TENDON OVERUSE PATHOLOGY: CLINICAL AND LABORATORY STUDIES

by

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ABSTRACT

Background: Painful tendon overuse pathology (tendinosis) is poorly understood. The objectives were to identify major cell populations within clinical tendinosis lesions, and to examine factors involved in the regulation of tendon cell death, survival, or proliferation. The overarching hypothesis was that both cell death and cell proliferation play roles in the development of tendinosis.

Methods:

1: Chronic patellar tendinosis tissue was compared with normal, pain-free patellar tendon using Western blot, immunohistochemistry and in situ hybridization. A variety of cell types were examined in relation to relevant features of soft tissue injury and repair including cellular proliferation and versican expression.

2: In adult male rats, early tendinosis was induced in the supraspinatus tendon by 4-16 weeks of mechanical loading (eccentric exercise). Tendons were analyzed morphologically using polarized light and transmission electron microscopy, and by immunolabeling for molecular markers of proliferation and survival.

3: The influence of IGF-I on tenocyte survival was tested in response to chronic hypoxia in a cell-culture setting.

Results

1: Tendinosis was characterized by proliferation of tenocytes, endothelial cells, and smooth muscle cells within a versican-enriched extracellular matrix. Mast cells were also more numerous in patient biopsies, whereas macrophages and lymphocytes were virtually

absent. VEGF expression was increased in endothelial cells from tendinosis tendons and was more marked in patients with shorter symptom duration.

2: Mechanical loading of the rat supraspinatus tendon by downhill running caused focal tendon lesions characterized by tenocyte proliferation, collagen disarray and glycosaminoglycan accumulation. Tenocytes in these areas of injury demonstrated a proliferative response which correlated with IGF-I expression and phosphorylation of ERK-1/2 and IRS-1.

3: Prolonged hypoxia of primary tenocyte cell cultures resulted in tenocyte apoptosis and caspase activation. Apoptosis could be prevented dose-dependently by IGF-I, which activated the PKB survival pathway

Conclusions

The current studies outlined predominant cell populations present in tendinosis lesions and identified factors which may be involved in regulating their death, survival and activity. These experiments have opened up new avenues of research into the pathophysiology of tendinosis.

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List of Abbreviations Used

APAAP	alkaline phosphatase anti-alkaline phosphatase
Caspase	Cysteinyl aspartate-specific proteinase
CD	cluster distribution
CDMP	cartilage derived morphogenetic protein
COX	cycloxygenase
DAB	3,3'-diamino-benzidine
ERK	extracellular regulated kinase
FITC	flourescein isothiocyanate
GAG	glycosaminoglycan
GRIP	glutamate receptor interacting protein
H&E	haematoxylin and eosin
HIF	hypoxia inducible factor
IGF	insulin-like growth factor
IL	interleukin
IRS	insulin receptor substrate
mGluR	metabotropic glutamate receptor
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NK	neurokinin
NMDA	N-methyl-D-aspartate
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E2
PI3K	phosphoinositide-3 kinase
PKB	protein kinase B
PO_2	partial pressure of oxygen
RCT	randomized control trial
αSMA	alpha smooth muscle actin
ser	serine
SP	substance P
SSD	saline sodium citrate
TEM	transmission electron microscopy
TGFβ	transforming growth factor beta
TGFβR	TGFβ receptor
TIMP	tissue inhibitor of matrix metalloproteinase
TNFα	tumour necrosis factor
TUNEL	Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VGluT	vesicular glutamate transporter
VISA	Victorian Institute of Sport Assessment
VISA-A	VISA - Achilles

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CHAPTER 1

Introduction*

OVERVIEW

Understanding of pathology provides a solid foundation for evidence-based prevention and treatment. In recent years, increasing recognition of the recalcitrant nature of overuse tendinopathies has stimulated research into all aspects of tendon science, including pathological investigations.

Human tendon pathology can be studied in various ways [72]. It can be defined according to its macroscopic features (gross pathology and imaging abnormalities), or its microscopic features using the light and electron microscope (histopathology). More recently, human tendon pathology has also been examined using molecular biology techniques. The advent of DNA arrays and RT-PCR (reverse transcriptase polymerase chain reaction) means that gene expression can be measured in normal and pathological tendons [5, 46]. The extent of tendon pathology can also be described or quantified according to functional outcomes or by mechanical testing.

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This chapter provides an overview of the literature on human tendon overuse pathology from a range of perspectives – macroscopic, microscopic, molecular and functional. Before painting this contemporary picture of tendon pathology, I briefly describe normal tendon from the same perspectives for comparison.

NORMAL TENDON

Tendons are load-bearing structures that transmit the forces generated by muscle to their bony insertion, thereby making joint movement possible [49]. The basic elements of tendon are cells, collagen bundles and ground substance (non-collagenous components of the extracellular matrix). Together the collagen and ground substance comprise the extracellular matrix. Collagen is arranged in hierarchical levels of increasing complexity beginning with tropocollagen, a triple-helix polypeptide chain that unites into fibrils, fibres (primary bundles), fascicles (secondary bundles), tertiary bundles and finally the tendon itself [67]. Mature collagen and its associated proteoglycans provide tendon with its tensile strength and shield the intra-tendinous cells and vessels from injury.

Macroscopic anatomy

The tendon is covered by the epitenon, a loose, fibrous sheath containing the vascular, lymphatic and nerve supply. More superficially, the epitenon is surrounded by paratendon, a loose, fibrous, fatty tissue with an inner synovial lining. The epitenon is continuous with the endotendon which houses the nerves and vessels and divides the tendon proper into fascicles. The myotendinous and osseotendinous junctions are highly

specialized regions where the tension generated by muscle fibres is transmitted to the tendon and bone respectively [49].

Light microscopic appearance

Under the light microscope, normal human tendon consists of dense, clearly defined, parallel collagen bundles. Collagen has a characteristic reflective appearance under polarized light known as birefringence. Between the collagen bundles are fibroblasts (tenocytes) with spindle-shaped nuclei and sparse cytoplasm. Tenocytes synthesize the ground substance and procollagen building blocks of the tendon matrix [10]. Some authors also comment on the presence of tenoblasts—more rounded cells that proliferate at sites of local remodeling [78]. A network of capillaries runs parallel to the collagen fibres in the endotendon [49].

Glycosaminoglycans (or GAGs – the main component of ground substance) can be stained for routine light microscopic evaluation. In normal tendon, GAGs provide structural support for the collagen fibres and also regulate the extracellular assembly of procollagen into mature collagen. Some GAGs are incorporated into collagen fibrils during the early, lateral assembly of fibrils [13]. Other GAGS are interfibrillar, maintaining a hydrated viscoelastic structure and allowing sliding of fibres and fascicles relative to one another (lubrication). At the light microscopic level, using Alcian Blue stain, ground substance is usually confined to the endo- and paratendon, although in some tendons seams of GAGs are visible between collagen bundles in the tendon proper [29]. For example, histological analysis of the rotator cuff tendon shows layers of loosely

organized alcian blue-stained material (GAG) running between the longitudinal collagen fiber bundles, which may be necessary to allow transmission of inhomogenous strains during glenohumeral (shoulder joint) stabilization [11].

Normal ultrastructural appearance

Normal human tendon, when examined under the electron microscope, displays prominent cross-striations of fibrils which are caused by the overlapping of laterally adjacent superhelices. The periodicity of the striations measures about 55nm in longitudinal section [26] and arises due to the quarter-overlapped lateral packing of collagen fibrils.

Despite the apparently simple appearance of tendon on light microscopy, electron microscopy reveals that the three-dimensional ultrastructure of human tendons is complex [50]. EM demonstrates that the collagen fibrils are highly organized, not only longitudinally but also transversely and horizontally; i.e. the longitudinal fibrils run not only parallel, but also cross each other to form spirals (plaits) which may uncoil and recoil during loading and unloading. In transverse sections, the longitudinal fibrils vary in diameter from 50 to 300nm, generally have a smooth (circular) profile and appear homogenous in content [26]. In longitudinal views, some GAGs appear randomly oriented whereas others are associated with collagen fibrils in a regular period corresponding to the banding pattern of collagen, indicating a highly ordered interaction between GAGs and collagen in the normal state [84].

