intratendinous tendon structure showing hypercellularity and proliferating vessels in the tendon tissue proper. The results of this kind of treatment is reported to have a success rate around 70% or better (Leppilahti et al. 1991; Schepsis et al. 1994; Morberg et al. 1997), but in a critical review by Tallon and colleagues this was questioned as they found that the studies reporting high success rates were generally of poor design (Tallon et al. 2001).

In this thesis, the radical surgical procedure performed on patients of these studies is henceforth referred to as "old surgical method", and implies open tenotomy, paratenon stripping and multiple longitudinal incisions in the same operation.

Novel variant of surgery – Minimal invasive treatment

A novel alternative to the radical surgical methodology described above, is a minimal invasive surgery based on the same idea as the sclerosing injections, where the newly formed vessels and accompanying nerves, ventral to the Achilles tendon, are targeted (Alfredson et al. 2007b). Through a small incision in the skin, the ventral part of the tendon is scraped free from the adhering tissues using ultrasonography and Doppler as guide (Alfredson et al. 2007b). This can all be done in local anaesthesia and the patient can return to physical activity 3-6 weeks after the procedure.

This kind of surgical treatment performed on some of the patients in this thesis is from here on referred to as the "new surgical method". From these patients biopsies were taken from the ventral side of the Achilles tendon, including parts of tendon tissue proper.

ISSUES OF SEX AND GENDER IN TENDINOPATHY

In all fields of medical science, a difference between the sexes are commonly described as there are well-described differences between how males and females – of all species – present and react to disease. Quite commonly, the terminology – as defined by the World Health Organisation (WHO) – is erroneously used with no distinction between sex and gender. According to these guidelines, sex is defined as "genetic/physiological or biological characteristics of a person which indicates whether one is female or male" and gender as "women's and men's roles and responsibilities that are socially determined" (WHO 1998; Wizemann 2001). Furthermore, the gender a person identifies with can change in the lifetime of the individual, as a persons own sense of gender can change over time (Wizemann 2001). This terminology aims to differ between the factors attributed purely to the biological presets of the sexes and how society, and culture, may affect the way in which we perceive suffering and disease based upon our socially determined identities. Also, it points to the importance of discerning how science and practice are influenced by these factors.

Concerning Achilles tendinopathy, when comparing male and female patients, some studies have reported a dominance of males with up to 89% of patients (Kvist 1991), while other – more recent studies – have described a patient material consisting of 55% females (Alfredson et al. 2005). When looking at different studies one finds varying ranges in-between these values (Nelen et al. 1989; Paavola et al. 2000; Ohberg et al. 2002; Paavola et al. 2002b). It has been shown that females generally have a prolonged period of complications and recovery, as compared with males undergoing the same surgical treatment (Maffulli et al. 2008). In one study evaluating eccentric training on patients with midportion tendinosis, the majority of the patients showing poor results were females (Fahlstrom et al. 2003).

Females with symptomatic Achilles tendinopathy have been shown to have a better microcirculation in the Achilles tendon than their male counterparts (Knobloch et al. 2008). On the other hand, the collagen synthesis rate is reported to be lower in female patellar tendons than in male counterparts (Miller et al. 2007). Further, the impact of menopausal hormone alterations has been associated with a decrease in collagen I leading to less tensile strength (Moalli et al. 2004). Menopausal women without hormone replacement therapy (HRT) with an active physical lifestyle are reported to

display a thicker tendon diameter as compared to those with HRT (Cook et al. 2007). In a recent cohort study (Gaida et al. 2010), comparing male and female patients with asymptomatic Achilles tendon pathology, a difference in the distribution of fat was found between the sexes. Males had predominantly a central fat distribution and women a peripheral distribution of fat (Gaida et al. 2010). These findings point to a relation between fat metabolism, or insulin resistance, and tendon pathology in males, whilst in women, the pathologic condition is more likely related to estrogenic effects, as such effects have been shown to prevent central fat distribution.

Concerning substance P, a substance of interest in this thesis, the sex hormones of both males and females have in animal studies been shown to influence the synthesis of this neuropeptide (Kream et al. 1987; Hart et al. 1998; Mowa et al. 2003) as well as the response to it (Bailey et al. 1989; Hart et al. 1999; Bradesi et al. 2001).

Experimental animal models 6

HISTORY

Experimental laboratory animals have been used throughout the history of mankind; the first records found of their use are those of Greek-philosopher-physicians around 300 years BC (Cohen et al. 1984). By performing dissections on animals, Aristotle (384-322 BC) could describe comparative anatomical features, while Erasistratus (304-258 BC) was the first on record to perform live animal experiments (Cohen et al. 1984).

In times of church supervision on rules of conduct concerning the treatment of human cadavers, animal dissections was the sole way for scientists of the day to understand the principles of human anatomy and physiology. An example is that of a Roman physician by the name of Galen (around 200 AD) who compared his animal findings with his knowledge of human patients as he was prohibited to perform human autopsies (Apuzzo 2000). The arabic physician Avenzoar practiced, and tested, his surgical techniques on animals before applying them on his human patients (Abdel-Halim 2005).

In modern times, starting in the 18th and 19th centuries, animal experimentation became all the more common and accepted, but has always been surrounded by debate and opposition. In response to this, the first animal protection laws were put in place by the British parliament, in the year of 1822, followed by the Cruelty to Animals Act (1876), specifically aimed at regulating animal testing (Sechzer 1981).

In Sweden, animal protective legislation followed suit not until the year of 1944. Prior to this, there was only a law prohibiting the abuse of farm animals. By 1988, the law was reformed to more strictly regulate the use of laboratory animal, and the focus of this legislation can be summarised in the principles of humane experimental techniques, as defined by Russel and Buch in 1959 (Russell et al. 1959). This entails the concept of the three R:s – Replace, Reduce, and Refine. In any experiment, one should always strive to replace the living animals with in vitro techniques whenever they are deemed to produce the same result. One should reduce the number of animals needed, in order to still attain statistical significance and sufficient testing of the hypothesis. There should always be an ongoing improvement of the procedure applied to the animals and thus a decrease of painful or distressing experiences. In fact, as stated above, according to Swedish law it is illegal to perform animal studies when there is a suitable method that can replace it (Swedish statutes, Animal Welfare Act 1988:534 (1988)).

In This Chapter

USE OF ANIMALS IN RESEARCH - GENERAL CONSIDERATIONS

Animal models are regularly used as means of developing new medical treatments as well as understanding basic mechanisms of pathology.

A search for alternative methods is always in the scope of most scientific researchers, and several models are available such as cell cultures, embryonic stem cells, videos, computerised models and others (Goldberg 2004). However, not all kinds of research can be applied to these models, as in-vivo conditions are in many cases irreproducible when considering all possible influences of the biological systems in play. Despite this, very good basic science is made possible in non-animal experiments (Goldberg et al. 1989; Balls 1994). Furthermore, the need for good experimental design, and use of correct statistical methods, are of utmost importance to live up to the three R:s (Festing 1994).

TENDINOPATHIES

In the study of tendinopathies, several animal models have seen the light of day, with smaller mammals, such as rats and mice, being the most commonly used (Warden 2007; Lui et al. 2010). Furthermore, larger mammals such as rabbits, dogs, goats and even horses have also at times been in use (Warden 2007; Lui et al. 2010). The study of tendinopathy in non-human primates is basically non-existent due to ethical considerations and high cost, but could otherwise be considered the ideal species from a translational standpoint (Warden 2007).

ANIMAL MODELS OF TENDINOPATHY - OVERVIEW

Generally speaking, there are two methods that can induce tendinopathic changes in a tendon and upon which the majority of animal models are based. These are a) mechanical, b) chemical (Lake et al. 2008).

The mechanically induced tendon changes are often considered more translational towards the human tendon conditions (Lake et al. 2008), as tendinopathies – including tendinosis – are considered to be related to repetitive mechanical loading. However, chemically induced models can be used to study tissue healing and inflammatory response, which is also thought to have an important role in tendinopathy (Lake et al. 2008).

Concerning the mechanical models, they can be further divided into active or passive participation models; where in the active case, the animals themselves are performing the repetitive motion considered to induce the tendinopathy, such as treadmill running (Soslowsky et al. 1996). Via this way, there are no confounding factors in the form of anaesthesia, electrical stimulation or inoculations of other kinds. In the case of passive participation models, the animal is usually anaesthetised and the induction of tendinopathy is induced by an exogenously applied mechanical loading of the tendon. This includes the model used in this thesis, as well as other similar methods where muscle stimulation, with or without combining movement of the joint on which the tendon works (Backman et al. 1990; Backman et al. 1991; Messner et al. 1999;

Archambault et al. 2001; Nakama et al. 2005; Nakama et al. 2006).

Some theories concerning the cause of tendinopathy in various locations include extrinsic factors, such as an "impingement" of the supraspinatus tendon by compression of the coracoacromial arch (Neer 1972; Neer 1983). Models trying to simulate these extrinsic alterations are also in use, usually by surgically altering the biomechanics of a joint (Soslowsky et al. 1996), and in some cases combining this with a mechanical, overuse-model of the type described above (Soslowsky et al. 2002).

Specific animal models of mechanically induced tendinopathy in use today, include a model of supraspinatus tendinosis, originally described by Soslowsky and collaborators (Soslowsky et al. 2000). It entails treadmill-running rats that run 1 km daily, five times a week, at a 10° decline. This set-up gives rise to a repetitive impingement of the supraspinatus tendon, which results in degenerative changes including irregular collagen structure and hypercellularity, after about 4 weeks (Carpenter et al. 1998; Soslowsky et al. 2000; Soslowsky et al. 2002). This is likely to be one of the most commonly used models today (Warden 2007).

Furthermore, there is a rabbit model of flexor digitorum profundus tendinosis in which, after sedation, the animal is electrically stimulated to produce contractions in the muscles of one forelimb (Nakama et al. 2005). This is done repeatedly for two hours a day, three days a week to a total of 80 hours. By resisting forefinger movements, loading of the tendon is achieved (Nakama et al. 2005). This model has shown both microtears of the tendon as well as increase in growth factor production to occur.

These mechanically induced models have in many studies been shown to mimic histopathological changes seen in human tendinosis, such as hypercellularity, increased vascularity, and upregulation of stress response genes (Backman et al. 1990; Backman et al. 1991; Messner et al. 1999; Archambault et al. 2001; Nakama et al. 2005; Nakama et al. 2006). Also, in some of the models, an acute inflammatory response, with expression of pro-inflammatory genes and the detection of infiltrating inflammatory cells was noted (Backman et al. 1990; Barbe et al. 2003; Perry et al. 2005).

The "chemically induced" tendinopathy models are often based on collagenase injections. This way of producing tendon changes is seen as one that causes minimal distress to the animals being studied. It has been described to induce tendon degeneration in combination with a classical inflammatory response (Silver et al. 1983; Williams et al. 1984). Collagenase injections are used in both the study of acute injury as well as tendinopathy. After causing a general matrix disruption with bleeding and fluid exudation, the tendon heals but with an abnormal structure, such as a lack of fibril alignment and diameter decrease.

Some studies base their tendon altering effects on substances that do not induce mechanical degradation, as in the collagenase model, in order to better study the effects of inflammation alone, on the tendon tissue. These substances include cytokines, carrageenan, which is a vegetable polysaccharide, and corticosteroids (Tatari et al. 2001; Marsolais et al. 2007). Prostaglandins have also been used, as they are up-regulated in exercise, and it has been speculated that they in turn induce an endogenous up-regulation of collagenase (Lake et al. 2008). It should here be pointed out that no elevation of endogenous prostaglandin E_2 has been seen in tendinosis tendons as compared to healthy tendons using microdialysis technique (Alfredson et al. 1999).

The chemical models used have shown effects such as increases in cellularity, and vascularity, tendon structure disruption and inflammatory responses (Lake et al. 2008).

THE RABBIT AS AN EXPERIMENTAL ANIMAL

In this thesis, the experimental animal of choice was the New Zealand White rabbit; partly because of the already established tendinopathy model, but also due to certain characteristics of the rabbits themselves.

The rabbit, oryctolagus cuniculus in Latin, is present in many different breeds, but one of the most widely used as laboratory animals is the New Zealand White, which holds a weight of 2-5 kg (van Zuthpen et al. 2001). It is a so called outbred species, which ensures genetic variability and therefore may have the limitation that a certain treatment given has a possibility of varying between individuals, and therefore has a risk of not showing statistically significant effects. On the other hand, this genetic variability ensures that any observed effect is not constrained to a certain strain of animals, as it would in an inbreed variant (Institute of Laboratory Animal Research 1996).

The New Zealand White rabbit has been popular in research due to its appreciated traits of docility, reproductive performance, growth, and good health (van Zuthpen et al. 2001).

Normally, rabbits are preferably group-housed, but this can in some cases be problematic after sexual maturity, as animals will defend their own territory (van Zuthpen et al. 2001). Also, having the animals in too large pens makes it harder for the animal handlers to pick up and handle the animal safely. This handling of the rabbits is actually one of the more important parts of keeping rabbits as laboratory animals. They are prone to injury if struggling; if the handler is using bad technique or if the rabbit is not used to being handled, there is a risk of them breaking their backs while in transport (van Zuthpen et al. 2001). Though they are considered docile animals, they are very easily frightened and have a high susceptibility to stress (van Zuthpen et al. 2001), which can cause problems when being handled (with respect to anaesthesia, analgesia, transportation, etc.).

As a laboratory animal, due to their size, rabbits are easy to give injections (subcutaneous, intramuscular, intravenously), as well as to sample blood from. The rabbit is generally considered to be quite similar to humans when looking at cellular and tissue physiology (Fox 1984).

CHARACTERISTICS OF THE RABBIT AS A TENDINOPATHY MODEL

As described in chapter 3 ('Comparative anatomy') there are some obvious differences between human and rabbit Achilles tendons. However, the rabbit is still a popular research animal concerning tendon research (Backman et al. 1990; Backman et al. 1991; Hart et al. 1999; Han et al. 2000; Archambault et al. 2001). The size of the rabbit tendon is clearly larger than that of small rodents such as mice and rats lending to greater ease of anatomical and histological studies, and the rabbit should be considered easier to handle than larger mammals such as horses and others (cf. below) (Warden 2007).

It has been shown that the impact of neuropeptides on tendon tissue is affected by the sex of the rabbit (Hart et al. 1998; Hart et al. 1999), wherefore in this thesis; the animals used were all of the same sex (female).

CHARACTERISTICS OF OTHER SPECIES USED IN TENDINOPATHY MODELS

Rats and Mice

Several different experimental rat models of tendinopathy have been demonstrated (Warden 2007; Lake et al. 2008). Rats, in the same way as mice, have short gestational periods and low costs concerning purchase and housing. Furthermore, the genome of rats and rodents are fully sequenced (Lander et al. 2001; Venter et al. 2001; Waterston et al. 2002; Gibbs et al. 2004); the only mammals species in which this have been done aside from humans, wherefore they are well suited for comparative gene expression studies (Warden 2007).

When looking at tendinosis specifically, the rat as a model may be considered as a superior one to that used on mouse concerning histological studies, as the tendons of rats are larger than those of mice. Additionally, concerning the shoulder, the rat shoulder shows striking similarity with the human counterpart from a functional anatomy point of view, making it higly suitable for supraspinatus tendinosis studies (Soslowsky et al. 1996)

Horses and dogs

Natural occurring tendinosis in horses and dogs are common phenomenon (Kasashima et al. 2004; Fransson et al. 2005), and biopsies taken from surgical treatment of these as well as studies on treatment alternatives have been done in the past. Specifically comparative ultrasonographical studies between horse and human have shown promising results (Boesen et al. 2007). However, due to high costs in handling and purchasing of these animals, experimental models are not widely used, and they are generally considered impractical as laboratory animals (Warden 2007).

Neuropeptides

7

BACKGROUND

In This Chapter

Neuropeptides have, like the name implies, classically been associated with neuronal functions and origin. They are made up of short sequences of amino acids which function by way of modulating synaptic activity, or act as primary neurotransmitters. Typically, neuropeptides are derived from large precursor molecules, which undergo posttranslational processing and modifications. The complexity of this system is huge, as a single neuropeptide precursor can result in single or multiple copies of a single neuropeptide. A multitude of individually distinct neuropeptides can be derived from a single precusor molecule, and depending on what cell type that is producing the neuropeptide, the precursor can be cleaved to yield different sets of peptides (Li et al. 2008).

The human genome has been shown to include around 70 genes coding for neuropeptide precursors, and from these over 100 individual neuropeptides, that are released from synapses in CNS, have been described (Burbach 2010). Not only is there a multitude of individual neuropeptides, but they are also known to react with more than one specific receptor, even if they have a preferred receptor of choice. These receptors are often of the G-protein-coupled type (Li et al. 2008), and many pharmaceutical companies are focused on finding medications acting on these receptors in order to affect pain, depression, nausea, and emesis etc. (DeVane 2001).

SUBSTANCE P (SP) AND ITS RECEPTOR(S)

SP is an 11 amino acid chain that belongs to the tachykinin family (Figure 6 & 7). It is derived from the preprotachykinin A gene, which gives rise to four precursor isoforms (α-, β-, γ-, δ-) (Burbach 2010). These, in turn, are all capable of producing the active peptide SP, but also neurokinin A, neuropeptide K and gamma (Carter et al. 1990; Burbach 2010). The α-isoform is more abundant in the brain, while β- and –γ forms are found primarily in the peripheral tissues (Kotani et al. 1986).

SP has historically been considered as confined to the central and peripheral nervous system (CNS and PNS), and the most studied effects are those it displays in these settings. It is released from afferent neurons in central and peripheral nerve endings. It is mostly stored in small-diameter

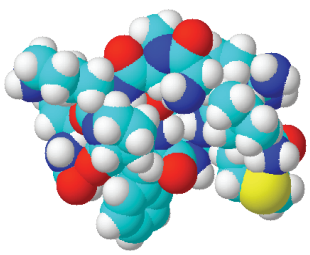


Figure 6. A three-dimensional model of substance P

Aδ- and C-fibres, from which it is released following nociceptive stimuli (McCarthy et al. 1989).

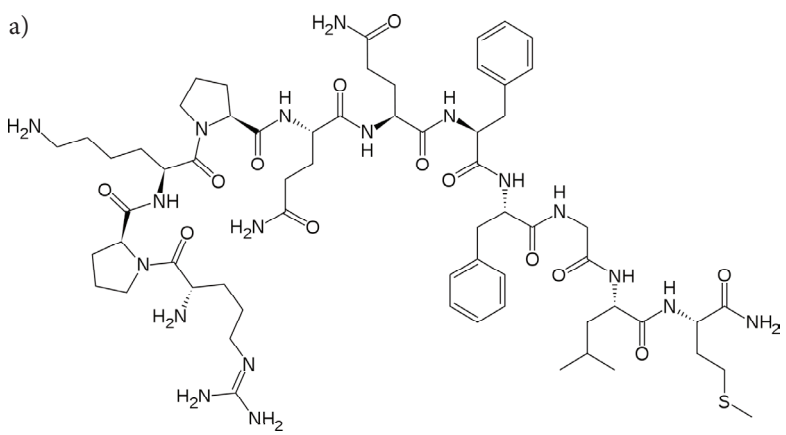
SP produced by neurones originates in the ribosomes (Harmar et al. 1980; Harmar et al. 1982; Keen et al. 1982) before being transported in vesicles (Merighi et al. 1988) to the terminal endings of axons, where the final enzymatic processing is done (Brimijoin et al. 1980).

While the preferred receptor for SP is the neurokinin-1 receptor (NK-1R), a tachykinin receptor, SP is well known to also react with NK-2 and NK-3 receptors, which are otherwise exhibiting preferences for neurokinin A and neurokinin B, respectively (Regoli et al. 1994). The same can be said about other tachykinins concerning their cross-reactivity with the NK-1R; all tachykinins, which share the common COOH-terminal amino acid sequence, have been shown to exhibit some degree of cross-reactivity among tachykinin receptors (Hardwick et al. 1997). The outcome is dictated by the availability of the receptor and the concentration of the peptide (Regoli et al. 1994).

There are studies that have shown that the NK-1R upon activation is internalised into the cell upon which it is expressed (Wong et al. 2005; Adelson et al. 2009). Ordinarily, this internalisation is followed by a recycling of the receptor to the cell surface, but in the case of chronic stimulation by SP, the NK-1R has been observed to be down-regulated entirely (Cottrell et al. 2006). Furthermore, it has been speculated that SP has a modulatory effect on its own release via so called auto-receptors (Malcangio et al. 1999). This could work in two ways, with SP increasing its own release through the production of inositol-1,4,5-trisphosphate and subsequent release of calcium ions, which facilitates the release of SP, or via negative feedback by inhibitory NK-1R auto-receptors (Malcangio et al. 1999).

Antagonists towards the NK-1R have been developed in hopes to treat migraine, depression, pain, and arthritis, in which conditions SP plays an important role (Garrett et al. 1992; Zubrzycka et al. 2000; May et al. 2001; Ebner et al. 2006). However, due to poor effects, there have been few effective NK-1R antagonist medications that have reached the market (Dray et al. 1998; Hill 2000). One example is the antagonist named MK-869, which was originally planned to treat major depressive disorders (Kramer et al. 1998) and

a)



b) H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2

Figure 7: a) Molecular structure of substance P
b) Amino-acid sequence of substane P

originally showed great promise, but was found not to be better than placebo in controlled clinical trials (Enserink 1999). Instead, it is used as an anti-emetic (Emend©) for cancer patients undergoing chemotherapy for which it works well (Rapoport et al. 2010).

In relation to pain

The involvement of SP in pain is well known, as the anatomical localisation of its synthesis has been found in (1) dorsal root ganglion, (2) dorsal horn neurons and (3) raphe nuclei (Helke et al. 1982; Jessell 1982). SP is released in the spinal cord upon noxious stimulation of the periphery (Helke et al. 1982), or by application of capsaicin – the substance that produces a burning sensation in hot peppers – which facilitate its release also in the periphery (Dray 1992). When SP has been released by capsaicin-induction, the afferent neuron is depleted for days, and up to weeks (Dray 1992), with a significant antinoceptive effect (Yamamoto et al. 1991). Further demonstrations of SP's involvement in pain transmission have been shown through antinoceptive effects of intrathecal injections of a SP receptor antagonist (Spantide) (Post et al. 1987).

In relation to inflammation

SP and other tachykinins are involved in what is called "neurogenic inflammation". Primarily the vasculature is affected by these neuropeptides, which facilitate vasodilation, extravasation of plasma proteins, and leukocyte adhesion to the endothelium. Antidromic stimulation of nerves has shown to lead to a peripheral release of SP, which gives rise to the above mentioned neurogenic inflammation, including peripheral neurogenic vasodilatation and plasma extravasation (Jancso et al. 1967; Lembeck et al. 1981). This neurogenic inflammation is promoted by release of the neuropeptides from capsaicin-sensitive nerve fibres. These mechanisms are thought to be important for removing toxic material by increase in blood flow and lymph drainage (Lundberg et al. 1983)

Other stimulatory agents, promoting the release of SP and other neuropeptides, include heat (> 43°C) and protons, as well as histamine, serotonin, lowering of pH, increased osmolarity, inflammation and tissue injury (Harrison et al. 2001).

Released SP has been shown to have pro-inflammatory actions in for instance the airways, and studies have shown this to be regulated by receptors with inhibiting functions, these influenced by histamine H_3, adenosine A_1, dopamine D_2 and others (Maggi 1991). Potential anti-inflammatory drugs could be substances with agonistic action on these receptors, but also antagonists for the tachykinin receptors, as there are NK-1R:s on inflammatory cells (Ho et al. 1997).

Furthermore, smooth muscle relaxation/contraction, inotropic and chronotropic effects on the heart and bronchoconstriction of the airways, among others, are also known to be affected by neuropeptidergic signals (Harrison et al. 2001).

In relation to wound healing and angiogenesis

In studies of skin burns and other trauma, there are several reports of an increase in SP activity in the wound (Dunnick et al. 1996; Schaffer et al. 1998) After damage to the tissue, sensory afferent nerve fibres release neuropeptides, such as SP, to mobilise an inflammatory response as well as the tissue repair via the above-mentioned neurogenic inflammation and fibroblast proliferation (Nilsson et al. 1985; Ziche et al. 1990; Schaffer et al. 1998). Furthermore, SP is considered a potent angiogenetic substance (Ziche et al. 1990), which in the case of wound healing assists in the revascularisation of the healing tissue.

The healing capabilities of SP have been shown in animal studies on the Achilles tendon of rats (Ackermann et al. 2002; Burssens et al. 2005b; Carlsson et al. 2010), where it stimulated nerve ingrowth, angiogenesis, and tendon tissue proliferation with collagen organisation during healing. Studies have also shown SP to have healing capabilities concerning the cornea of mice and rabbits when given intravenously (Hong et al. 2009), as it induced mobilisation of CD29+ stromal-like cells.

A double-edged effect of SP has however been described, since the regenerative forces exerted by SP in certain cases can lead to adverse effects such as excessive scar tissue formation (Jing et al. 2010). In hypertrophic scar tissue, an increased number of SP-positive nerve fibres has been described (Crowe et al. 1994; Scott et al. 2005) that could possibly cause the hypertrophy, as a result of the reparative characteristics mentioned above in combination with a possible down regulation of apoptosis in certain fibroblasts (Jing et al. 2010). The similar phenomenon can be seen in Dupuytren's contracture of the palmar fascia (Schubert et al. 2006), and in interstitial cystitis (Selo-Ojeme et al. 2004) – a condition of increased scarring of the urinary bladder – both of which are characterised by sprouting of SP positive nerve fibres that stimulate proliferation of fibroblasts and the genesis of fibrosis. One could consider the same effects at play in the development and sustaining of tendinosis. In fact, Hart and others (Hart et al. 1995; Scott et al. 2008b) have proposed that neurotransmittors released in close proximity of the tendon could stimulate mast-cells to degranulate and to release mediators for angiogenesis and fibroblast proliferation.

Another concern is touched upon by Hong and collaborators, who mention that malignant cell lines which are displaying increased expression of NK-1R in combination with the cell-mobilising capabilities mentioned above could be a basis for tumour metastasis stimulation, and must be considered before any substance P directed therapies are applied (Hong et al. 2009).

In relation to tendinopathy

The importance of SP-positive nerve fibres in the nociception and healing of tendons has been speculated upon (Ackermann et al. 2003) and it has been suggested that SP-positive nerve fibres could be one of the contributing factors in the genesis of tendinosis (Schubert et al. 2005; Lian et al. 2006). These nerves are closely related to the vessels growing into the tendon (Bjur et al. 2005), but they appear to occur infrequently inside the tendon tissue proper although some studies have reported an increase of intratendinous, SP-positive nerve fibres (Schubert et al. 2005). In animal models of overuse an increase of SP in the paratenon of rats has been shown (Messner et al. 1999).

Further information on the possible importance of SP in tedninosis development is warranted.

PRODUCTION OF SIGNAL SUBSTANCES, INCLUDING SP, BY NON-NEURONAL CELLS

In the last decades, much effort has been put into mapping non-neuronal expression of what has otherwise been considered as substances exclusive to neuronal cells.

Neurotransmittors, such as acetylcholine (ACh), have been found to be an important product of not only immunological cells (Kawashima et al. 2003), but also epithelial cells in airways and epidermis (Grando et al. 1993; Grau et al. 2007), as well as smooth muscle cells and endothelial cells (Horiuchi et al. 2003; Wessler et al. 2008). The human placental villi, otherwise devoid of cholinergic neurons, have also been demonstrated to produce, store and release ACh (Olubadewo et al. 1978).

Also concerning the neuropeptides, expressed primarily throughout the nervous system, non-neuronal cells in tissues such as gonads, intestine and vulvae hypodermis (Li et al. 2008) have been shown to produce them as well. There are also many reports of neuropeptides being produced by glial cells in the CNS, and these are thought to be of importance in local autocrine/paracrine interactions (Ubink et al. 2003).

Concerning SP, high dose capsaicin-treatment of mice has shown that only a 50% depletion of tissue SP-levels was obtained, giving rise to the idea that there must be other sources of production than purely neuronal (Erin et al. 2004). In fact, expression of SP has been shown in human fibroblasts, lymphocytes, platelets (Lai et al. 1998; Bae et al. 2002; Jones et al. 2008) and the above-mentioned glial cells. Also, an endogenous production of SP has been described for epithelial cells and keratocytes in the cornea, and SP is believed to be part of an autocrine and paracrine system at this site (Watanabe et al. 2002).

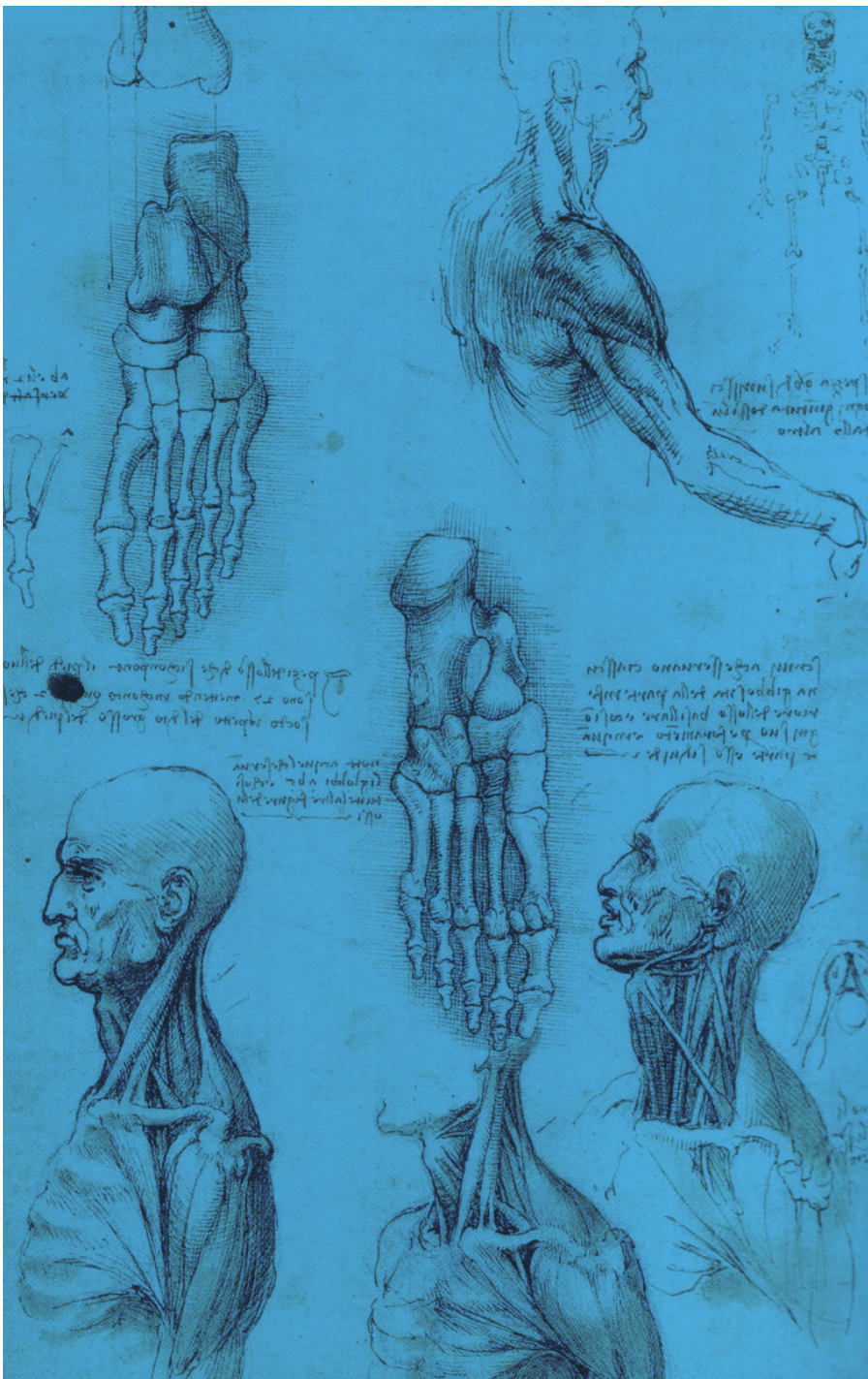
PRODUCTION OF SIGNAL SUBSTANCES BY TENOCYTES

The findings described above has led the tendon research field to look closer at the mechanisms involved in the processes of tendinopathy, with a biochemical model of tendinopathy originally suggested by Khan and co-workers (Khan et al. 2000) in focus, in combination with ideas that neurogenic mechanisms are of importance (Hart et al. 1998; Hart et al. 1999).