The ultrastructure of epitenon and paratendon reveals a woven network of longitudinal, oblique and transverse collagen fibrils. This arrangement appears to serve as an effective buffer to resist longitudinal, transverse, horizontal and also rotational forces during movement and activity [25].

With respect to the ultrastructure of organelles, it appears that normal tenocytes contain sparse polyribosomes and rough endoplasmic reticulum, a Golgi complex and secretory vesicles that serve to synthesize and transport matrix molecules to the cell surface. Tendon sheaths, when present at sites of increased friction, are populated by synoviocytes identical in appearance to those of synovial joint linings. These are characterized by numerous subplasmalemmal, pinocytic and secretory vesicles [81]. Macrophages with abundant lysozomes are occasionally seen. Adipocytes and mast cells are normally encountered only in the loose connective tissue (endotendon, peritendon) rather than the tendon proper.

Normal tendon biochemistry: human data

There is an increasing recognition that tendon remodeling may play important roles in both healthy and pathological tendon. Biochemist David Eyre is renowned for discovering the collagen cross-links now increasingly used in bone metabolism research [27]. His earliest studies of cross-links were in tendon [28]. Hydroxypryridinium crosslinks increase as tendon ages, and can be used to estimate turnover rates. A limiting factor at present is that crosslinks are plentiful in all forms of cartilage, bone, dentin,

ligament, tendon, fascia, intervertebral disc, lung, gut, cervix, aorta, and vitreous humor. Thus, changes in serum or urine levels of collagen crosslinks are not specific for tendon.

The crosslinks within and between mature collagen molecules provide mechanical stiffness. They also provide resistance to breakdown by proteases; this resistance contributes to the remarkably long half life of collagen in tendon (greater than 100 days) [85]. The patellar tendon has a significantly higher content of dihydroxylysinonorleucine than other tendons, suggesting that there are tendon-specific differences in cross-links [36]. The functional role of these different specific cross-links, however, remains unclear and provides fruitful material for future investigations.

Whereas cross-linked collagen provides tensile strength and stiffness, ground substance contributes to the viscoelastic properties of tendon and the lubrication and spacing that is essential for inter-fibrillar gliding and cross-tissue interactions. It also provides a medium for the diffusion of dissolved nutrients and gases. Although the proteoglycans and GAGs make up less than 1% of the dry weight of tendon, they contribute greatly to the volume and viscoelasticity of the tissue by maintaining water within the tissues [49]. The water molecules are entrapped by the negatively charged GAGs with their preponderance of hydrophilic hydroxyl groups.

GAGs are linear polysaccharides that, with the exception of hyaluronic acid, are covalently bound to a protein core, forming a proteoglycan. The main GAGs of tendon are dermatan sulphate, chondroitin sulphate, keratan sulphate and heparan sulphate. The

proteoglycans in tendon include small (decorin, biglycan, lumican) and large (versican, aggrecan) molecules. Decorin, the best characterized, is so named because it appears to 'decorate' the outside of fibrils. This dermatan-sulphate rich proteoglycan with its single GAG side chain forms "shape modules" that form interfibrillar bridges, thereby maintaining the normal registry and alignment of tendon [54, 84]. In human tendons, the major chondroitin sulphate proteoglycans appear to be versican and aggrecan [89]. Aggrecan is also linked to keratan sulphate and hyaluronan, and is present in both tensional and compressed regions of tendon, but more abundant in compressed regions [71]. Versican binds to hyaluronan forming large aggregates, and is reported to be predominantly localized in the perivascular matrix, around nerve structures, as well as in the pericellular matrix around tenocytes where it buffers the loads transmitted from the fibrillar matrix [77].

Tendon is not a uniform structure

Tendons are not all identical but vary in their anatomy, physiology, and thus not surprisingly, their molecular consituents [64]. Tendons in compressed regions tend to express proteoglycans more typical of fibrocartilage [89]. For example, the sulphated GAG content of the normal cadaver supraspinatus tendon is between three and ten times greater than in the common biceps tendon [75]. The major GAG of the supraspinatus is chondroitin sulphate with a smaller proportion of dermatan sulphate. In contrast, the common biceps tendon contains predominantly dermatan sulphate with less chondroitin sulphate [75]. The increased amount of GAG in the supraspinatus may serve to resist

compression and to separate and lubricate collagen bundles as they move relative to each other (shear) during normal shoulder motion [11].

In addition to the variation among different tendons, there are striking differences in the composition of tendons along their length [64]. In the Achilles tendon and its insertion, types I, III, V and VI collagens, decorin, biglycan, fibromodulin and lumican have been reported in both the mid-tendon and the fibrocartilage, although their relative expression differed with site [89]. The expression patterns of versican and aggrecan were complementary; versican mRNA was present in the mid-tendon and absent from the fibrocartilage, while aggrecan mRNA was present in the fibrocartilage and absent from the mid-tendon [89]. This is puzzling, given that versican expression is reportedly upregulated by compressive loading [61], and is highly expressed in the surface layer of synovial joint cartilage [62]. It is likely that the range and distribution of extracellular matrix molecules detected in tendons may reflect the ability of tendon cells to respond adaptively to the differing types and magnitudes of forces that act upon their various regions [64].

HUMAN TENDON PATHOLOGY

There have been many recent advances in the description of overuse human tendon pathology. Thus, the following section reviews the macroscopic pathology, summarizes histological and immunohistological changes, and describes insights from biochemistry and molecular biology into cell-matrix abnormalities of tendon that has suffered overuse injury.

Macroscopic overuse tendinopathy – tendinosis

Macroscopically, painful tendon affected by overuse (e.g., Achilles, patellar, lateral elbow, rotator cuff tendinopathy) loses its normal glistening white appearance and becomes grey or brown. It may be hard to the feel and sometimes grates when the surgeon's scalpel cuts through it.

A second, gross feature of tendinosis is a general proliferation of capillaries and arterioles. Alfredson, Cook, and others have suggested that this abnormal tendon vascularity (neovascularization, as seen on color Doppler) is associated to some degree with tendon pain, although there is not a perfect correspondence between pain and the presence of neovessels on colour Doppler [16, 17, 39-41, 68]. A novel approach to evaluating tendon vasculature in tendinosis is called orthogonal polarization spectral imaging. This method has been applied to the rotator cuff tendon during arthroscopy of the shoulder. Biberthaler and colleagues [12] visualized and quantified, in vivo, the microcirculation of the painful rotator cuff during arthroscopic surgery. They found that at the edge of the lesion, the functional capillary density was significantly reduced compared with controls. This finding was confirmed by histology [12]. This provides impetus for further research that aim to clarify the role of tendon vascularity in pathogenesis and association with pain production.

Light microscopic findings in tendinosis

In tendinosis, light microscopy reveals abnormalities in collagen, in ground substance, and among tenocytes and endothelial cells [47]. A characteristic feature is collagen

disruption, disarray and disorientation. Some collagen fibres appear to separate giving the impression of loss of their parallel, tightly packed orientation. There is a decrease in fibre diameter, and a decrease in the overall density or packing of collagen [60]. Collagen and capillary microtears can also occur and these may be surrounded by erythrocytes, fibrin and fibronectin deposits [9]. There is an increase in thinner, Type III collagen and increased collagen solubility. These changes lead to decreased birefringence of the tendon under polarized light microscopy [47]. The acid Alcian Blue stain consistently demonstrate an increase in GAGs (sometimes referred to as mucoid degeneration) which can exist in association with vessels, with fibrocartilagenous changes at the enthesis, or diffusely interspersed throughout the tendon [65].