In recent years, the idea of a neurologically based view on tendinopathy took a new turn, with findings of locally produced substances, classically considered to be of neuronal origin. These studies of locally produced substances was initiated by findings of higher levels of glutamate and the related receptor, NMDAR1, in tendons with tendinosis, as compared with normal tendons, in a microdialysis study by Alfredson and collaborators (Alfredson et al. 1999). Following studies on the Achilles and patellar tendons, have delineated the production of substances such as acetylcholine and catecholamines, as well as receptors related to these, i.e. M_2- and α_1-receptors, in tenocytes (Danielson et al. 2006; Danielson et al. 2007a; Danielson et al. 2007b; Danielson et al. 2007c; Bjur et al. 2008a; Bjur et al. 2008b). Furthermore, the receptor for NPY – the Y1 receptor – has recently been described in the tenocytes and blood vessel walls of the Achilles tendon (Bjur et al. 2009). In addition, tenocytes express markers fovuring glutamate release (Scott et al. 2008a). All these studies, in combination with findings of NK-1R in close relation to the Achilles tendon (Forsgren et al. 2005), led to the inquiries postulated in this thesis.

II AIMS

Part II
Chapters



"Science is a way of thinking much more than it is a body of knowledge."

Carls Sagan

Hypothesis and Aims

8

HYPOTHESIS

In the project of this thesis it was hypothesised that the neuropeptide substance P (SP) – known to affect pain signalling, vascular regulation, and fibroblast proliferation – plays a role in the processes that occur in Achilles tendinopathy. Based on the earlier studies on non-neuronal signalling systems in tendons performed by the research group, it was furthermore hypothesised that a source of SP affecting the Achilles tendon might be the cells (tenocytes) of the tendon tissue itself, in addition to SP-containing nerves predominantly located in the paratendinous tissue surrounding the tendon. Particularly the paratendinous tissue ventral to the Achilles tendon – being the target of several new treatment regimens and a previously histologically uncharted region – was considered highly interesting for the processes of Achilles tendinopathy and possible SP influences.

In This Chapter

AIMS

I. In order to delineate the characteristics of the loose paratendinous connective tissue ventral to the human Achilles tendon, the area was investigated regarding:

- general innervation patterns (PGP 9.5)
- sensory innervation patterns (SP/CGRP) and the distribution of the SP-receptor NK-1R
- sympathetic innervation patterns (TH) and the distribution of α_1-AR

II. In order to test the hypothesis that the tenocytes of the tendon tissue itself can produce SP, which in turn can exert autocrine/paracrine effects within the tendon, human Achilles tendon tissue from healthy individuals and tendinopathy patients was investigated regarding:

- intratendinous distribution of SP and SP mRNA
- intratendinous distribution of the SP-receptor NK-1R and NK-1R mRNA

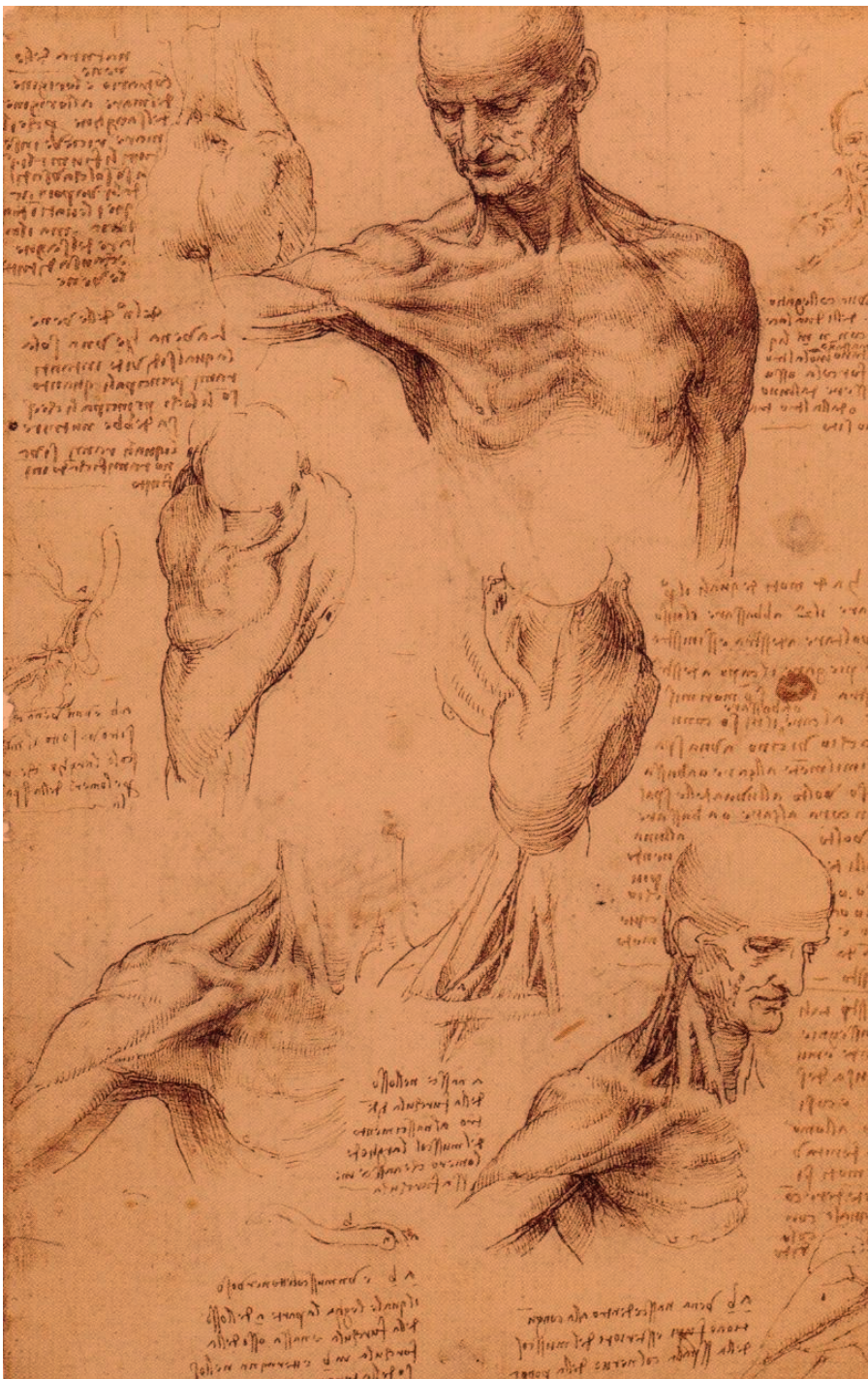
III. In order to be able to experimentally test the hypothesis of SP involvement in tendinopathy (see IV), a tendinosis-inducing animal model (rabbit) was re-established and the model's validity was objectively evaluated.

IV. In order to evaluate the effects of SP on tendon tissue, as well as its possible role in tendinosis-development, the outcome of exogenously administered SP on cell proliferation and vascular patterns in the Achilles tendon was investigated using the rabbit model.

III

MATERIAL & METHODS

Part III
Chapters



"No man should escape our universities without knowing how little he knows."

J. Robert Oppenheimer

Material

9

In This Chapter

Tendinosis patients

Normal controls

Animals

All human and experimental animal subjects that are included in the papers of this thesis are summarised in Table 1 & 2 respectively. The table concerning patients and controls also include a note on the surgical method that was used for obtaining the samples examined, and if any treatment had been given prior to surgery.

TENDINOSIS PATIENTS

A total number of 28 patients with a long duration of pain ("chronic", cf. 'Inclusion criteria') in their Achilles tendon was included in this thesis (for details, see Table 1). They had been diagnosed as having midportion Achilles tendinosis via clinical examination as well as ultrasonography or MRI. Clinical findings where those of a tender and swollen midportion of the afflicted tendon, and a significant loss of function due to pain during physical activity. When examining the region with swelling and pain using ultrasonography (or MRI), a widening of the tendon, irregular tendon structure, and hypoechoic regions were visible. Concerning MRI, increased signaling could be seen in combination with the widened tendon. Using colour-Doppler during ultrasonography (used in the majority of the tendons), a high blood flow in the afflicted tendons was visualised.

A subgroup of patients (n=10) had been given treatment with sclerosing Polidocanol injections prior to surgery (Lind et al. 2006) with poor clinical results.

All subjects, regardless of being subjected to sclerosing injections or not, had undergone conservative treatment with an eccentric calf muscle training program (Fahlstrom et al. 2003). Furthermore, all subjects included were otherwise healthy, on no medication, and not smoking. There was a mixture of recreationally active and non-active subjects.

NORMAL CONTROLS

Voluntary individuals with pain-free, and clinically normal tendons, showing normal tendon thickness and a lack of structural changes on ultrasonography, were included as controls. A total of 7 controls were included, all with a low to moderate physical recreational level (jogging, work-out, walking, bicycling etc.).

Table 1. Human subjects

Code	Sex	Age	Papers				Notes
Controls n=7; mean age: 42.4 years; range: 33-47							
AK 3	Female	47 years		II			
AK 4	Female	47		II			
AK 5	Female	47		II			
AM 1	Male	33		II			
AM 2	Male	36		II			
AM 3	Male	46		II			
AM 4	Male	41		II			
Tendinosis patients n=28; mean age: 46,8; range 26-67							
ATK 1	Female	46		II			Old surgical method
ATK 5	Female	47		II			Old surgical method
ATK 8	Female	54		II			Old surgical method
ATK 9	Female	56		II			Old surgical method
ATK 10	Female	50		II			Old surgical method
ATK 12	Female	50		II			Old surgical method
ATK 13	Female	42		II			Old surgical method
ATK 15	Female	52		II			Old surgical method
ATK 17	Female	54	I	II			Sclerosed, New surgical method
ATK 18	Female	39	I				Sclerosed, New surgical method
ATK 19	Female	49	I				Sclerosed, New surgical method
ATK 20	Female	57	I				Sclerosed, New surgical method
ATK 22	Female	56	I	II			Sclerosed, New surgical method
ATK 23	Female	57	I	II			Sclerosed, New surgical method
ATK 24	Female	47	I				New surgical method
ATM 5	Male	39		II			Old surgical method
ATM 6	Male	54		II			Old surgical method
ATM 7	Male	37		II			Old surgical method
ATM 8	Male	42		II			Old surgical method
ATM 9	Male	46		II			Old surgical method
ATM 11	Male	42	I				Sclerosed, New surgical method
ATM 13	Male	41	I				Sclerosed, New surgical method
ATM 16	Male	36	I				Sclerosed, New surgical method
ATM 17	Male	67	I	II			New surgical method
ATM 18	Male	34	I	II			New surgical method
ATM 19	Male	29	I				New surgical method
ATM 20	Male	61	I	II			New surgical method
ATM 21	Male	26	I	II			Sclerosed, New surgical method

ANIMALS

These studies included a total of 35 female, New Zeeland White rabbits that were subjected to different training regimens in the experimental tendinosis-inducing model used (cf. below). All animals had been obtained from a licensed laboratory animal breeder, and were kept in an organised animal housing complex. They were kept in ordinary cages, allowing free movement. The laboratory where the rabbits were kept maintained a 12:12 hour light-dark cycle, and they had food and water ad libitum.

The animals were delivered to the laboratory when they had reached a weight of approximately 4 kg, which resulting in an age ranging from 6 to 9 months. This places their age around when the species is considered to be skeletally mature (Woo et al. 1986) however some have reported this to occur at an older age (Hart et al. 1995). No control of an epiphyseal closure was performed so this cannot be confirmed in this study.

An additional 5 rabbits were originally included in the experiment. They had, however, adverse effects to the sedation and for this reason four of them consequently did not survive, after the first training session. The fifth was lost before its second training session, also related to the sedation process. No discomfort was noted as a result of the training, with the exception of a slight limp after the first sessions.

Table 2. Rabbit groups

Group	N	Mean weight at start	Papers			
Untrained controls	6	4.5**			III	IV
One week	6	4,1			III	IV
Three weeks	6	4,1			III	
Six weeks	6	4,4			III	
1 week + SP-injection	6	4,6				IV
1 week + NaCl-injection	5	4,2				IV

**) missing weight data in three cases

Experimental design

10

In This Chapter

INCLUSION AND EXCLUSION CRITERIA FOR PATIENTS AND CONTROLS

The criteria for Achilles tendinosis patients were:

Inclusion:
- More than 3 months of pain in the Achilles tendon, i.e. chronic (Kettunen et al. 2002)
- Clinical symptoms of a tender and thickened midportion of the Achilles tendon with verified structural changes by ultrasonography or MRI.

Exclusion:
- Medications for other disease
- Known acute or chronic inflammatory disease
- Radiating pain in the lower limb due to disease or injury
- Disease or injury affecting the lower extremities
- Smoking habits

The criteria for controls:

Inclusion:
- No history of Achilles tendon pain
- Normal clinical and ultrasonographical findings

Exclusion:
- Medications for other disease
- Disease or injury affecting the lower extremities
- Smoking habits

EXPERIMENTAL ANIMAL STUDIES

Set-up and design

The New Zeeland white rabbits were subjected to repetitive exercise using a modified application of an established tendinosis-inducing apparatus (Backman et al. 1990). The machine was constructed to hold three rabbits simultaneously. The machine was affecting the right leg while the left leg

was left unattached to the machine.

The tendinosis-like changes were generated as a result of a movement consisting of passive dorsi- and plantarflexion of the right ankle in combination with a concentric contraction of the right triceps surae muscle induced by electrical stimulation (see below). In order to prevent excessive movement of the animal, a band was placed around the pelvis. This also prevented ankle movement of the left leg.

The ankle movement of the right leg was achieved using pneumatic pistons in which the range of motion was set to approximately 9.5 cm, which translates to 20-25° dorsiflexion and 35-40° plantarflexion. This had earlier been described as the optimal range (Backman et al. 1990).

Simultaneously with the passive plantarflexion, a contraction of the right triceps surae muscle was achieved by placing two surface electrodes (paediatric electrodes 40 426A; Hewlett Packard, Andover, Massachusetts, USA) on the calf muscle. The electrodes were placed 2 cm apart. It was made sure that the current did not transfer directly between the electrodes via the administered conductive gel. The electricity was synchronised with the piston movement by way of a microswitch (type 14E 10; Disa Elektronik A/S Herlev, Denmark) in order to induce a contraction of the muscle at the same time that the ankle was plantarflexed.

A single impulse, with duration of 0.2 ms was delivered 85 ms after initiation of the plantarflexion. The amplitude ranged from 35-50 V depending on the response from the rabbit; the laboratory personnel made sure that the triceps muscle contracted and adjusted the amplitude thereafter. The frequency of movement was set at 150 movements per minute (2.5 Hz). All rabbits underwent training for 2 hours every second day.

Anaesthesia was administered by intramuscular injections of commercially available fentanylfluanison (Hypnorm) (0.2-0.3 mL/kg) and diazepam (5mg/ml, 0.2 mL/kg). A booster of 0.1 mL/kg of fentanylfluanison was given every 30-45 minutes during the experimental procedure to maintain the anaesthesia. Post training, subcutaneously administered buprenorphine (Temgesic) (0.01-0.05 mg/kg) was given as analgesia after each 2-hour training session. One day after the last session (in the case of the exercised animals) the rabbits were anaesthestised by sodium pentobarbital (Mebumal) (60 mg/kg) intraperitoneally and then sacrificed by an overdose of sodium pentobarbital.

The rabbits included in this thesis were divided into different groups with varying length of their exercise regimens (see Table 2). The basic set up was as follows: One week, three weeks or six weeks of exercise. An additional group was a control group that had no exercise in the apparatus at all. Two other groups did also obtain injection treatments in conjunction with the exercise; either SP (10^{-8} µmol/mL, Sigma, S6883) or 0.9% NaCl. The two injection groups exercised in the machine for one week. The injections were given in the tissue surrounding the tendon, primarily ventral to the Achilles tendon, but were perfused along the entire length of the tendon. Both types of injections had a volume of 1 mL, and were administered for 3-5 minutes, just after each training session.

All groups originally had 6 rabbits included, but due to a deceased rabbit in a late stage of the experiment, the saline injected group only had 5 rabbits completing the training regimen. The rabbit losses in the other groups (n=4) occurred early in the experimental protocol, and could thus be replaced.

Tissue samples

11

In This Chapter

SAMPLING OF HUMAN TISSUE

Surgically obtained biopsies were taken under strict sterile conditions. Concerning the tendinosis patients, the samples were taken during surgical treatment, either under general anaesthesia or using a local anaesthetic (4-5 mL Prilocaine hydrochloride, 10 mg/ml; Södertälje, Sweden). Biopsies from the controls were taken using a local anaestetic (4-5 mL Prilocaine hydrochloride, 10 mg/ml; Södertälje, Sweden).

From the tendinosis patients, the biopsies were obtained during two different types of surgical procedure. From 13 patients the biopsies corresponded to samples from the midportion of the Achilles tendon (2-6 cm from the calcaneal insertion), at the central/dorsal parts of the tendon obtained during the "old surgical method" (cf. 'Introduction'). This surgical procedure was done under general anaesthesia. From 15 patients the biopsies were taken from the ventral part of the tendon and adjacent parts of the paratendinous tissue. This, more recently developed surgical procedure ("new surgical method", cf. 'Introduction') was performed under local anaesthesia, by performing a straight longitudinal incision lateral to the Achilles midportion. This approach was guided by visualisation of the tendon and the paratendinous changes, using ultrasonography with colour Doppler. The soft tissue was released from the ventral side of the tendon (scraping), and during the operation biopsies were taken from this soft tissue and the ventral parts of the tendon. This method of surgery is considerer minimal-invasive (cf. 'Introduction').

Biopsies from the controls were taken from a corresponding position in the Achilles tendon (2-6 cm from the calcaneal insertion). Due to ethical considerations, a dorsal approach was always used and the Achilles tissue samples corresponded to superficial parts of the tendon.

RABBIT BIOPSIES

Immediately after the animals had been sacrificed, the entire triceps surae, with attached Achilles tendon complex, was collected from both legs of the rabbits in all groups but one (in the NaCl group only the injected/exercised leg was collected). Biopsies were taken from three parts of each Achilles tendon: a distal part, close to the calcaneus (part A), a part of the midportion (part B), and a part from the proximal portion of the tendon,

close to the myotendionus junction (part C). Samples were approximately 5x5 mm in size. These were intended for sectioning and staining, using the techniques described in chapter 12.

TISSUE PREPARATION

Directly after the biopsies were obtained, they were transported to the laboratory to be directly frozen or subjected to a chemical fixation, prior to freezing, in a 4% solution of formaldehyde in 0.1 phosphate buffer, pH 7.0 at 4°C overnight, followed by a thorough wash in 10% sucrose-containing Tyrode's solution. Both the fixed and unfixed biopsies were mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL, USA) and frozen at -80°C in liquid nitrogen-chilled propane. In most cases, an unfixed and a fixed biopsy were processed from each patient/animal.

SECTIONING

Using a cryostat, series of sections were cut and mounted on chrome-alun gelatin precoated slides, dried and processed for immunofluorescence or stained with hematoxylin-eosin. Sections in study I and II were of mostly longitudinal orientation, in relation to the tendon direction, while the sections in study III and IV were cut exclusively transversely.

In order to prevent free RNAse:s to destroy the mRNA in sections to be used for in situ hybridisation, the cryostat knife was washed in 70% EtOH in DEPC-H_2O. These slides were mounted on Super Frost Plus slides (nr. 041300, Menzel-Gläser).

Sections used for general morphological evaluation or immunofluorescence staining were cut at a thickness of 7 µm while the ones used for in situ hybridisation were cut at 10 µm.

12

Histological, immuno-histochemical and *in situ* hybridisation methods

In This Chapter

STAINING FOR EVALUATION OF GENERAL MORPHOLOGY (H&E)

All the examinations of general morphology in this thesis were based on hematoxylin-eosin (H&E) stainings. These were performed on sections parallel to those undergoing immunofluorescence staining or in situ hybridisation from the same biopsy. The staining of basophilic structures, such as cell nuclei and nucleic acid, is achieved by the haematoxylin component that gives a blue color. Cytoplasmic content and extracellular proteins are stained using a eosin dye, giving various shades of red.

IMMUNOHISTOCHEMISTRY

Immunofluorescence (TRITC/Alexa) methods

The method of immunofluorescence was the main method of detecting signal substances and receptors immunohistochemically, as well as enzymes and other protein structures, in all the studies (I-IV). A battery of antibodies was used, including antibodies against: SP (I,II,IV), α_1-AR (I), CD31 (III-IV), CGRP (I), NK-1R (I,II,IV), PGP9.5 (I), TH (I). The antibodies were of polyclonal type with the exception of the antibody to CD31 and one of the SP-antibodies, which both were monoclonal.

The staining procedure started with a pre-treatment in acid potassium permanganate for 2 minutes, if the sections were to be stained for SP, α_1-AR, CGRP, PGP9.5, and TH. This procedure is considered to aid in antigen retrieval and has been shown to enhance specific immunoreactions (Hansson et al. 1995). Following this, the sections were washed in phosphate-buffered saline (PBS) three times of five minutes each, before being incubated in 1% Triton X-100 detergent solution, for 20 minutes. This was the initial step for sections that were not pre-treated with potassium permanganate, i.e. sections to be processed for NK-1R and CD31.

The incubation in Triton X-100 was followed by a wash in PBS (three times, five minutes each) before normal serum was applied to the sections for 15 minutes, in order to reduce background staining. Depending on

what animal the primary antibody had been raised in, the normal serum came from swine, donkey or rabbit (corresponding to the species of the secondary antibody). The sera were in all cases diluted 1:20 in PBS with 0.1% bovine serum albumin (BSA) supplement; the exception being cases in which sections were stained with goat primary antibodies, when the serum was only diluted in normal PBS to avoid cross-reactions of the goat serum with bovine immunoglobulins present in BSA. Following the incubation in normal serum, the primary antibody was applied to the sections in a humid chamber, either at 37°C for 1 hour, or at 4°C overnight.

After incubation with the primary antibody, the sections were once again rinsed in three 5-minute washes of PBS before once again being incubated in the respective normal serum (cf. above) followed by a 30-minute incubation with a secondary antibody at 37°C, in a humid chamber. The secondary antibody was marked with either a tetramethylrhodamine isothiocyanate (TRITC)-molecule or Alexa Fluor. A TRITC-conjugated swine anti-rabbit IgG (R0156, Dako, Glostrup, Denmark), diluted to 1:40 in PBS with 0.1% BSA, was used when the primary antibody had been raised in rabbits; when the primary antibody came from mouse, a TRITC-conjugated rabbit anti-mouse IgG (R0270, Dako, Denmark) diluted, 1:40, was used. The third secondary antibody used was Alexa Fluor (Invitrogen, CA, USA), a donkey anti-goat antibody, conjugated with a green dye, which has a longer sustainability than a FITC-molecule (fluorescein isothiocyanate), but uses the same spectrum for detection. This was used at a dilution of 1:300 and whenever the primary was raised in goat.

The incubation with secondary antibody was followed by final 5 minute x3 washes in PBS before the sections were mounted with coverslips, using Vectashield®, Hardset™ (Vector Laboratories Inc., CA, USA)

Primary antibodies

All the antibodies used in the immunofluorescence stainings are presented in Table 3. For further information on each antibody, please see respective paper, as referred to in Table 3.

Control stainings

In order to verify the specificity of the primary antibodies used in these studies, rigorous testings were done. These included preabsorption using the antigen, when available, for which the antibody had been designed (Table 2). The preabsorbed antibodies were then used in parallel with non-preabsorbed antibodies for ordinary immunofluorescense staining (cf. above).

Control stainings were also performed on "reference tissue" such as human colonic tissue and rat heart, whenever a new antibody was introduced in the lab.

Furthermore, control stainings were applied in which the staining procedure was performed with the exclusion of the primary antibody (substituted with PBS), in order to rule out any cross-reactions of the secondary antibody.

Table 3. Primary antibodies

Antigen	Code	Source	Type	Dilution	Tissue	Method	Papers			
α_1-AR	PC160	Oncogene, Boston, MA, USA	Rabbit	1:50-1:100	Fixed	TRITC	I			
CD31	M0823	Dako, Glostrup, Denmark	Mouse	1:100	Unfixed	TRITC			III	IV
CGRP	PEPA27	Serotec, Oxford, UK	Rabbit	1:200	Fixed	TRITC	I			
NK-1R	NB300-119	Novus, Cambridge, USA	Rabbit	1:100	Unfixed	TRITC	I	II		
NK-1R	s5220	Santa Cruz, CA, USA	Goat	1:50	Fixed	ALEXA				IV
PGP 9.5	7863-0504	Biogenesis, Poole, UK	Rabbit	1:500	Fixed	TRITC	I			
SP	8450-0004	Biogenesis, Poole, UK	Rabbit	1:100	Fixed	TRITC	I	II		
SP	8450-0505	Biogenesis, Poole, UK	Mouse	1:50-1:100	Fixed	TRITC		II		
SP	H-061-05	Pheonix Pharmaceuticals, Belmont, CA, USA	Rabbit	1:200-1:300	Fixed	TRITC		II		
SP	S-184	RBI, Natick, MA, USA	Rabbit	1:100-1:300	Fixed	TRITC		II		
TH	P40101	Pel-Freez, Rogers, Arkansas, USA	Rabbit	1:100	Fixed	TRITC	I			

Table 4. Preabsorption antigens

Preabsorption antigen	Code	Source	Used with antibody	Dilution	Papers			
CGRP	C2806	Sigma, St Louis, MO, USA	PEPA27	20 µg/mL	I			
SP	S6883	Sigma, St Louis, MO, USA	all SP antibodies	20-150 µg/mL	I	II		
NK-1R	S5220 P	Santa Cruz, CA, USA	S5220	50 µg/mL				IV

Table 5. Normal sera

Serum	Code	Source	Dilution	Papers			
Swine	014-000-121	Jackson I.R. West Grove, PA, USA	1:20	I	II		
Rabbit	X0902	Dako, Copenhagen, Denmark	1:20		II	III	IV
Donkey	017-000-121	Jackson I.R. West Grove, PA, USA	1:20				IV

Table 6. Secondary antibodies

Secondary ab	Code	Source	Used against primary antibody	Dilution	Papers			
Alexa Fluor® 488, conjugated donkey anti-goat	A-11055	Invitrogen, CA, USA	Goat	1:300				IV
TRITC conjugated rabbit anti-mouse	R 0270	Dako, Copenhagen, Denmark	Mouse	1:40		II	III	IV
TRITC conjugated swine anti-rabbit	R 0156/ Z0259	Dako, Copenhagen, Denmark	Rabbit	1:40	I	II		

IN SITU HYBRIDISATION

In situ hybridisation was performed using a modified version of a well-established in situ hybridisation protocol (Panoskaltsis-Mortari et al 1995). We used commercially available digoxigenin (DIG)-hyperlabelled oligonucleotide probes (ssDNA) for marking mRNA in the tissue, and detection was achieved with an alkaline phosphatase (AP)-labelled anti-DIG antibody (Roche, Germany, 11 093 274 910).

All the solutions, and their components, used in the following steps are summarised in Table 7.

Fixed, or unfixed, tissue was freshly sectioned in 10 µm thick slices and left to air-dry at room temperature (RT) for 30 minutes, before post-fixation, for 60 minutes, in 4% paraformaldehyde (PFA) in 0.1 M sterile filtered PBS. The sections were then rinsed two times in 2xSSC for ten minutes each. Following this there was an 8-minute incubation in 0.2 M HCl at RT, which serves as a method of inhibiting endogenous AP activity. The sections were acetylated through a room tempered, 15-minute, incubation in a mixture of 195 mL diethylpyrocarbonate (DEPC)-water; 2.7 mL triethanolamine, 0.355 mL concentrated HCl and 0.5 mL acetic anhydride (which was added after the slides were placed in the cuvette with the rest of the mixture). Yet another 2xSSC wash was performed followed by administering the respective ssDNA probes (Table 7). These probes (50-100 ng) were administered on the sections, after being denatured (in a 1.5 mL Eppendorf tube) for 5 minutes in 80°C in 15 µL hybridisation solution and cooled on ice. The slides were covered with cover slips and sealed using nail polish, and then incubated overnight at 56°C

At the start of the second day, the sections were rinsed in 2xSSC for two washes of ten minutes at RT, followed by incubation in STE-buffer for another five minutes at RT. 100 µL of RNase A solution was administered onto the tissue sections and kept in 37°C for 30 minutes. Another wash with 2xSSC, this time with 50% added formamide and set in a heating chamber of 56°C for 20 minutes, was performed, followed by two 5-minute washes in 1xSSC, and finally two 5-minute washes in 0.5xSSC, all of which were at RT. After this, the sections were transferred into buffer 1 (see Table 8) where it was kept for five minutes before being incubated in the same buffer, with an added 4% horse normal serum, for 1 hour in a humid chamber at RT. 100 µL of anti-DIG-AP, diluted 1:500 in buffer 1 with horse normal serum, was added to the slides and incubated for another hour in the humid chamber at RT. Another series of washes followed; two ten-minute washes in buffer 1, and two five-minute washes in buffer 2 (see Table 7). The last step on day two was adding sterile-filtered (22 µm) substrate solution in which the sections were incubated upside-down, to avoid deposits in the tissue, at 4°C overnight.

The third, and final day, began with stopping the colour reaction by placing the slides in buffer 3 (see Table 7). A staining with 5% methyl green was done by washing the sections in a series of steps: 30 seconds in 75% ethanol, followed by 30 seconds in 95% ethanol, and then 4-5 seconds in 0.5% methyl green and finally dipped in pure (99.5%) ethanol three times. The sections were mounted with small cover slips using Pertex.