There is great variation in the cellular density and vascularity in tendinosis. In some areas tenocytes are abnormally plentiful, have rounded nuclei giving them an ovoid or chondroid appearance, and may stain positively with nuclear proliferation markers. In contrast, other areas of the tendon may contain fewer tenocytes than normal while others demonstrate occasionally necrotic or apoptotic features [37]. A more frequent finding, however, is a simple absence of tenocytes due possibly to an earlier episode of cell death in that region [58]. Regions of prominent microvessels are frequently observed [31, 55, 73]. Rarely, there is infiltration of lymphocytes and macrophages [47].

These light microscopic findings appear to be relatively consistent across many clinically relevant overuse tendinopathies [53], and constitute the constellation of features known as tendinosis. This is not a new discovery as Perugia et al. [69] noted the "remarkable

discrepancy between the terminology generally adopted for these conditions (which are obviously inflammatory since the ending 'itis' is used) and their histopathologic substratum". It is also worth noting that whether tendon is classified as having undergone complete rupture, surgical transection, partial tear, or microtrauma as a result of age or overuse, the end-results of repair processes in the tissue, following resolution of the inflammatory phase, have a very similar appearance in terms of the main features of tendinosis as detailed above [8, 9, 87].

Relationship of histologic abnormalities and mechanical properties of human tendon

Few studies have measured the mechanical consequences of tendinosis. Sano [79, 80] examined the relationship between the degrees of histologic abnormality at the supraspinatus insertion, the tensile strength, and the site of failure in cadaveric tendon (average age: 62 years; range: 39-83 years.) Having applied a tensile load to tendon failure, they found that the histologic score of degeneration was negatively correlated with the ultimate tensile stress at the insertion. Disruptions of tendon fibers including granulation (i.e. scar) tissue were located mostly in the articular half of the tendon and appeared to reduce the tensile strength of the tendon and thus, constitute a primary pathogenic factor promoting rotator cuff tear.

Ultrastructural changes in tendinosis

EM studies of pathological tendon have reported a wide variety of cellular and matrix abnormalities. In some areas, abnormal tenocytes contain pronounced rough

endoplasmic reticulum and a well-developed Golgi apparatus with cisternae dilated with electron-dense material (implying increased proteoglycan and protein production) whereas in other areas tenocytes are few or absent. Necrotic and apoptotic tenocytes have both been reported [37]. Such cell injury is usually associated with observable damage or degeneration of collagen fibrils. A large, classic electron microscopy study of tendinopathy [51] described: (a) hypoxic degeneration, (b) hyaline degeneration, (c) mucoid degeneration, (d) fibrinoid degeneration, (e) lipoid degeneration, (f) calcification, (g) fibrocartilaginous and bony metaplasia. This nomenclature has not been externally validated or widely adopted, and there appears to be a degree of overlap between these qualitative descriptions of tendon overuse pathology and historical pathological labels. It seems likely that these various types of degenerative appearance observed by Kannus and Jozsa may speak to the phenotypic plasticity of fibroblasts following soft tissue injury-repair [15, 82].

Tendinosis – insights from biological investigations

The above mentioned histological findings suggest the possibility of pathological, adaptive, and/or repair processes occurring in tendon overuse injuries.

Contemporary molecular biology techniques provide unique insights into cellular mechanisms that may play a role in the development of tendinosis. Although many questions remain, a general model has emerged in which tendon cells are continuously remodeling and responding to soluble signals, mechanical signals, and signals from the extracellular matrix in an ongoing feedback loop. This cell-matrix feedback loop could

conceivably go awry at several key points, leading to cell transformation, accumulation or breakdown of particular matrix components, and altered tendon function (Table 1.1). Alternately, the findings may represent the end-result of an inflammatory-repair process.

A central question in the molecular biology of tendon is to identify the chain of events leading to pain and tissue abnormality in tendinosis [52]. Fu and colleagues [35] reported an increased basal expression of cyclooxygenase-2 in tendinosis biopsies, as well as an increase in the production, in vitro, of prostaglandin E_2 (PGE_2) by tendinosis cultures. In contrast, Alfredson et al. [4, 6], using in vivo microdialysis, failed to detect an elevation of PGE_2 in patients with Achilles tendinosis and lateral elbow tendinopathy. While the first group of researchers concluded that PGE_2 may play a role in the chronic phase of tendinosis, the second concluded the opposite. In attempting to resolve this discrepancy, it should be noted that the increase in PGE_2 production by cultured tendinosis cells was rather small (less than 50%) and this may have been undetectable in vivo, particularly with a small surgical sample size. In contrast to Fu and colleagues [35], Graham Riley's laboratory [18] has reported that cultured Achilles tendinosis specimens did not generate any substantial baseline PGE_2. PGE_2 production was strongly induced by interleukin-1 (IL-1) [18], however. Following uphill exercise by individuals with no evidence of Achilles tendon pathology, COX2 levels were increased in the Achilles mesotendon (the loose connective tissue anterior to the paratendon) [56]. Furthermore, a COX2 inhibitor administered before exercise abolished the normal local rise in paratendinous blood flow, suggesting that blood flow in tendon may be physiologically regulated by local

production of PGE_2. Thus, the role of PGE_2 in tendon and paratendon pain remains unclear.

In support of the hypothesis that PGE_2 does not offer a complete explanation of tendinosis pain, Alfredson et al. [2, 3] reported increased lactate (two-fold greater than controls) and glutamate in the tendinosis lesion. Both of these substances have been associated with nociception and inflammation [14, 38, 48]. In addition, an increased presence of sensory nerves containing the nociceptive and pro-inflammatory substance P has been identified in painful tendons (Table 1.1). Intriguingly, several of the markers for sensory and autonomic nerves (e.g. substance P, tyrosine hydroxylase, acetylcholine esterase) have been detected not only in the nerves which accompany the prominent microvessels in tendinosis, but also in the tenocytes themselves, particularly in Achilles and patellar tendinosis patients, including their receptors [7, 20-23, 32]. Thus, it is possible that there may be different molecular mechanisms of pain at different points in the development of tendinosis, and that sprouting nerves and/or tenocytes may interact in previously unappreciated ways in modulating pain and blood flow in tendinosis.

The finding of increased lactate by microdialysis in the patellar, Achilles and extensor carpi radialis brevis tendons implies the possible presence of anaerobic conditions in the tendinosis lesion. Relative avascularity within tendons has long been considered a possible etiologic factor in predisposing to tendinosis and/or tendon rupture at particular anatomic locations in the Achilles and supraspinatus tendon, and a recent review of the available evidence by Benjamin and coworkers led them to conclude that "vascularisation

does affect the tensile strength and so rupture vulnerability of the healthy Achilles tendon" [88]. Clarifying the relationship between anaerobic or hypoxic conditions, pain, and the other cellular and matrix abnormalities described below require further research, particularly in the patellar tendon.

Achilles tendinosis and tendon rupture are both associated with increased mRNA levels of vascular endothelial growth factor (VEGF) [5, 70]. Immunohistochemistry further showed that VEGF could be identified in tenocytes of ruptured and fetal Achilles tendons, but not in those of normal adults [70]. In microvessels, the VEGF receptor VEGFR-1 was also seen. High VEGF levels have also been measured in homogenates from ruptured adult tendons whereas levels were lower in fetal tendon and negligible or absent in normal adult Achilles tendons [70]. These results prove the presence of an angiogenic peptide in injured human tendons, and suggest a role for VEGF in the angiogenic response of injured tendons. The spatial and temporal regulation of such observed variations in growth factor expression requires further study in the context of tendinosis.

Transforming growth factor beta (TGFβ) and platelet derived growth factor (PDGF) have both been implicated in patellar tendinosis, and may play a role in the abnormal cellularity and in the altered production and remodeling of the extracellular matrix [34, 78]. Rolf et al [78] assessed cellularity with respect to cell proliferation and the expression of platelet-derived growth factor receptor β. Tendinosis tissue had increased cellularity ($P<0.001$) compared with controls, and also a higher proliferative index, based

on proliferating cell nuclear antigen (PCNA) staining (P:<0.001). There is also evidence to suggest that following exercise (uphill running) in humans, TGFβ levels are locally upregulated in or around the Achilles tendon, as determined by microdialysis measurement from the mesotendon [45]. Thus, there may be some overlap in regulatory events underlying cellular proliferation and collagen metabolism in tendinosis and in tendon undergoing adaptation to exercise, including TGFβ-induced MMP synthesis and collagen synthesis [59].