Table 7. In Situ hybridization solutions
(Modified from Panoskaltsis-Mortari et al 1995)

Solution	Ingredients
Diethylpyrocarbonate (DEPC)	0.1% vol/vol, autoclaved
4% paraformaldehyde	2 g of PFA dissolved in RNase-free H_2O (25 mL) with added phosphate-buffered saline (PBS) (25 mL)
0.2 N HCl	1:60 dilution from 12 N HCl in DEPC-H_20
0.25% Acetic anhydride/0.1M triethanolamine-HCl buffer	0.93 g triethanolamine-HCl powder dissolved in 45 mL DEPC-H_2O with 6 drops of 10 N NaOH added (pH 8). Diluted to 50mL with DEPC-H_2O
STE-buffer	500 mM Nacl, 1 mM EDTA and 20 mM Tris-HCl, pH 7.5.
RNAse A solution	40 ug RNAse A/mL STE
20x SSC	3 M NaCl and 0.3 M sodium citrate, pH 7.0 - autoclaved
2xSSC, 50% formamide	25mL formamide (Sigma Chemical, St.Louis, MO, USA), 5 mL 20x SSC. Diluted to 50 mL with double distilled H_2O (ddH_2O)
Buffer 1	100mM Tris-HCl, pH 7.5 and 150 mM NaCl. Diluted to 1 L with double distilled H2O (ddH_2O). Filtered.
Buffer 2	100 mM Tris-HCl, ph 9.5, 100 mM NaCl and 50 mM $MgCl_2$. Diluted to 1 L with ddH_2O. Filtered.
Substrate solution	1 mL buffer 2, 20 µL NBT/BCIP (Roche, Mannheim, Germany), 10 µL levamisole solution (1 mM) (Vector Labs, Burlingame, CA, USA)
Buffer 3 (stop buffer)	10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. Diluted to 1 L with ddH_2O. Filtered.
Hybridization solution	500 uL formamide (Sigma, St.Louis, MO, USA), 200 uL 20xSSC, 50 uL of 20xDenhardt's solution (Sigma), 50 uL of heat-denatured herring sperm DNA (10mg/ml), 25 uL bakers yeast RNA (10mg/ml) and 175 uL of dextran sulfate 50%

Probes

All the antisense probes used are presented in Table 8. As negative controls, the corresponding sense probes were used. Positive control probes used are presented in Table 9. Further information on the mechanisms of each substance can be found in the respective paper, as referred to in Table 8 and Table 9.

Table 8. ssDNA antisense probes

Probe	Code	Source	Dilution	Tissue	Papers			
Antisense probe[1], recognising human SP mRNA	GD1001-CS (custom made)	GeneDetect®, New Zealand	50-100 ng in 15 µL hybridization solution	Fixed		II		
Antisense probe[2], recognising human TACR1 mRNA	GD1001-DS (custom made)	GeneDetect®, New Zealand	50 ng in 15 µL hybridization solution	Fixed		II		
Antisense probe[3], recognising rabbit VEGF mRNA	GD1001-DS (custom made)	GeneDetect®, New Zealand	50 ng in 15 µL hybridization solution	Unfixed			III	
Antisense probe coctail[4], recognising rabbit TACR1 mRNA	GD1001-DS (custom made)	GeneDetect®, New Zealand	50 ng in 15 µL hybridization solution	Unfixed				IV

1 GreenStar* DIG_{10}-hyperlabelled oligonucleotide probe.
Sequence: CGTTTGCCCATTAATCCAAAGAACTGCTGAGGCTTGGGTCTCCG.
2 GreenStar* DIG_{10}-hyperlabelled oligonucleotide probe.
Sequence: TGACCACCTTGCGCTTGGCAGAGACTTGCTCGTGGTAGCGGTCAGAGG
3 GreenStar* DIG_{10}-hyperlabelled oligonucleotide probe.
Sequence: TGCTGGCCCTGGTGAGGTTTGATCCGCATGATCTGCATGGTGACGTTG
4 GreenStar* DIG_{10}-hyperlabelled oligonucleotide probe coctail.
Sequence #1: GGCTGCACGAACTGGTTAGACTCAGAGGTGTTGGTGGAGATGTTGGGG,
Sequence #2: TGGAGCTTTCTGTCATGGTCTTGGAGTTGCTGCGAGAGGAGCCGTTGG,
Sequence #3: TGACCACCTTGCGCTTGGCAGAGACTTGCTCGTGGTAGCGGTCAGAGG

Table 9. Positvive controls, ssDNA probes

Probe	Code	Source	Dilution	Tissue	Papers			
Antisense probe[1], recognising all species β-actin	GD5000-OP	GeneDetect®, New Zealand	50 ng in 15 µL hybridization solution	Fixed/unfixed		II		IV
Poly(dT) probe	GD4000-OP	GeneDetect®, New Zealand	50 ng in 15 µL hybridization solution	Unfixed			III	IV

1 GreenStar* DIG_{10}-hyperlabelled oligonucleotide probe.
Sequence: CGTTTGCCCATTAATCCAAAGAACTGCTGAGGCTTGGGTCTCCG.

MICROSCOPIC EXAMINATIONS

A Zeiss Axioscope, equipped with epifluorescence optics and an attached Olympus DP70 digital camera, was used to evaluate the tissue samples, after staining for H&E, immunofluoresence and in situ hybridisation. Evaluation was performed by at least two examiners (G.A., S.F., P.D., A.S., J.E.-S., and/or L.B.), however the evaluaters varied from paper to paper, and at times different evaluators looked at different characteristics in the tissue (cf. paper III and IV).

When semi-quantitative assessment was made of the sections (papers I & II), each examiner made their assessments independently and a consensus was later formed concerning the immunoreaction patterns. When the quantification of tenocytes was done in papers III & IV, two examiners individually took pictures at three different locations in each section and counted each visible tendon cell in those pictures. A mean of the three pictures was calculated for each of the examiners, and the same procedure was undertaken for all three parts of the rabbit Achilles tendon (A, B and C) (paper III & IV). The mean for the entire tendon (A, B, and C) was then calculated, as Friedmann's test showed no significant difference inbetween the three parts.

The vascularity of the Achilles tendons of the rabbits (paper III & IV) was graded using a blinded method, for which the identification number of each slide was covered using paper slips and new code numbers were given. The entire slide was examined, and the intra-tendinous vascularity was assessed using a modified version of an established tendinosis scale – the Bonar scale (cf. paper III, IV). One of the examiners (G.A.) did this latter evaluation, and performed a re-test of 25% of the slides, before the identity of the slides was decoded. See paper III-IV concerning grading and group analysis.

Statistics

13

In This Chapter

GENERAL

Statistical calculations were applied for study III and IV, when quantifying the number of cells in the tendon tissue, as well as for the semi-quantitative evaluation of vascularity.

As the number of rabbits was low (a maximum of 6 rabbits per group), the population could not be assumed to be normally distributed, and consequently non-parametric statistics were applied.

All statistical calculations were performed in a computer program (SPSS 11.0 and PASW Statistics 18.0, for Macintosh), with significance predetermined at $p<0.05$.

FRIEDMAN TEST

Friedman test is a non-parametric test, similar to the parametric repeated measures ANOVA. It is used to detect differences across multiple test attempts, and the values are transformed into ranks by columns before being calculated (Friedman 1937; Friedman 1939). The Friedman test was used in these studies to calculate whether there was any significant difference between the different parts of the same tendon of the same rabbit.

WILCOXON SIGNED RANK TEST

Wilcoxon signed rank test is a non-parametric test designed to test for differences between two related samples, and is used when the distributional assumptions of a paired Student's t-test cannot be satisfied (Wilcoxon 1945). This test was used in this thesis to check if there was any significant difference between the two legs of the same rabbit.

KRUSKAL-WALLIS ONE-WAY ANALYSIS OF VARIANCE

Kruskal-Wallis test is one of testing equality of medians between different groups. It is a non-parametric method using ranks to replace original data. It can be considered as an extension of the Mann-Whitney U test to

include more than two groups (Kruskal et al. 1952). Kruskal-Wallis one-way analysis of variance was used to look for any significant difference between the groups examined, before moving on with the Mann-Whitney U (pair-wise) test.

MANN-WHITNEY U TEST

The Mann-Whitney U test is a non-parametric test used to calculate whether two independent groups have equally large values. It also employs the use of ranks (Fay et al. 2010). The Mann-Whitney U test was used to test for any significant difference between two groups of rabbits at a time. The obtained p-value was multiplied with the number of pair-wise tests performed (Bonferroni correction).

INTRACLASS CORRELATION - ICC

The intraclass correlation is readily used to assess the consistency and reproducibility of measurements made by observers. When a ICC score is 1, it means that there is a total agreement between the examinations (Koch 1982). In this thesis, a two-way mixed model with a consistency type was used when comparing the two researchers quantifying the number of cells in the rabbit Achilles tendon. In order to test the intrarater-reliability of the researcher grading the vascularity a two-way mixed model of absolute agreement was used. In both cases, the average measures ICC was applied.

Ethical considerations

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In This Chapter

ETHICS CONCERNING HUMAN STUDIES

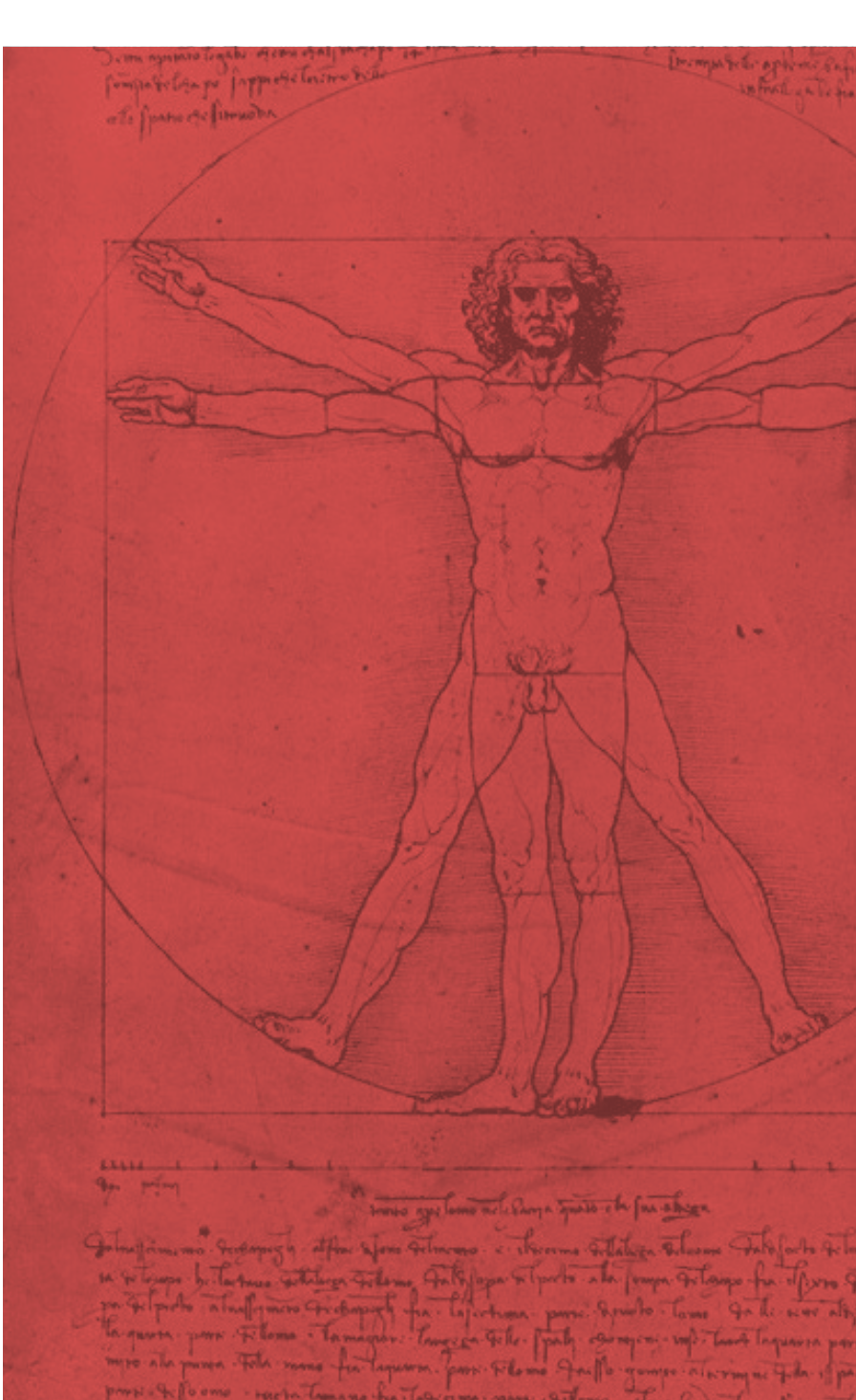
The studies on human subjects included in this thesis was approved by the former Committee of Ethics at the Faculty of Medicine, at Umeå University (prior to 2004), as well as the Regional Ethical Review Board in Umeå (established 2004), Sweden (project number 04-157M). The principles stated in the Declaration of Helsinki 2000, 5th revision, were followed when performing all the experiments, when in contact with participating subjects, and in the study implementation and design.

ANIMAL ETHICS

The regional ethical committee on animal ethics in Umeå, Sweden, has approved all the animal studies of this thesis (project number A34/07 and A95/07). A licensed breeder bred all animals for the sole purpose of being used in animal experiments. The housing of the rabbits was approved by the Board of Agriculture.

IV RESULTS

Part IV
Chapters



"A man should look for what is, and not for what he thinks should be."

Albert Einstein

Human studies

15

MORPHOLOGY (STUDY I & II)

General tissue morphology

Using H&E-staining, the morphological aspects of the tendon and surrounding tissue could be delineated.

Paratendinous tissue including loose connective tissue with vascular structures and nerves were observed. In-between the tightly bundled, and parallel aligned, collagen fibres of the tendon tissue proper, endotenon streaks were observed in the sections. These endotenon structures contained small vessels as described in the 'Introduction'. Blood vessels were more frequently occurring, and of larger dimensions, in the paratendinous tissue than in the tendon tissue proper.

The biopsies from tendinosis patients included paratendinous tissue, as well as parts of the tendon tissue itself. Both the old and new surgical methods, as described in the Introduction, were used.

The ventral, paratendinous, tissue from patients was comprised primarily of richly vascularised loose connective tissue and showed signs of innervation. There was also to some extent fat tissue to be found in the biopsies. Visually, no difference could be detected when comparing the patients that prior to surgery had received sclerosing Polidocanol injections with the ones that had only received conservative treatment, including eccentric training.

When comparing the tendon tissue proper of the Achilles tendon of the patients described above, to that of controls, it was noted that the control tendons were comprised of tightly bundled collagen fibres and tenocytes that were tightly fitted in-between the bundles. There were some fine vascular structures coursing between the bundles, being located in the endotenon network surrounding the tendon fascicles. Large blood vessels occurred in the epitenon and paratenon.

The tendinosis patients, on the other hand, often showed a more disrupted collagenous structure as compared to the controls. Also, a relative hypervascularity was noted in these biopsies, as the occurrence of vessels intratendinously was more obvious than in the healthy controls.

In This Chapter

Tenocytes

The tenocytes in the tendon samples from the controls were tightly squeezed in-between the collagen bundles. They had an elongated slender appearance, and showed for the most part a straightened form, being in alignment with the collagen.

The shape of the tendon cells from tendinosis patients was, on the other hand, often irregular; some being rounded, or "swollen", due to having a larger nuclear component as seen by the blue haematoxylin staining. Others where still quite slender, but did often have a wavy appearance and not the straightened form like the tenocytes in the control tendons. The similar visual characteristics of tenocytes were sometimes observed in part of the control tendons. Tendons of tendinosis patients were, in general, more hypercellular than those of controls, although some variation occurred between the tendons.

NERVE-RELATED CHARACTERISTICS OF THE VENTRAL PARATENDINOUS TISSUE IN CHRONIC ACHILLES TENDINOSIS (STUDY I)

In order to describe the general, sensory, and sympathetic innervation patterns in the ventral paratendinous tissue targeted in the novel Achilles tendinosis treatment regimens (sclerosing injections and surgical scraping), markers for general (PGP9.5), sensory (SP, CGRP), and sympathetic (TH) innervation, and the receptors NK-1R and α_1-AR, were used. We also wanted to investigate whether there was any difference between patients that had recieved sclerosing Polidocanol injections prior to surgery, as compared to those who had not.