The abnormal extracellular matrix in tendinopathies has been characterized in detail for some tendons, although the underlying cause of the alterations has not been conclusively identified. Supraspinatus tendons from patients with a chronic rotator cuff rupture have significantly increased concentrations of hyaluronan, chondroitin and dermatan sulphate compared with "normal," cadaveric, supraspinatus tendons [75]. The authors suggested this finding may represent an adaptation to an alteration in the types of loading (tension vs compression vs shear) which act on the rotator cuff tendons in the shoulder [75]. Conversely, they further suggest that pathological factors such as low oxygen tension or the auto- and paracrine influence of growth factors may also be important in the altered matrix following rupture [75].

Injured human tendons appear to possess an intrinsic healing capability characterized by long-term upregulation of procollagen production. Riley et al. reported that the composition of tendon specimens from patients with chronic tendon rupture was consistent with that of newly synthesized matrix, even in relatively old tendon specimens

[76]. Hamada et al. [43] used in situ hybridization localize cells containing type-I procollagen mRNA in chronically and more-recently torn rotator cuff tendon. In biopsies from complete-thickness tears, actively synthesizing cells were significantly more abundant at the proximal tendon stumps in the specimens that were obtained less than 4 months after trauma, compared with those obtained 4 months or more after trauma. In the less substantial tears (partial thickness), the number of active cells was maintained even in long-standing tears. In larger tears, hypocellularity and fibrocartilage metaplasia were more common, leading to the suggestion that as the size of tendon injury increases, the repair potential decreases [63].

In addition to altered collagen and proteoglycan synthesis, altered matrix remodeling is a feature of tendinosis. Riley and colleagues reported an increased activity of MMP enzymes including MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13 in human rotator cuff pathology [74]. Higher rates of extracellular matrix turnover in the non-ruptured supraspinatus may be part of an adaptive response to the mechanical demands on the tendon, or conversely due to effects of injury such as fibrosis or the ingrowth of nerves and vessels. Fu et. al. [33] reported similar results when studying the expression of procollagen type I, MMP1 and tissue inhibitor of metalloproteinase 1 (TIMP1) by immunohistochemistry in human patellar tendinosis tissues and healthy patellar tendons. In situ gelatin zymography was used to detect collagenolytic activities. The production of MMP1, TIMP1 and collagenolytic activities was also compared in cell cultures from tendinosis samples and controls. Tendinosis tissues and cultures showed an increase in

the expression level of MMP1 and a decrease in that of TIMP1, a condition favoring collagen degradation.

The role of TGFβ has been scrutinized with respect to tendon overuse injury because it is a cytokine strongly associated with matrix remodeling [30]. TGFβ isoforms (β1, β2 and β3) and their signalling receptors (TGFβR1 and TGFβR2) were reported to be present at sites of blood vessels both in normal and in injured Achilles tendons [30]. Pathological tendon showed an increase in cell numbers and in the percentage of TGFβ2- and TGFβR2-expressing cells. TGFβR1 was restricted to blood vessels and was absent from cells in the fibrillar matrix. In their discussion, the authors cite evidence that TGFβ signalling may be dependent on TGFβR1. If this were the case, it could explain why chronic tendon lesions fail to resolve and suggests that the addition of exogenous TGFβ might have little effect on chronic tendinopathy, other than to stimulate cells normally restricted to the endo- and paratendon. The implications of this important finding remain to be explored further.

Another member of the TGFβ superfamily which is implicated in tendon injury and repair is cartilage-derived morphogenetic protein-1 (CDMP1). CDMP1 appears to be expressed in the torn rotator cuff [66]. The localization and expression of CDMP1 was examined by in situ hybridization and immunohistochemistry. Active cells synthesizing the alpha-1 chain of collagen Type I mRNA were localized predominantly in the torn edge, and minimally evident in tissue that was more distant from the torn edge. CDMP1 had a similar distribution as the alpha-1 chain of collagen Type I. These data provide the

first observational evidence that CDMP -1 was activated specifically at the site of the torn rotator cuff tendon and was associated with increased collagen synthesis.

The different cellular environment of tendon insertions, as opposed to the tendon proper, might influence the process of matrix remodeling at the tendon-bone insertion. Gotoh and colleagues [42] examined the contribution of IL1β, cathepsin D, and MMP1 to osteochondral destruction at the osseotendinous junction. Strong immunoreactivity was found in torn supraspinatus insertions, but not in the insertions of intact tendons. Macrophages and multinucleated giant cells showed immunoreactivity for all three mediators and were often found at the interface between the osteochondral margin of the enthesis and granulation (scar) tissue, suggesting that they may be involved in osteochondral destruction. The authors concluded that the presence of scar in tendon may contribute to the development of rotator cuff tears by weakening the insertion.

Gene arrays have recently been used to examine tissue from four patients with Achilles tendon disorders, to identify changes in the expression of genes that regulate cell-cell and cell-matrix interactions. The greatest difference between the normal (post-mortem) and tendinosis tissue samples was the downregulation of MMP3 (stromelysin) in all the tendinosis samples. The expression levels of type I and type III collagen were significantly higher in the degenerate compared to the control samples [46]. The downregulation of MMP3 has also been identified in a cDNA array and confirmed by real-time PCR by Alfredson et al [5]. Ireland et al. speculated that the downregulation of

MMP3 may contribute to the increased levels of GAGs that are characteristic of tendinosis [46].

Gene arrays have also been used to examine the extent of inflammatory cell and cytokine expression in Achilles tendinosis. There was no increase in the mRNA for typical markers of lymphocytes, monocytes, and granulocytes [5]. In addition, the RNAs for IL1 and TNFα, important cytokine mediators of inflammation, were not detected. The authors noted that surgical biopsy studies represent the end stage of tendon pathology so there remains a need to examine tissue from earlier stages of the disease process where inflammation may play a more prominent role.

What role does apoptosis play in tendinopathies?

Because areas of hypocellularity are a prominent feature in some advanced tendinosis lesions, the question arises as to the cause and mode of cell death, and their overall importance in the pathological process. There are two forms of cell death, apoptosis and necrosis. Necrosis, or "accidental" cell death, generally results from conditions where the integrity of the cell membrane is lost (e.g. direct trauma, toxins, ischemia), and is characterized by spillage of the cellular contents into the extracellular space. In contrast, apoptosis or "programmed" cell death is unleashed by dedicated enzymes (caspases) in response to various cellular stresses including DNA damage, hypoxia, oxidative stress, or loss of cell-matrix contact [1, 24]. Apoptotic and necrotic tenocytes are occasionally observed in tendinosis of the elbow [37]. A study conducted with our Norwegian collaborators Lian, Bahr and Engebretsen during the first years of my PhD training

showed that tenocyte apoptosis is not a prominent feature in patellar tendinosis biopsies, but that nonetheless there was a statistically significant increase due to the presence of scattered apoptotic cells throughout the tendinosis biopsies, typically in association with the hypervascular (i.e. tendinosis) regions [57]. Following tendon rupture, a very high percentage of apoptotic fibroblast-like cells has been reported for the supraspinatus [90]. Thus, the role of apoptosis at various disease sites and stages remains as controversial for tendinosis as it does for osteoarthritis [1]. In theory, treatment with growth factors, anabolic levels of mechanical loading, or stem cell implantation might have the potential to prevent ongoing apoptosis, and stimulate remaining cells to proliferate and align, thereby restoring and organizing the lost tendon cells [19, 86]. However, as patellar tendinosis is generally thickened and hypercellular, as opposed to osteoarthritic cartilage which is thin and hypocellular, the rationale of transplanting further cells into the tissue is debatable.

OBJECTIVES

The overall objectives of the current thesis were:

(1) to identify and quantify specific cell populations within the patellar tendons of athletes with patellar tendinosis, in comparison to athletes with healthy patellar tendons;

(2) to examine tenocyte regulatory events in a standard laboratory model of tendon overuse injury;

(3) in primary tenocyte cultures, to characterize events modulating cell death and proliferation in response to physiologic stimuli.

SPECIFIC AIMS

For Objective 1 (PAPERS I - IV), the specific aims were to compare, in patients with tendinosis and in patients with normal tendon:

a) the distribution of mast cells (mast cell tryptase), endothelial cells (CD31), α-smooth muscle actin containing cells, and the density of mast cells and of microvessels;
b) the distribution and protein levels of versican and its relationship to observed features of tendinosis pathology (microvascular hyperplasia, cellular proliferation and/or inflammation, fibrocartilage formation)
c) the cellular distribution of vascular endothelial growth factor-A (VEGF)
d) the cellular distribution of vesicular glutamate transporter 2 (VGluT2)

For Objective 2 (PAPER V), the specific aims were to examine a validated rat model of supraspinatus tendinosis with regard to:

a) the time course of tendinosis development using a valid histopathology scale
b) the rates of tenocyte tenocyte apoptosis and proliferation over time;
c) the expression of IGF-I and the activation of IGF-I signaling in tenocytes

For Objective 3 (PAPER VI), the specific aims eres to assess:

a) the ability of IGF-I to prevent tenocyte apoptosis using a standardized assay (hypoxic primary tenocyte cell culture);
b) the ability of tenocytes to activate PKB in response to IGF-I stimulation

HYPOTHESES

1. Versican expression is greater in patellar tendinosis tissue than in normal control tendon. Versican expression is qualitatively associated with histological features of tendinosis including cellular proliferation.
2. Mast cell density is greater in patellar tendinosis tissue than in normal control tendon.
3. VEGF expression is more extensive in tendinosis biopsies than in normal control tendon.
4. VGluT2 is expressed in specific anatomic structures within tendon (i.e. nerves, vessels, tenocytes, or other cell types) and its expression is greater in tendinosis tissue than in normal control tendon.
5. Downhill running for 4-16 weeks duration will induce features of tendinosis in the rat supraspinatus tendon including apoptosis and cellular proliferation.
6. Prolonged hypoxia will result in apoptosis and necrosis of tenocytes.
7. IGF-I will exert a pro-survival effect on primary tenocyte cultures in the setting of hypoxia-induced cell death.

Table 1.1 Molecular findings in chronic, non-ruptured tendinopathies

Molecule	**Potential significance of change**	**Reference**
Tendon		
↑ Collagen I mRNA	↑ collagen synthesis	[46]
↑ MMP2 mRNA	↑ matrix turnover	[5, 46]
↑ Collagen III	↓ tensile strength	[47]
↑ fibronectin mRNA	↓ tensile strength	[5]
↑ GAG, ↑ chondroitin sulfate	↑ tendon swelling ↑ MRI signal	[75]
↓ MMP3 mRNA	GAG accumulation	[46]
↓ dermatan sulfate, ↓ decorin mRNA	abnormal fibril size regulation	[5]
↑ lactate	↑ anaerobic metabolism	[2]
↑ COX2	↑ collagen synthesis	[34]
↑ TGFβ	↑ GAG synthesis ↓ GAG degradation	[34]
↑ VEGF mRNA	↑ angiogenesis	[5]
↑ PDGFR, ↑ PCNA	↑ tenocyte proliferation	[5]
↑ IGF-I	↑ tenocyte proliferation	[44]
↑ CD31	↑ microvessels	[12]
↑ SP	Pain, edema	[83]

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CHAPTER 2

Increased Versican Content is Associated with Tendinosis Pathology in the Patellar Tendon of Athletes with Jumper's Knee*

INTRODUCTION

Tendinopathy is a common source of chronic musculoskeletal pain [18]. The histology of tendinosis – the underlying pathology in many cases of tendinopathy - is well described and certain histopathological features of tendinosis appear to be consistent across tendons [21], but the molecular changes in tendon composition have been investigated less extensively. Imaging abnormalities include hypoechoic areas on greyscale ultrasound and high signal on magnetic resonance imaging (MRI), corresponding to an increase in glycosaminoglycans [9]. Cross-sectional studies of tendon from competitive athletes have demonstrated that such abnormal tendon morphology on imaging – even when not associated with symptoms -- is associated with a 4.2 times greater risk of developing into symptomatic tendinopathy compared with a sonographically normal tendon [9]. Thus, changes in the extracellular matrix may represent an early step in the pathogenesis of tendinosis.

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Unlike the pathology of osteoarthritis, which is characterized by a loss of glycosaminoglycan and decreased DNA content, biochemical assays of tendinosis have demonstrated an increased presence of glycosaminoglycan and DNA. Chondroitin sulphate is the major glycosaminoglycan in tendinosis tissue and the accompanying increase in DNA content has been primarily attributed to fibroblasts and vascular cells (endothelial and smooth muscle cells) [14, 20]. Therefore, the excessive chondroitin sulphate found in biochemical assays of tendinosis could be produced by resident fibroblasts (tenocytes) or by vascular cells, or both [34]. Furthermore, the core proteins to which the glycosaminoglycan is likely bound have not yet been examined to our knowledge.

Versican is the most abundant of the large chondroitin sulphate proteoglycans in tendon [44]. Immunohistochemical studies of normal tendon and of collagenase-digested tenocyte arrays have found versican in the endotendon around vessels, and in the pericellular matrix around tenocytes [5, 35]. In an experimental model of subfailure ligament injury, versican mRNA in resident fibroblasts was reported to be acutely upregulated [32]. This finding may be consistent with versican's role in creating a hydrated, anti-adhesive matrix that promotes cellular proliferation and migration following injury [22]. Versican expression is also upregulated in bovine tendon by *in vitro* compressive loading [36], a mechanical stimulus which has been hypothesized to occur at the inferior patellar pole during loaded knee flexion [1, 17]. Thus, both injury or altered mechanical loading could lead to distinct patterns of increased versican synthesis

in tendon, and the distribution of versican in tendons may give insight into the underlying mechanisms.

Therefore, we aimed to characterize the quantity and distribution of versican in the patellar tendon of athletes presenting with tendinosis. We hypothesized that versican would be present in excess in patellar tendinosis, and that it would be distributed in the extracellular matrix surrounding tenocytes, endothelial cells, and/or smooth muscle-containing cells.

METHODS

Subjects

Tendon tissue was obtained from patients with documented patellar tendinosis (n=21) and from control participants (n=10). The patients (18 males and 3 females) were athletes who had experienced pain for longer than 3 months and were unable to participate in sport at their pre-injury level. The average Victorian Institute of Sports Assessment (VISA) score for patients was 42 (15-65) indicating significant functional limitation (0=very poor function and 100=perfect function and no pain) [42]. All patients in the present study also had MRI abnormalities at the proximal aspect of the patellar tendon confirmed by a board-certified musculoskeletal radiologist and a specialist orthopaedic surgeon. The control group (7 males and 3 females) was selected from patients undergoing intramedullary nailing for low-energy tibia fractures and who had no current or previous knee pain. None of the patients or controls had previous surgical treatment in or around the knee, corticosteroid injections in or around the knee, serious

traumatic injury affecting the knee, or any rheumatic or degenerative knee condition. The average age of subjects was 30 years (24-34 years, n=21) in the patient group and 29 years (19-43 years, n=10) in controls. All subjects gave their written, informed consent. The study was approved by the local Committees for Research Ethics in Australia, Norway and Canada.

Biopsy procedure

The surgical technique and biopsy handling was identical in the two groups. Biopsies were taken from the proximal bone-tendon junction, and the tendon tissue was excised using a full thickness wedge-shaped incision, widest at the patellar pole (1 cm) and narrowing distally (2-3 cm in length). Biopsies were either immediately frozen in liquid nitrogen and stored at -80°, or chemically fixed in Zamboni's solution for 4 to 24 h and then washed in 0.1 M phosphate-buffered NaCl, pH 7.2, with 15% sucrose (weight/volume) (PBS) and 0.1% natriumazide. After fixation, the biopsies were stored in PBS at 4°C, dehydrated, paraffin embedded and serially sectioned at 5 μm thickness.