To achieve this, biopsies from the ventral paratendinous tissue from a total of 15 individuals with chronic painful Achilles tendinosis, of whom 10 had been given sclerosing polidocanol injections prior to surgery, were studied using immunofluorescence and H&E-staining.

Main results:

No difference could be delineated concerning the patterns of immunoreactions for the two groups (those who had received sclerosing injections and those who had not).

PGP9.5 immunoreactions could be seen in nerve fascicles as well as in the frequently occurring perivascular innervation throughout the loose connective tissue ventral to the Achilles tendon (the paratendinous tissue, cf. general tendon histology), and in the epitenon. To some extent, PGP9.5 immunoreactive nerve fibres were also noted in the endotenon septa, and thus entered deeper into the tendon tissue proper. However, it should be noted that the degree of innervation in the endotenon region was found to be quite sparse. The majority of the nerves were found outside the tendon tissue proper. Not all blood vessels were supplied with PGP9.5 perivascular innervation, and the existing perivascular innervation was found to be primarily located around arteries and arterioles. The main localisation was found to be in the media-adventitia junction of the vessel walls (Study I, Figure 2) .

The innervation of the tissue ventral to the human Achilles tendon was comprised of both sympathetic as well as sensory fibres. The nerve fascicles and the perivascular fibres showed primarily reactions conforming to the sympathetic nervous system, with a

high degree of TH immunoreactions, but there was also sensory innervation to be seen, demarcated by the SP- and CGRP antisera.

Receptors for signal substances could be found throughout the specimens; α_1-AR and NK-1R were present on nerve fibres as well as in blood vessel walls.

PRESCENCE OF SUBSTANCE P AND THE NEUROKININ-1 RECEPTOR IN TENOCYTES OF THE HUMAN ACHILLES TENDON (STUDY II)

We wanted to examine the possibility of a local, non-neuronal, production of substance P and the occurrence of its preferred receptor, NK-1R, in the tendon tissue proper of the human Achilles tendon. Furthermore, potential differences between tendinosis patients and healthy controls in this regard were investigated.

We collected biopsies containing tendon tissue proper from a total of 27 individuals, 20 of whom had chronic painful midportion Achilles tendinosis, and 7 of whom were healthy controls. In this study, a few tendon biopsies were taken during surgical treatment (scraping) of the ventral soft tissues ("new surgical method"), but most patient samples were collected using the "old surgical method" (cf. 'Introduction'). All samples were stained using imunofluorescense with antibodies directed towards SP and NK-1R. Also, some samples were stained using in situ hybridisation to look for SP and NK-1R mRNA.

Main results:

When initially immunostaining for SP, there were indications that the tenocytes of the Achilles tendon exhibited SP themselves. This was observed via stainings with four different SP-antibodies. However, when preabsorption of the antibodies was performed, the reactions could not be abolished. Thus, the conclusion was that specific SP-immunoreactions were not detectable.

As in study I, SP-positive nerve fibres were found, and these reactions could be abolished using preabsorption of the primary antibody.

In order to further evaluate whether the tenocytes have the capability to produce this neuropeptide themselves, in situ hybridisation was applied. Using an antisense probe directed towards human SP mRNA, we found that the tendon cells in fact showed SP mRNA. A sense probe, which is a negative control, verified the specificity of the experiment. According to our semi-quantitative assessment, the tendon cells from tendinosis patients exhibited reactions more frequently than the healthy controls.

Concerning the NK-1R receptor, immunofluorescence staining showed reactivity in blood vessel walls, both in the vessels of the paratendinous tissue, the paratenon, and in the intratendinous vessels (in the endotenon). Reactions were also seen in nerve fascicles both in the endotenon and in the paratenon and paratendinous tissues. Of utmost importance was the finding that the tenocytes expressed NK-1R. The semi-quantitative estimation showed that samples from tendinosis patients exhibited a higher amount of NK-1R than the healthy controls, as judged by three independent evaluators. Corroborative results were found when staining was done with in situ hybridisation. The tenocytes thus showed NK-1R mRNA, most particularly so for tendinosis tendons.

Experimental model

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In This Chapter

MORPHOLOGY (STUDY III & IV)

General tissue morphology

As with the studies on human Achilles tendon tissue, H&E-staining was used to visualise the general morphological characteristics of the rabbit Achilles tendon and surrounding tissues.

In the case of the rabbit tendon tissue, the tissue was, as stated in 'Material & Methods', sectioned in a transverse fashion. Although this approach varied from that used for human Achilles tendons (longitudinal direction), the benefit of it was that of clearly visualising the individual tendon fascicles as well as (in most cases) the paratenon and parts of the paratendinous tissue.

As with the human tendons, the collagen structure inside the control rabbit tendons was tightly bundled, and thin layers of endontenon separated the bundles. In the endontenon septa, vessels coursing in-between the tendon fibre bundles (as described in the 'Introduction') could be seen. A higher vascularity was noted for the paratenon as well as in the paratendinous tissue, in which quite large nerve fascicles could be seen in some samples, than in tendon tissue proper.

Tenocytes

The tenocytes, being transversely cut, appeared as quite small round cells, but the thickness could vary. In some parts of the tendon, there were areas with high cellularity.

TENDON TISSUE CHANGES IN THE TENDINOSIS-INDUCING MODEL; TENOCYTE HYPERCELLULARITY AND VASCULAR PROLIFERATION (STUDY III)

In order to experimentally test the effects of SP, we needed a suitable model of tendinosis. Therefore, we wanted to verify that the Backman tendinopathy model (Backman et al 1990) is a reliable model for tendinopathy and tendinosis, and to test whether the contralateral leg is a reliable control.

For this experiment, a total of 24 rabbits were included and subjected

to exercise/stimulation. These were divided into four groups: a control (untrained), a 1-week, a 3-week and a 6-week group. The 1-, 3- and 6-week groups were exercised by passive flexion-extension in conjuncture with electrical stimulation and a contraction during the plantar plexion, every other day, for the duration of the respective timeframe. Exercise was done in the in vivo tendinopathy model (Backman et al 1990), and was followed by histological examination of the rabbits' Achilles tendons. Cellularity was quantified using H&E-staining and manual counting of cells. Vascularity was graded using a modified Bonar scale, after CD31-immunofluorescence staining. Finally, in situ hybridisation, detecting VEGF, was performed to verify angiogenesis.

Main results:

The general morphology of the tendon tissue was changed in relation to how long the groups had been exercised. Tendinosis-like changes, like abnormal tenocyte morphology and disruption of collagen structure, were mainly noted in the 3 and 6-week groups. Some variability appeared between individual animals, but generally the most prominent changes were seen in the rabbits that had been exercised for 6 weeks.

Quantification of tenocyte number showed a significant increase after 3 weeks of training. The same result was seen concerning vascularity, which was noted to be significantly increased when combining all rabbits which had trained for more than 1 week in one group, as compared to those who had trained for 1 week or less.

The resting, contralateral leg showed similar changes concerning both cellularity and vascularity, and therefore the contralateral leg was deemed inappropriate as a control.

In situ hybridisation detected VEGF mRNA in blood vessel walls; more evidently so in the 6-week group, as compared to the control group.

SP EFFECTS ON TENDON TISSUE IN THE EXPERIMENTAL RABBIT MODEL (STUDY IV)

With our experimental, animal model, we wanted to check whether exogenously administered SP could accelerate the development of tendinosis-changes. In order to do this, we included a total of 24 rabbits. They were grouped into an untrained control group, a 1-week group, a SP-injected group and a saline-injected (NaCl) group. The two groups receiving injections were also submitted to one week of training according to the Backman tendinopathy model. The outcome was studied in the same way as study III, with quantification of tenocytes as well as grading of the vascularity. Furthermore, immunofluorescence and in situ hybridisation stainings for NK-1R were made.

Main results:

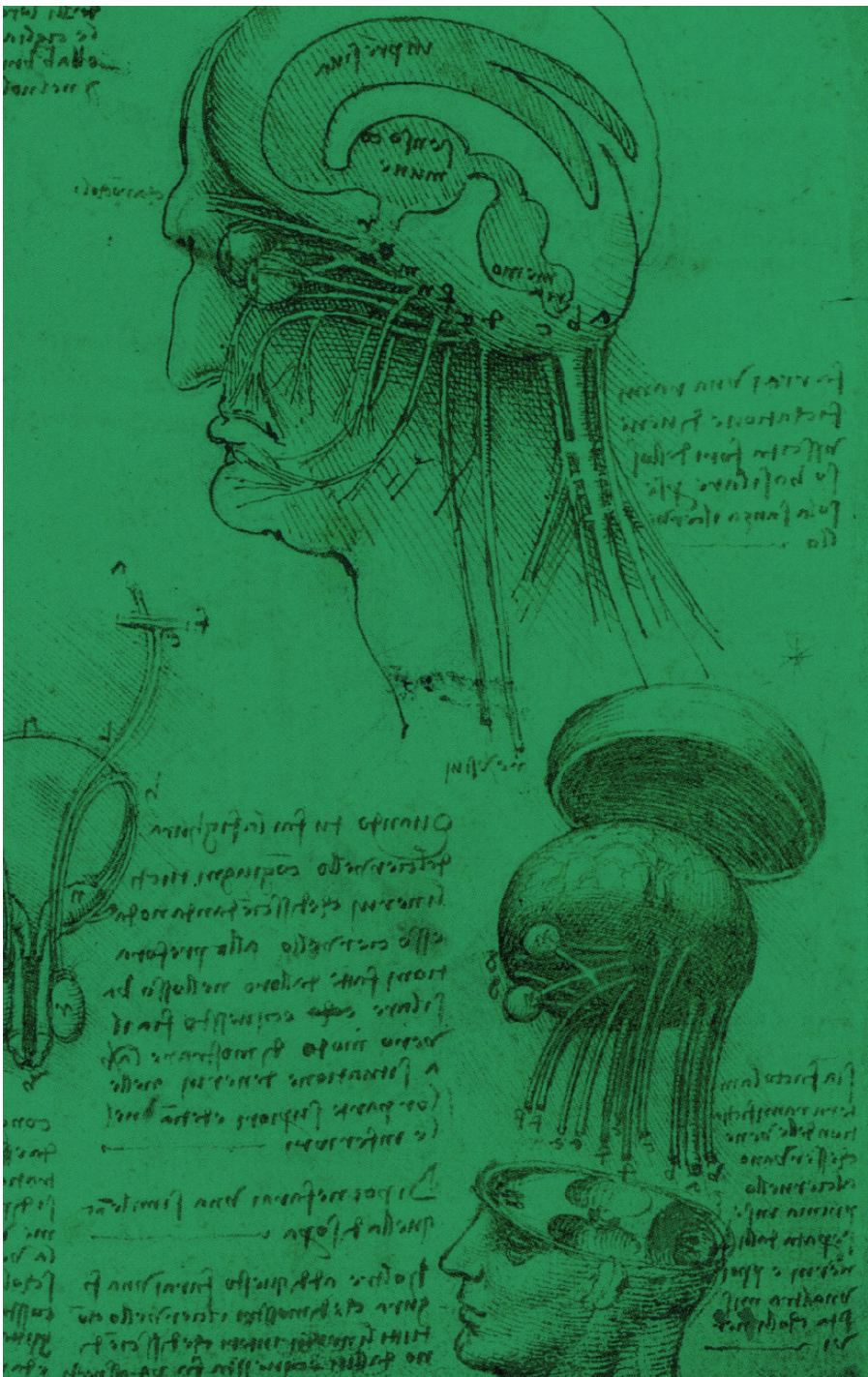
The general morphological findings were similar to those in study III, but when exogenously administering of SP was done, there was a significant increase in the number of cells found inside the tendon tissue as compared to the non-injected, 1-week group and the untrained controls. Concering vascularity, there was an increase in the SP-injected group as compared to the untrained controls. Also, an influx of inflammatory infiltrates was noted in the paratendinous tissue, primarily in close proximity of the myotendinous junction.

Strangely, the same results as in the SP-injected group were found in the NaCl-injected group concerning the number of tenocytes. However, the vascular response of the NaCl-injected group did not differ from the non-injected, 1-week group or the untrained controls.

Immuno- and in situ hybridisation staining showed the presence of NK-1R on the rabbit tenocytes as well as in blood vessel walls and in nerve fascicles.

V DISCUSSION

Part V
Chapters



"By all means let's be open-minded, but not so open-minded that our brains drop out!"

Richard Dawkins

17 Opening remarks

The studies of this thesis set out to find an increased understanding of tendinosis pathology and of the contemporary treatment methods of Achilles tendinosis directed at the ventral paratendinous tissue. This was done by delineating morphological characteristics of the tendon tissue proper of the Achilles tendon and of the tissue ventral to the tendon itself, with particular focus on the neuropeptide SP and its preferred receptor, the NK-1R. The biochemical hypothesis concerning tendinopathy, as described by others (Hart et al. 1995; Khan et al. 2000; Danielson 2009), was at the core of this thesis, and by looking at the neuronal as well as non-neuronal sources of SP, and at the localisation of NK-1R as seen histologically, the possible functional role of SP in tendon pathology was investigated. The effects of SP, regardless of neuronal or non-neuronal origin, were furthermore studied using an experimental animal overuse model that showed increased cellularity and vascularity in the Achilles tendon, in combination with paratendinous inflammation, after exogenous administration of SP.

18 Methodology – strengths and limitations

In This Chapter

STUDIES ON HUMAN ACHILLES TENDONS

The methods used in the studies in this thesis were primarily based on histological examination, using immunohistochemistry and in situ hybridisation to clarify the expression patterns of proteins and mRNA, respectively. Additionally, morphological stainings with H&E were applied in order to delineate the general appearance of the histology.

The results obtained by these methods are based on the evaluation by several observers and a plenitude of control stainings throughout the scientific procedure, such as reference tissue staining, blocking peptides for antibodies, combination of techniques (immunohistochemistry and in situ hybridisation), and blinding of observations. Furthermore, great experience concerning the methodologies in use exists within the research group, including understanding pitfalls, and the knowhow as how to avoid these. These facts give some credibility to the accuracy of these studies.

The collaboration between laboratory work and the clinical side is also of great importance, as clinical examinations as well as US (in majority) and MRI were the basis for the definition of the patient category (tendinosis). Furthermore, the experience of tendinosis treatments was of great importance as a background for the thesis.

STUDIES ON THE EXPERIMENTAL ANIMAL MODEL ("BACKMAN MODEL")

Methodological considerations regarding the model itself

As for the experimental animal model, researchers from the construction of the machine and its set up (Backman et al. 1990; Backman et al. 1991) were involved in the present study design and the start-up of the experiment to ensure a high reproducibility and accuracy. Still, new information concerning the principles for the model was attained during the experiments performed for this thesis, which should be considered for future studies.

In other research groups who have attempted to recreate the results from the original "Backman model" (Backman et al. 1990) it has been

discussed that the age of the animals used in the original paper (6-9 months) might be inadequate due to the rabbits not being skeletally mature (Hart et al. 1995). However, other groups contradict this (Woo et al. 1986; Maeda et al. 2010), claiming that the age is appropriate in the way that the animals are skeletally mature, and exhibit closed epiphysis. Nevertheless, aspects concerning age should definitely be taken into consideration, as several studies have shown that age affects the mechanical properties of the healing of tendons and ligaments (Woo et al. 1990; Hefti et al. 1991; Gillis et al. 1995; Maeda et al. 2010) as well as the metabolism of tendon cells in in vitro studies; the cells from skeletally immature animals showing a higher metabolism than cells from older animals (Floridi et al. 1981; Almekinders et al. 1999).