We applied Bonar's classification system [21] to confirm the presence of tendinosis in patients, and its absence in controls, using tendon sections stained with Alcian blue and haematoxylin & eosin (H&E). Distinct features of tendinosis (fibrocartilage metaplasia, neovascularization, fibroblastic hypercellularity) were independently identified by two investigators (AS, JLC) blinded to group. The inter-rater reliability for identifying these features was 100%. Patient biopsies that yielded tissue sections at least $1cm^2$ in size (10 of 21 biopsies) were used for more extensive qualitative observations regarding the relation of versican expression to features of tendinosis.

Detection and quantification of versican in tissue sections

Western blot methodology

Tendon samples were extracted from samples of tendon with 4M guanidium chloride and 50 mM sodium acetate buffer (50 mM sodium acetate, 100 mM disodium sulphate; pH 5.8) containing proteinase inhibitors for 72 hours at 4°C. The protease inhibitor cocktail was prepared as a 10X concentrate and added to the buffered guanidine hydrochloride solution to give final concentration of 8 mg/l soybean trypsin inhibitor, 0.1 M aminohexanoic acid, 0.01 M ethylenediaminetetra-acetic acid, 1.0 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulfonyl fluoride, 2.0 mM iodoacetic acid and 0.02 % (w/v) sodium azide. The proteoglycans present in the extract were isolated by ion-exchange chromatography on Q-Sepharose, deglycosylated with chondroitinase ABC and keratanase, and separated by SDS-polyacrylamide electrophoresis as previously described [38]. Handling and processing of patient and control biopsy material was identical. The gels were then analysed by Western blotting using the LF99 antibody (a kind gift of Dr Larry Fisher, NIH) and 2B1 (Seikagaku, Japan) to versican. The LF99 antibody was raised against a peptide corresponding to the N-terminus of versican (LHKVGKSPPVRC) [3]. This epitope is cleaved during versican maturation, and therefore represents newly synthesized proteoglycan. The 2B1 antibody was raised against proteoglycans extracted from a human yolk sac tumour, and later identified as specifically recognizing an epitope close to the C-terminal domain in the versican core protein [19]. Samples equivalent to 2μg wet weight of tissue were applied to each well.

Quantitative immunohistochemistry

The distribution of versican was examined in tissue sections using the 2B1 antibody followed by a commercially available avidin-biotin-HRP (horseradish peroxidase) visualization system (Vector Labs). Stained tissue sections were systematically scanned at 20x magnification by a technician who was unaware of the labeling code using the BLISS microscope workstation (Bacus Laboratories, Lombard, U.S.A.). Image Pro Plus (version 4.5.1.22 from Media Cybernetics, San Diego, California, USA) was used to highlight positive DAB end-reaction product using the same threshold file for all sections. Values are expressed as the average number of positive pixels per field.

Labeling of proliferating cells and vascular cell types

Proliferation of endothelial cells is a key event in angiogenesis. Therefore, we used monoclonal antibodies against markers for endothelial cells (CD31; DAKO JC70A), and proliferating cells (Ki67; BD Pharmingen B56) to label cell types in normal and patellar tendinosis tissue. CD31 (platelet endothelial cell adhesion molecule) has been commonly used to identify microvascular endothelial cells [4]. α-SMA (Biomeda 1A4) has been shown to identify the contractile cytoskeleton in the media of arterioles, as well as pericytes and myofibroblasts [11, 40, 41]. Ki67 is a nuclear protein expressed in proliferating cells, with peak expression during M phase [25].

Determination of microvessel parameters

Microvessels (defined here as vessels with diameter <50 μm or smaller) from the 10 most proximal viewing fields (40x objective, 0.35mm^2 total area) were outlined using digitized, systematically scanned micrographs of H&E sections. All individual vessel

profiles were manually traced in Image Pro Plus and counted with the digital counting tool. Vessel density was defined as the number of profiles per mm^2. Vessel thickness and lumen were determined by measuring vessel profiles in the transverse plane. Thickness was defined by the external borders of the adventitia, whereas the lumen diameter was defined by the internal borders of the intima. To obtain a sufficient number of vessels for analysis of thickness and lumen (n=303), both circular and oblique vessel profiles were included as long as the plane of section passed completely through the vessel; diameters were thus obtained using the least diameter method, which minimizes measurement error due to oblique sectioning [8].

Statistical analysis

To compare the amount of versican immunostaining in control and patient samples, the average number of positive pixels was subjected to a *t*-test with significance pre-determined at α=0.05, after ensuring the data were normally distributed and did not violate the assumption of equal variance (Levene's test). Results are expressed as mean $\pm$ standard deviation (SD). Vessel density was compared in the same way. Vessel diameters were not normally distributed, and the distribution appeared to differ between patients and controls; therefore the non-parametric Kolmogorov-Smirnov test was used to test the hypothesis that the distributions were drawn from different populations. Analysis was carried out using SPSS version 13.0 (SPSS Inc., Chicago, U.S.A.).

RESULTS

Comparison of versican expression in normal tendon and tendinosis

Western blot analysis of proteoglycan samples extracted from patellar tendon samples demonstrated that in pathological tissue there were clearly increased levels of versican present in pathological tissue (Figure 2.1). Most of the versican labeled by the 2B1 antibody appeared to be catabolic products of the proteoglycan [38]. Labeling with the LF99 antibody was prominent in gels obtained from patient samples but was barely detectable in normal tendon, and appeared to preferentially identify a high molecular weight form of versican.

Semi-quantitative immunohistochemistry independently confirmed that versican was present in both normal and pathological tendon, but was significantly increased in patients ($2.63x10^5 \pm 9.0 \times 10^4$) compared to controls ($1.97x10^5 \pm 6.0x10^4$, $p=0.042$).

Versican distribution in patellar tendinosis – qualitative observations

Tendons from patients with patellar tendinosis demonstrated three main patterns of versican expression (Figure 2.2). A proximal-distal pattern of these tendinosis features could be detected in 9 of the 10 patient biopsies which had sufficient tissue for extensive qualitative observation (as described in Methods).

In the most proximal part of the tendon (Zone 1), there were prominent regions of fibrocartilage. These regions extended into the tendon proper up to several millimeters in length and width. In Zone 1, versican was more prominent in tendinosis tissue than in

control tissue in the vicinity of more rounded, chondrocytic-appearing tenocytes, sometimes in alternating layers consistent with classic descriptions of fibrocartilage. Bordering this zone distally in all cases was a region of vascular hyperplasia and endothelial proliferation (Zone 2), in which versican was also enriched (Figure 2.2).

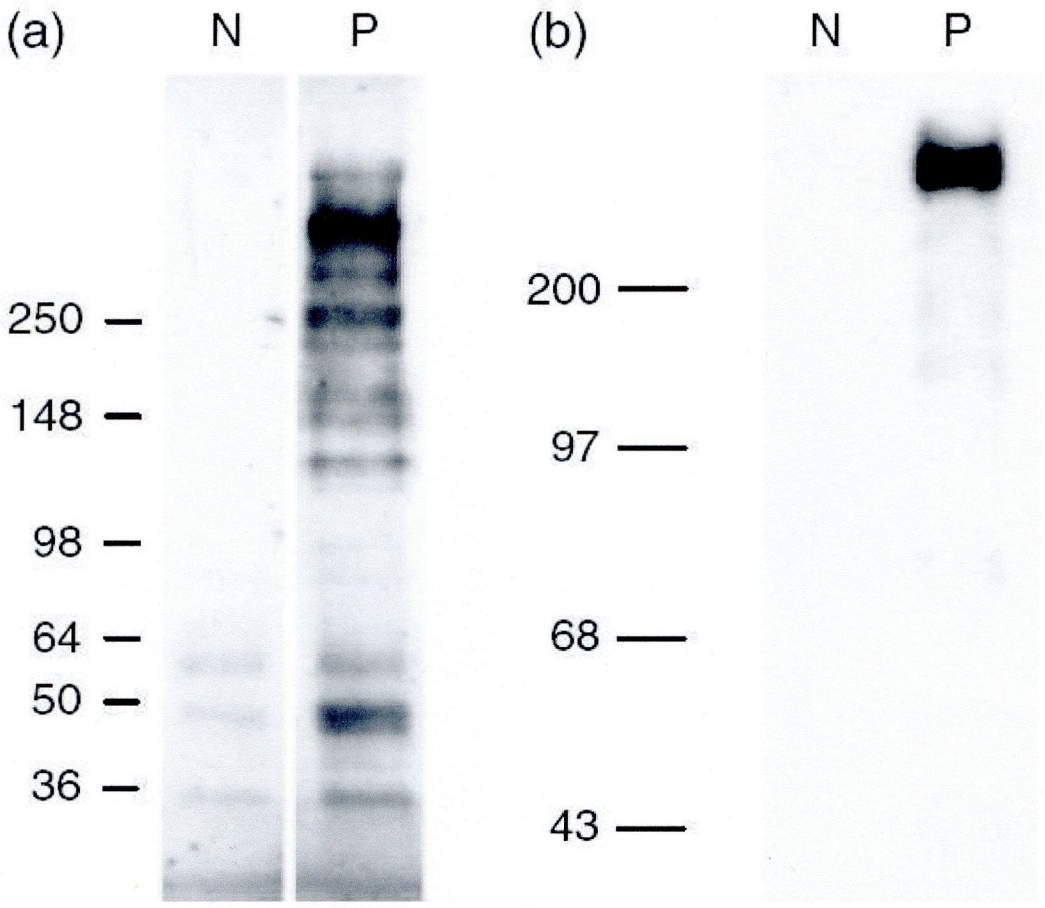

Figure 2.1 Detection of versican in normal and pathological tendon. Western blots of purified tendon proteoglycan extracts (2 mg/lane) were probed with 2B1 (versican C-terminal domain – panel A) or LF-99 (N-terminal domain – panel B). Lanes represent normal (N) and pathological (P) human patellar tendon samples. Molecular weights are shown in kilodaltons. Similar results were obtained in gels from five different patients.

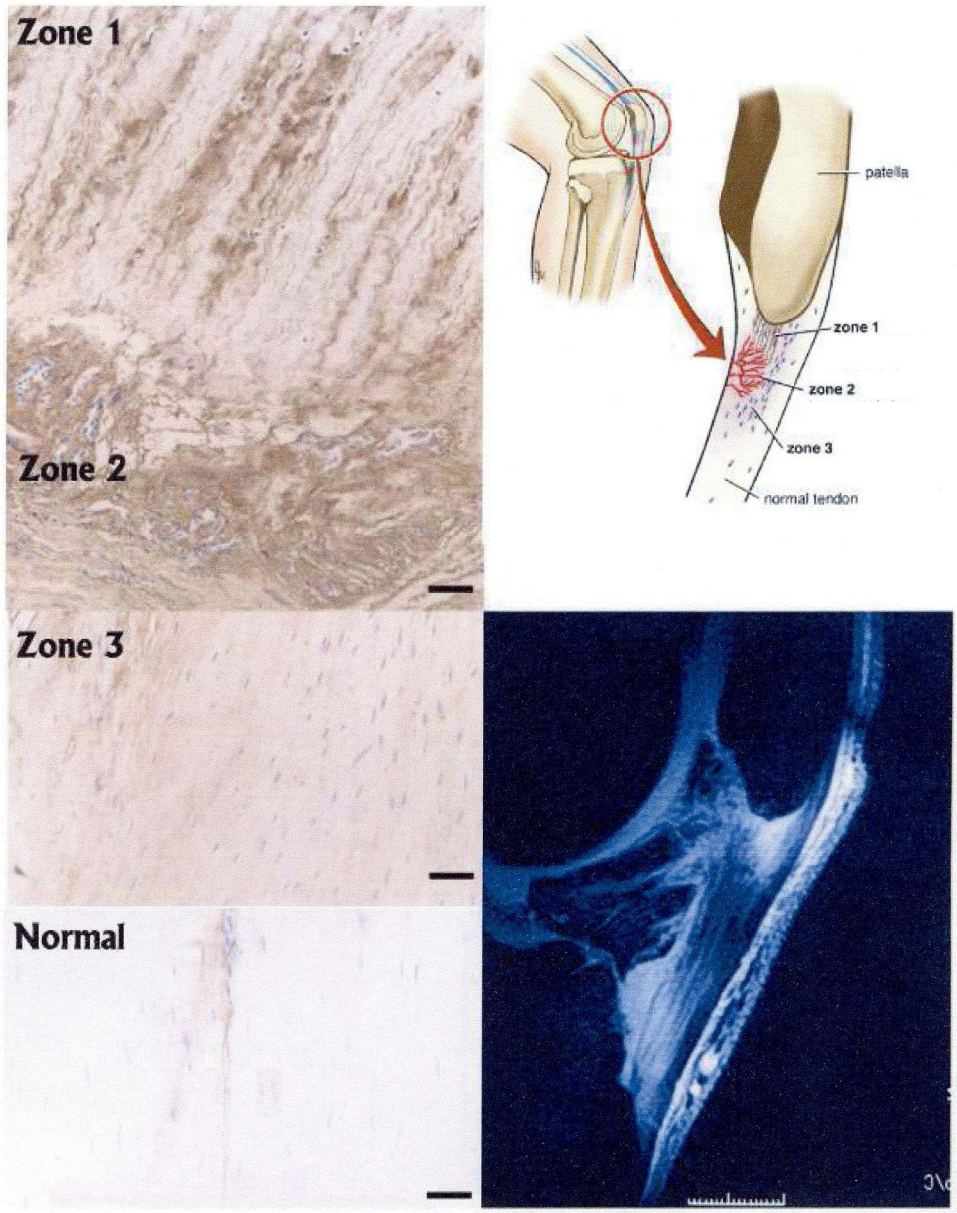

Figure 2.2 Representative micrographs demonstrating zones of versican deposition in association with specific features of tendinosis pathology. MRI from a jumper's knee patient is shown for orientation. Zone 1 contains banded distribution of versican (brown staining) in association with chondrocytic cells (Hematoxylin counterstain), bordered distally by microvascular hyperplasia (Zone 2). Zone 2 is interspersed and bordered by tenocyte hypercellularity and a generalized increase in versican content (Zone 3). In surrounding normal tendon versican staining is fainter and is localized to the endotendon. Scale bar on micrographs = 50 μm, on MRI = 1 cm.

Moving further distally, Zone 3 was characterized primarily by increased tenocyte density but otherwise normal tendon structure (Figure 2.2). In these regions, a greater quantity of versican was detected which was distributed evenly throughout the extracellular matrix of the tendon proper, i.e, there was no focal increase in versican in Zone 3.

Finally, large regions of the anterior and distal patellar tendon in patients with tendinosis were frequently normal and demonstrated no general or focal increase in versican (Figure 2.2). As in uninjured tendon from control samples, tendon from patellar tendinosis patients contained versican predominantly around microvessels in the endotendon running longitudinally with the principal line of action of the tendon [5].

Association of versican with vascular hyperplasia

In the hypervascular region (Zone 2), versican formed an expanded glycosaminoglycan-rich matrix penetrated by CD31+ microvessels exhibiting positive Ki67 nuclear labeling in the intima (Figure 2.3A-C). In microvessels from both normal and tendinosis samples, versican expression was typically complementary to α-SMA (i.e. absent from the tunica media) (Figure 2.3D,E). However, in some larger arterioles of tendinosis biopsies, versican was present in association with medial and/or intimal hyperplasia (Figure 2.4), giving the vessel walls an irregular, thickened appearance. In patellar tendinosis tissue samples, α-SMA-positive fibroblasts (i.e. myofibroblasts) were present in areas of versican-rich tissue, often adjacent to proliferating microvessels (Figure 2.5), but this was not the case in normal tendon.