Another important note is that the tendinopathic changes described in the original paper (paratenon inflammation, altered cell nuclei, fibrillation etc.) (Backman et al. 1990) have not been recreated (Archambault et al. 2001). The same lack of plentiful tendinopathic changes was reported in a similar study on rat (Messner et al. 1999), in which only epi- and paratenon inflammation could be induced. In the rabbit study by Archambault and colleagues, another frequency of loading was applied, i.e. half that of the "Backman model", to better simulate "slow hopping" of laboratory rabbits. Continuous measurements of the tendon loading were made for 11 weeks, to monitor the exact loading applied, but still no significant tissue changes were seen at the end (Archambault et al. 2001). It was theorised that the model could not produce sufficient muscle force to cause tendon damage (Archambault et al. 2001). Concerning electrical stimulation, the muscle contraction in the Archambault study was achieved by use of implanted nerve cuff electrodes on the tibial nerve (Archambault et al. 2001). The voltage used for the nerve stimulation by Archambault and co-workers was 1-3 V, as compared to the 35-50 V used in the "Backman model", and the current in the Archambault study was stimulating the nerve directly as compared to the situation in the Backman study, in which it passed through the skin and muscle of the rabbit (cf. 'Experimental design') via surface electrodes. The authors of the Archaumbault study discussed whether the lack of an inflammatory infiltration of the paratenon might be due to the length of the experiment before the animals were sacrificied (11 weeks), the theoretical acute phase having passed. However it could also be due to the different procedures in their study design, as compared to the "Backman model".

One might speculate on whether antidromic nerve stimulation is responsible for a peripheral SP release and initiation of neurogenic inflammation in the model. Studies have shown that low amplitude (less than 5V) antidromic stimulation of C-fibres, which have high thresholds of activation (≥5V) (White et al. 1985; Drdla et al. 2008), is not sufficient to cause peripheral SP release (White et al. 1985; Lindberg et al. 1986). It could be speculated that increased voltages as well as a higher frequency of movement in the kicking apparatus are required to produce tendinosis-like changes. Perhaps it is not the overtraining-component alone in the "Backman model" that produces the tendon changes described in this thesis, but also neurogenic effects by the possible release of SP and other factors, due to the electrical stimulation. Here it should be recalled that also the contralateral tendon, i.e. the tendon subjected to neither exercise nor electrical stimulation, was also affected (cf. 'Bilateral tendon tissue changes'). Thus, other factors in play must be considered.

Is the proper outcome assessed?

Proliferation of tendon cells is considered one of the hallmarks of tendinosis pathology (Khan et al. 1999), and the number of intratendinous cells is quite easily assessed using an ordinary haematoxylin and eosin staining. However, one cannot be completely certain of the cell type visualised. Although the characteristics of all cells quantified in these studies differed clearly from those of inflammatory- or vascular cell-types, the exact phenotype of these cells has not been evaluated by the use of any cell markers in the studies of this thesis. This is simply due to the fact that there are no currently established specific markers for tenocytes (Riley 2008). A subpopulation of the cells counted may be tenoblasts, i.e. not mature tenocytes, or the cells could in part be mesenchymal stem cells recruited to the tissue to assist the tendon in coping with the stress exerted upon it. In fact, some tendon cells show clear stem like features, having the capacity to differentiate into different cell types (Salingcarnboriboon et al. 2003).

Although the variables concerning blood vessels assessed in the experimental studies of this thesis are the ones generally considered most appropriate to judge when grading tendinopathy in animal models (Lui et al. 2010), the pattern of vascularity considered pathological seems to differ from study to study. The Bonar scale (Cook et al. 2004), used in a modified form in this thesis, is based on the occurrence of a proliferation of vessels longitudinally to the tendon orientation (Maffulli et al. 2000). However, others tend to look at this differently and describe irregularly coursing vessels as the pathological finding in tendinopathy (Williams 1986; Khan et al. 1999).

In the studies of this thesis, transverse sections of the rabbit tendons were used, and the vascularity considered pathological was primarily based on the number of vessels running in parallel with the tendon fibres in accordance with Maffulli and collaborators (Maffulli et al. 2000). Nevertheless, irregular vascularity was also deemed pathological, but one needs to be humble and acknowledge the fact that longitudinal sectioning of the tendons, or another method of scoring vascularity, could also have been applied with rationale.

The possible effects of increased pressure caused by injections

Perhaps the most unexpected finding in this thesis was that of the changes seen after administering NaCl as a control for the SP-injections in the animal model. The subsequent hypercellularity in response to what is generally considered a "non-effect injection" raises an important question about the methodology: Are the injections themselves, regardless of substance injected, affecting the tissue?

One could speculate whether the subsequent increase in pressure following the repeated injections (of 1 ml) into the paratendinous tissue of the rabbit would affect the Achilles tendon in such a way that it responds with, among other things, a cellular proliferation. An alternative explanation might be that NaCl promotes endogenous SP production, which has been shown in a mouse model in which intradermal injections of NaCl into the ear caused an increase in tissue SP (Chen et al. 2007).

The vascular response found in the SP injected group, and not in the NaCl injected group, may be a secondary phenomenon to the marked inflammatory response of the SP in the paratenon.

Statistical considerations

In the experimental animal studies of this thesis, neither the number of tendon cells per area, nor the vascular grading, in a normal rabbit population were known. Also, the extent and exact nature of the effect on these variables by use of the "Backman model " was not possible to forsee. This prevented an a priori power analysis. Therefore, the studies in this thesis should be considered as pilot-studies in nature, since a quantification of tendinosis changes as performed here has not been done before.

A further depth to this issue is that of upholding the three R:s of ethical consideration concerning animal studies as described in the 'Introduction'. One should therefore reduce, replace and refine the animal study to the greatest extent possible. It would therefore be irresponsible at the initiation of a pilot animal study to include "too many" animals when the outcome is unclear. At the same time, the sacrifices made in this study would be meaningless if it did not contain the statistical power to neither prove nor disprove the null hypothesis.

One method of increasing the possibility to detect differences between groups, and which was used concerning the vascularity scoring in this thesis, is to combine groups showing the same trend. Hereby it is possible to increase the power by producing a larger sample size – in this case, grouping animals that were exercised for 3 or more weeks and grouping those who had less than 3 weeks of exercise, instead of comparing all four groups (controls, 1, 3, and 6 weeks) one by one with less sample size per group. Furthermore, as no statistical difference was found in-between the legs of the same rabbit, a mean value of the vascularity of both tendons was used in the groups, whenever available, in order to create a combined vascularity score for each rabbit.

19 Possible effects of paratendinous sympathetic innervation in tendinopathy

As is the case concerning the dorsal aspects of the patellar tendon (Danielson et al. 2008), the tissue ventral to the Achilles tendon displayed innervation with a marked sympathetic component, as well as sensory innervation. Although primary afferent nociceptors lack catecholamine sensitivity in normal physiological conditions (Janig et al. 1996), it has been described that there might be a mechanism involving sympathetic stimulation of afferent nerves via adrenergic receptors following tissue trauma or nerve damage (Janig et al. 1996). This phenomenon is believed to explain pain-dominated diseases such as rheumatoid arthritis as well as complex regional pain syndrome (CRPS) – in which reflex sympathetic dystrophy and causalgia are included (Kidd et al. 1992; Baron et al. 1999). When the efferent sympathetic supply to the afflicted region is blocked, the pain is relieved (Baron et al. 1999), giving further strength to the idea that sympathetic signalling is involved.

The findings in this thesis do indeed suggest the possibility of an involvement of sympathetic modulation of the pain signalling in chronic tendinopathy of the Achilles tendon, as both sympathetic and sensory nerve structures were observed in the paratendinous tissue ventral to the Achilles tendon. Also, there was an apparent occurrence of the adrenergic α_1-AR, not only in the blood vessel walls, but also on the nerves in the tissue, including larger nerve fascicles, which suggests an importance of the sympathetic nervous system in the nerve signalling of the tissue. Similar findings were made in the loose connective tissue dorsal to the patellar tendon with tendinosis (Danielson et al. 2008). It should here be pointed out that both these regions are the main focus of the contemporary treatment methods for tendinopathy directed at paratendionus structures – treatments that accomplish pain-relief in many patients (Alfredson et al. 2007b; Willberg et al. 2008). It is thus obvious that there is a morphologic correlate for the occurrence of effects on both blood vessels and nerves via treatments (scraping; injections) directed to these regions.

On a final note concerning the paratendinous innervation, the inadequate tissue healing described in the 'Introduction' as a phenomenon of the pathology in tendinosis, may in fact be sustained by the sympathetic nervous system. It has, in fact, been shown that a chronic sympathetic stimulation diminishes an organism's ability to heal wounds (Perez et al. 1987; Wucherpfennig et al. 1990).

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The possible role of the neuropeptide SP in tendinosis development, paratendinous inflammation, and tendon healing

In This Chapter

BASIS FOR SP EFFECTS

The studies of this thesis have shown that the tissue ventral to the Achilles tendon contains nervous and vascular structures which express the preferred receptor for SP, NK-1R, but also that the tendon cells of the Achilles tendon proper display this receptor, and furthermore that these cells show features favouring SP production. This suggests several pathways for the SP system of the Achilles tendon (Figure 8): There can be effects of neuronally originating SP on the vessels and nerves outside the tendon, but also such effects on the blood vessels and tendon cells inside the tendon. Furthermore, locally produced SP by the tendon cells could affect the cells themselves and other adjacent cells in an autocrine and paracrine manner, as well as possibly activate the NK-1R:s expressed on the vascular and nervous structures located close by. Concerning locally produced SP, it should however be said that in non-neuronal cells neuropeptides are produced in much less concentration than in neurons (Ubink et al. 2003), and thereby most likely only affecting cells close by. Nevertheless, it has been speculated that neuropeptides can course for some distances within a tissue (Grady et al. 1996), and this aspect together with the wide distribution of NK-1R seen in and around the tendon, point to the idea that SP in fact can exert its effects for considerable distances concerning the Achilles tendon.

When considering the possible mechanisms of both neuronal SP and non-neuronal SP one must take into account the stability of SP in the tissue. The rate at which SP is degraded in the tissue is highly dependent on the ability of availiable degrading enzymes, such as angiotensin converting enzyme (ACE), endopeptidases, enkephalinase, and trypsin, which are all thought to degrade SP released from nerve endings (Rissler 1995; Scholzen et al. 2001). It is interesting to note, that following certain trauma, there is not only an up-regulation of neuropeptides, but also an impaired inactivation of neuropeptides, which predisposes to a longer

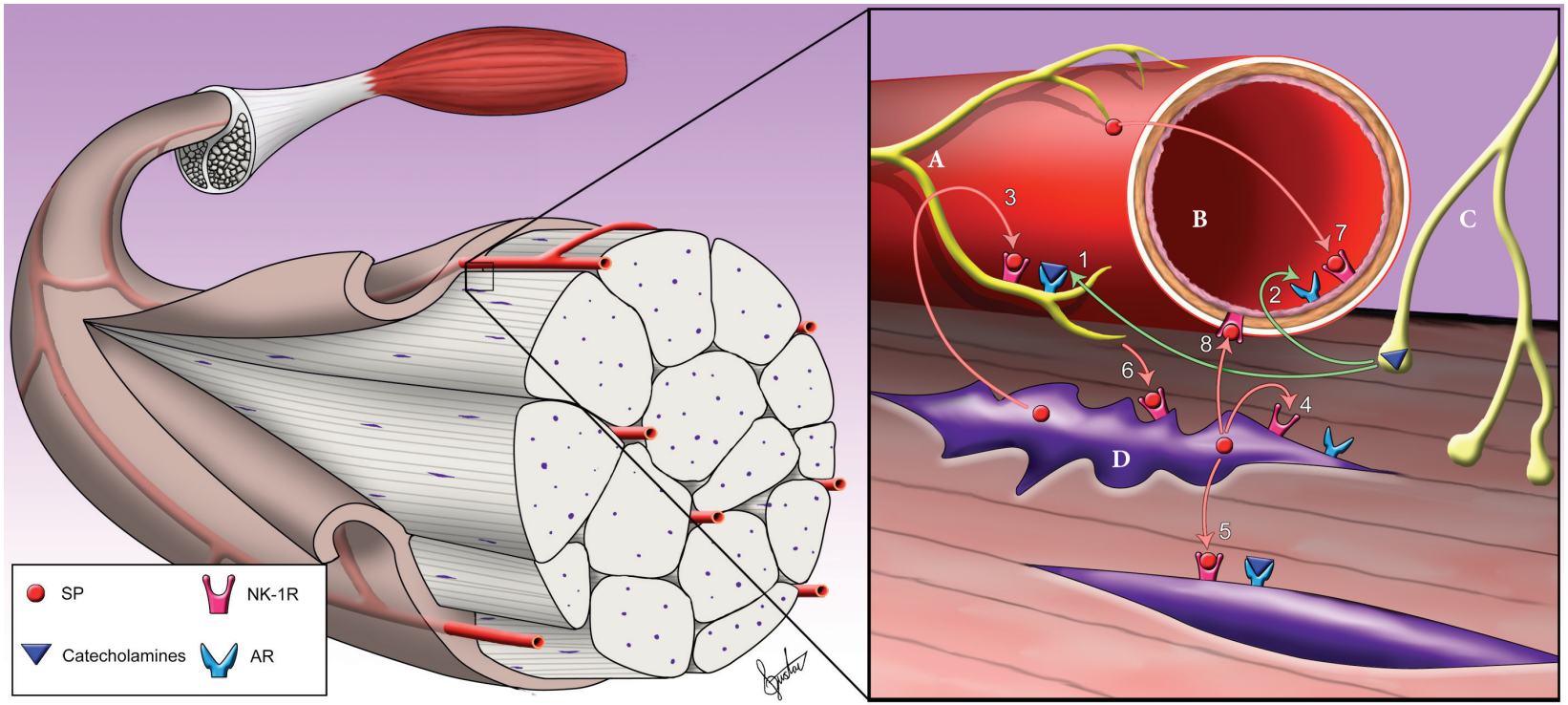


Figure 8. Schematic drawing of Achilles tendon tissue illustrating the possible pathways of the substance P (SP) system of the tendon, as well as a summary of potential influences/interactions of paratendinous innervation. The illustration is based on the findings of patterns for nerves, SP, and receptors in the present studies. The nerve fascicles containing SP-reactive fibres (A), often seen to accompany blood vessels (B), express the SP preferred receptor, the neurokinin-1 receptor (NK-1R), as well as adrenergic receptors (AR), subtype α_1. The nerve fascicles containing sensory innervation (SP/CGRP) are hereby receptive to stimulation by both SP and catecholamines. The latter are released from nerve endings of the sympathetic nerve fibres of the tendon (C) which, in addition to affecting the signalling of nerves, possibly including pain signalling (1), can influence neighbouring tendon blood vessels (2), since also blood vessels display adrenergic receptors. The source of the SP that can stimulate the NK-1R:s on the nerves may be the tendon cells, tenocytes (D), since these express SP mRNA. However, more likely than this tenocyte-to-nerve pathway (3), is that the non-neuronal SP produced by a tenocyte affects the cell itself in an autocrine way (4), or neighbouring tenocytes in a paracrine fashion (5), since tenocytes also express NK-1R. SP is known to stimulate cell proliferation, and the studies of this thesis show that this applies to tendon cells. SP released from the sensory nerves is likely to do that (6). However, among the other known efferent effects of SP is also stimulation of angiogenesis and blood vessel regulation. It is hereby interesting to note that NK-1R is also found in blood vessel walls, which means that the SP of the tendon, whether originating from sensory nerves (7), or more speculatively the tenocytes (8), can affect also the vascularity of the tendon. The studies on the experimental model confirm that SP can increase tendon vascularity.

duration for the neuropeptides to be active in the tissue (Birklein et al. 2008).

It should here be pointed out, that as for the possible production of SP in non-neuronal cells (tenocytes), these studies could only verify this to the extent that mRNA for SP was detected in these cells. The immunoexpression of SP in tenocytes was extensively investigated, but the results were negative, in contrast to the case for nerve structures, which were found to be immune-positive for SP. There are several possible explanations for this discrepancy. As has already been stated, SP of non-neuronal cells, in contrast to that of neuronal cells, has been described to be expressed in very low quantities (e.g., (Ubink et al. 2003)). In other words, it is possible that the immunohistochemical method used for SP detection in the present studies is not sensitive enough for detection of the SP protein in the tenocytes. The in situ hybridization used, on the other hand, might detect very small quantities of SP mRNA. Furthermore, it must be admitted, that the presence of mRNA for a certain protein, does not with certainty coincide with the production of the protein in question. Nevertheless, many aspects favour that tenocytes actually do produce SP, not least the facts that non-neuronal production of SP in other collagen producing cells has previously been described (Watanabe et al. 2002) and that the tenocytes express NK-1R.

THE POTENTIAL IMPLICATIONS OF SP IN TENDINOSIS

Linking the importance of SP to tendinosis can be done by looking at the characteristics of the condition, which conform to pain, neovascularisation and changed vasoregulation, as well as proliferation of tenocytes (Khan et al. 1999). SP has been shown in multiple studies to be closely related to these events, including an involvement in pain regulation (Post et al. 1987), blood flow and angiogenesis (Ziche et al. 1990; Harrison et al. 2001), and tenocyte proliferation (Burssens et al. 2005b) giving it a hypothesised role in the pathology of tendinosis. The present findings, including an up-regulation of NK-1R in human tendinosis and effects of SP in the animal model, do in fact strengthen such an idea.

One could speculate, that the increased expression of NK-1R, seen on tenocytes of tendinosis tendons, occurs in response to a high availiability of SP in the tissue, as SP has been shown to upregulate the expression of NK-1R following its internalisation (Velazquez et al. 2002). The opposite could also be true, as cells which lack stimulation by neuronal signaling are known to upregulate the receptors on its surface (Tsay et al. 1989), likely in order to better react to the low levels of ligands.

In the discussion concerning the relevance of the NK-1R:s in tenocytes, it should also be remembered that the recently discovered tachykinins endokinins and hemokinin 1 show high affinity for NK-1R (Kurtz et al. 2002; Page et al. 2003). Therefore, information on the expression patterns of these tachykinins for tenocytes would be welcome.

In any case, the fact that a marked occurrence of NK-1R can be seen for tenocytes suggests that there is a continuous stimulation of SP (and possibly other tachykinins as well) on these cells, presumably of autocrine/paracrine type. This may then be a sustaining factor in tendinosis. It should here be recalled that SP could co-work with other nerve signal substances. It is observed that the tenocytes, especially in tendinosis tendons, exhibit receptors for catecholamines (Bjur et al. 2008b) and acetylcholine (Danielson et al. 2006). Recently, it has also been noted that the tenocytes are equipped

with NPY1 receptors (Bjur et al. 2009). Altogether, it would seem as if various signal substances of nerve transmitter character affect the tenocytes, especially in situations with tendinosis. Also other substances such as growth factors can have effects on the tenocytes. It has been observed that the tenocytes of the human Achilles tendon exhibit the p75 receptor for neurotrophins (Bagge et al. 2009). The cells also express the neurotrophins (NGF, BDNF) themselves (Bagge et al. 2009).

SP AND INFLAMMATORY CHANGES

Considering the widely accepted theory of microtrauma to the tendon tissue as a genesis to tendinosis changes, one would expect that there should be an inflammatory response, perhaps limited to the paratenon, in the early stages of tendinopathy. Studies on the inflammation pattern in different stages of the rabbit model are under investigation. Whether or not this has any direct effect on the transformation into a chronically painful syndrome, or whether other mechanisms are to blame, remains unsaid.

It should here be recalled that SP is known to be able to induce both neurogenic inflammation, i.e. vascular leakage and vasodilation, as well as non-neurogenic inflammation with pro-inflammatory effects on lymphocytes, leukocytes, macrophages as well as mast cells (Black 2002). The administered SP in our experimental animal model showed an inflammatory response, as evidenced by infiltration of inflammatory cells in the paratenon, and also an effect on the vascularity of the tendon.

SP AND TENDON HEALING

It is possible, that the proposed effects of SP in tendinosis development (cf. 'The potential implications of SP in tendinosis') are related to an attempt of self-healing of the tendon tissue.

Burssens and collaborators have done a study on the clinical implications of peripherally released SP, from C-fibers, by using burst TENS stimulation of human tendons undergoing healing, after suturing of tendon ruptures (Burssens et al. 2005a). In this study, the experimental TENS-group was found to have a faster production and organisation of collagen as compared to the control group. Whether or not these results can be considered to be a faster healing response, and whether the effects were all due to SP-release, remain unsaid. However it does indicate that SP released by electrical stimulation can have healing effects in human tendons.

In what is considered a normal healing process, of ruptured rat tendons, there is a sprouting of nerves into the rupture region, which retract after healing is completed (Ackermann et al. 2002; Carlsson et al. 2010). However, in our human studies on tendinosis, there is sparse innervation of the tendon tissue proper which makes the occurrence of a persisting nerve sprouting inside the tendon less likely for tendinosis, although other studies report this (Schubert et al. 2005). On the other hand, we have shown that SP-containing nerve fibres in combination with a marked sympathetic component are found in the paratendinous tissue. The importance of this is discussed below:

In the typical wound response in the skin, sensory nerves initiate an inflammatory response by SP release, as well as signal the event to the CNS (Ader 2007). Further on,

SP from sensory nerves stimulate fibroblasts (in this case the tenocytes) to generate matrix to close the wound (Nilsson et al. 1985) and may also stimulate angiogenesis (Seegers et al. 2003) . The sympathetic nervous system is however considered not beneficial for the wound healing, wherefore the sensory component is up-regulated during the healing process while the sympathetic is not (Reynolds et al. 1995).

One possible reason for the tendon tissue proper changes seen in tendinosis could be that the neuronally originating SP, as well as the possible local SP production of the tenocytes, participate in the development of the histological characteristics, whether a part of the pathology or a compensatory response thereof. The observations in the rabbit model favour this. At the same time, the increased sympathetic component hinders a complete healing of the tendon (leading to normalised collagen structure etc.). Although the incomplete healing still needs an explation, the sympathetic component discussed above, in combination with the locally produced signal substances, may be of great importance.

In any case, it seems that interventions directed towards the region on the ventral side of the Achilles tendon, containing blood vessels and the described nerves, lead to a comparatively complete healing-process in the tendon, as studies have shown improvement not only in the form of decreased pain (Alfredson et al. 2007b), but also and decreased tendon thickness (Lind et al. 2006).

21 Bilateral tendon tissue changes

One of the most unexpected findings in the studies of this thesis was the occurrence of distinct bilateral effects by using the "Backman model". Not only did the rabbits included in the study show bilateral increase in the number of cells in the tendon tissue proper, but there was also an increase in vascularity in the non-stimulated leg. Some explanations to mechanisms in play have been elucidated in paper III, but further discussion is in order.

It is a well-known fact that some pathological conditions in humans display bilateral characteristics, rheumatoid arthritis (RA) being one (Arnett et al. 1988). Whenever an inflammatory process is activated in a joint in RA, the corresponding joint of the contralateral limb is often also inflicted. Several groups have showed the phenomenon of bilateral effects, often as an unexpected result when using experimental models with the contralateral limbs as a control (Koltzenburg et al. 1999; Shenker et al. 2003).

Shenker and colleagues found that by intradermaly injecting SP-releasing capsaicin unilaterally they could elicit a bilateral response of hyperalgesia and allodynia in the forearm of human test subjects (Shenker et al. 2008). There are in fact several conditions in which a bilateral response has been observed, and in a review by Shenker, the importance of considering this fact in performing good studies is stressed (Shenker et al. 2003). In that review, it is also pointed out that this phenomenon has been noticed in experiments having control groups that received no intervention, like the control group in the experimental studies of this thesis. The occurrence of bilateral responses are considered to be mediated by the nervous system, rather than a general circulatory or systemic effect, as studies which block the nociceptive nerves thought to be involved – by way of surgery (Coderre et al. 1985; Levine et al. 1985), capsaicin (Levine et al. 1985; Levine et al. 1986; Kidd et al. 1995) or local anaesthesia (Levine et al. 1985; Bileviciute-Ljungar et al. 2000) – abolished contralateral effects. It seems logical that the nervous system is involved in RA (Levine et al. 1986; Kidd et al. 1995), as the symptoms are very localised and not generalised for all joints of the body.

In respect to the findings of the present studies and the theories of central neuronal mechanisms in bilateral symptoms, it is interesting to note, that it has been suggested that astrocytes and microglia located in the vicinity of the dorsal horns of the spinal cord, may be activated by, among other transmitters, SP to start a 'cascade ' of reactions maintain-

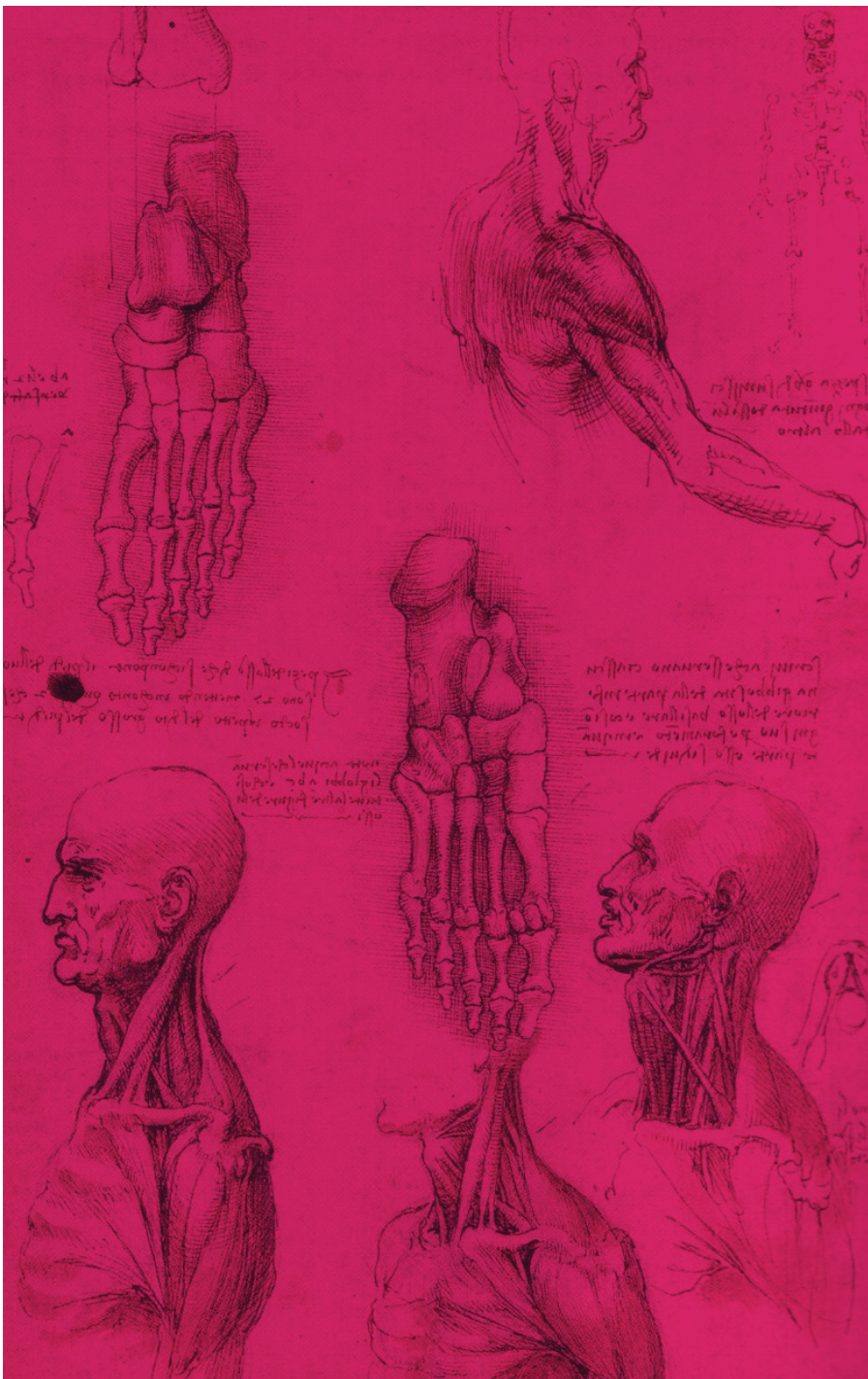
ing and possibly spreading pain (Hansson 2006). It has, in this way, been hypothesised, that astrocyte networks could even mediate the extending of pain to the contralateral side (Hansson 2006). Further investigation concerning possible glial cell involvement in tendinopathies would be welcome.

In certain studies, the occurrence of contralateral effects is explained as a result of the characteristics of the experimental model, the neurological explanation not being considered. One such example is the study on tendinosis induced in a rat model of repetitive strain (Fedorczyk et al. 2010), where contralateral changes in grip strength as well as increase in SP immunoreactivity were accredited to the use of the control limb as a postural support.

Bilateral symptoms are often seen in Achilles tendinosis patients. Thus, patients displaying tendon pathology in one extremity often have contralateral pathology (Shalabi 2004) or debute later on with painful symptoms from the contralateral tendon (Paavola et al. 2000). This makes the experimental findings of this thesis clinically relevant.

VI CONCLUSIONS

Part VI
Chapters



"The fool doth think he is wise, but the wise man knows himself to be a fool"

William Shakespear

Conclusions

22

The studies of this thesis have shown that:

- The paratendinous tissue found ventral to the Achilles tendon, in patients with chronically painful Achilles tendinosis, is highly vascularised. In close relation to the blood vessels, there is a notable sympathetic innervation, but also a sensory innervation. Receptors for transmitters of both the sympathetic and sensory nervous systems are found in blood vessel walls and nerve fascicles in this tissue.

- The preferred receptor for the neuropeptide SP, the NK-1R, is expressed, not only in blood vessel walls and nerve fascicles of the paratendinous tissue, but also on the tenocytes of the tendon tissue proper in human Achilles tendons, and especially so in patients suffering from tendinosis.

- The possible origins of SP targeting the different NK-1Rs are SP-positive nerve fibres in the paratendinous tissue, but also the tenocytes themselves since these cells express mRNA for SP.

- The "Backman model" can produce objectively verified tendinosis-like changes, like hypercellularity and increased vascularity, in rabbit Achilles tendons, after a minimum of three weeks of the exercise protocol.

- Exogenously administered SP triggers an inflammatory response in the paratenon of the rabbit Achilles tendon and accelerates the development of the intratendinous tendinosis-like changes, occurring after only one week of the exercise protocol.

- The contralateral leg of the animals in the "Backman model" is not a reliable control as bilateral changes are induced.

Based on the findings of this thesis, it is concluded that:

- Functions of, and interactions between, the peripheral sympathetic and sensory nervous systems may be of importance in Achilles tendinosis at the level of the ventral paratendinous tissue, i.e. the site toward which recently developed tendinosis treatments are directed. It cannot be ruled out that these interventions affect the neural elements, in addition to blood vessels, as pain relief is accomplished in a majority of patients given these treatments.

- SP, produced from nerves preferably located outside the tendon and/or by tenocytes within the tendon, may have important effects. These could include effects on blood vessel regulation, pain sensation and/or tendon tissue metabolism, as NK-1R is found in blood vessel walls, in nerve fascicles and on tenocytes of the human Achilles tendon.

- In the light of previously known SP-effects, such as effects on cell proliferation and angiogenesis, it is not far-fetched to propose an involvement of SP in the tendinosis changes seen in patients with Achilles tendinopathy, such as hypercellularity and increased vascularity.

- Experiments with the "Backman model" for rabbit Achilles tendon overuse show that SP induces hypercellularity and vascular proliferation in tendon tissue, thus strengthening theories of SP playing a role in tendinosis.

- Central neuronal mechanisms are likely to be involved in tendinosis pathology, as changes are seen in the contralateral Achilles tendon of the experimental animals.

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