Quantitation of microvessel density and thickness

Vascular density was 5.6 $\pm$ 9.7 vessels per mm^2 in controls, and 15.6 $\pm$ 16.7 vessels per mm^2 in patients (p=0.026). The distribution of vessel diameters was significantly different in patients (p=0.035) (Figure 2.6) due to an increased presence of small

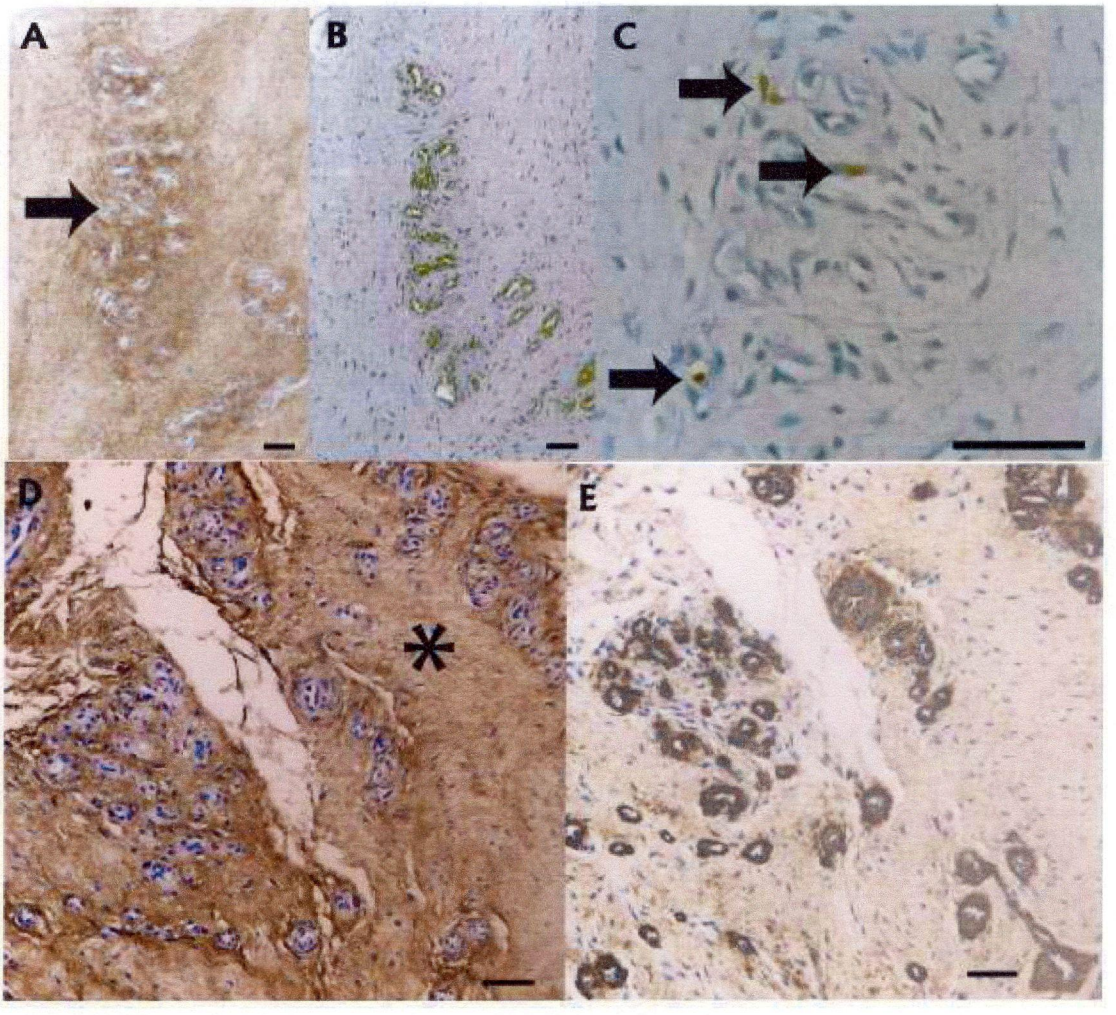

Figure 2.3 Association of versican with vascular proliferation. (a) Diffuse versican labeling (brown) around an abnormally dense capillary bed within the tendon proper, with more intense labeling directly adjacent to the vessels (arrow). (b) CD31 labeling endothelial cells serial to (a). (c) Higher power view of the same region showing Ki67 labeling of endothelial cells in the microvascular walls. (d) Intense versican labeling (asterisk) in a region of arteriolar proliferation. (e) Serial to (d), confirming the presence of smooth muscle actin (brown) in the intima media. Scale bars =50 μm.

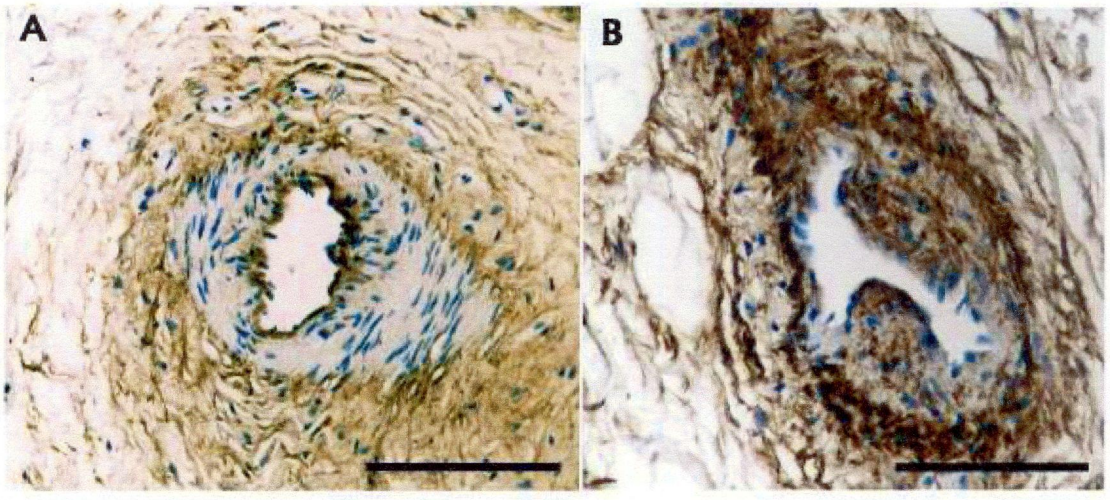

Figure 2.4 Versican distribution in arterioles from normal tendon and tendinosis tissue. (a) Normal arteriole– versican is localized to the surrounding connective tissue, and to a single layer of endothelial cells (tunica intima). (b) Abnormal arteriole – versican surrounds an irregularly thickened endothelial layer and extends into the smooth muscle layer (tunica media), and is also markedly increased in the outer layers. Scale bar = 50 μm.

diameter microvessels (capillaries, venules and arterioles). The distribution of lumen diameters was not different in patient and control samples.

DISCUSSION

The major novel finding of this study is that elevations in versican levels contribute to the increased extracellular matrix volume in symptomatic patellar tendinosis. Versican expression was associated with distinct regions of hypervascularity, hypercellularity, fibrocartilage, as well as morphologically normal tendon. Thus, several distinct cell populations and processes appear to contribute to the expression of versican in the patellar tendon with features of tendinosis. This finding extends previous work which demonstrates increased expression of aggrecan, biglycan and type II collagen in tendinosis lesions [10].

Turning first to the zone of fibrocartilage at the inferior pole of the patellar tendon in patient samples (Zone 1), versican was more prominent in the matrix surrounding rounded, chondrocytic cells. Directly adjacent to the region of chondroid metaplasia, a zone of hypervascularity (Zone 2) was consistently observed in patient samples. This was characterized by increased versican expression both in the perivascular matrix and within the vessel walls. This proximal-distal pattern of fibrocartilage bordered by

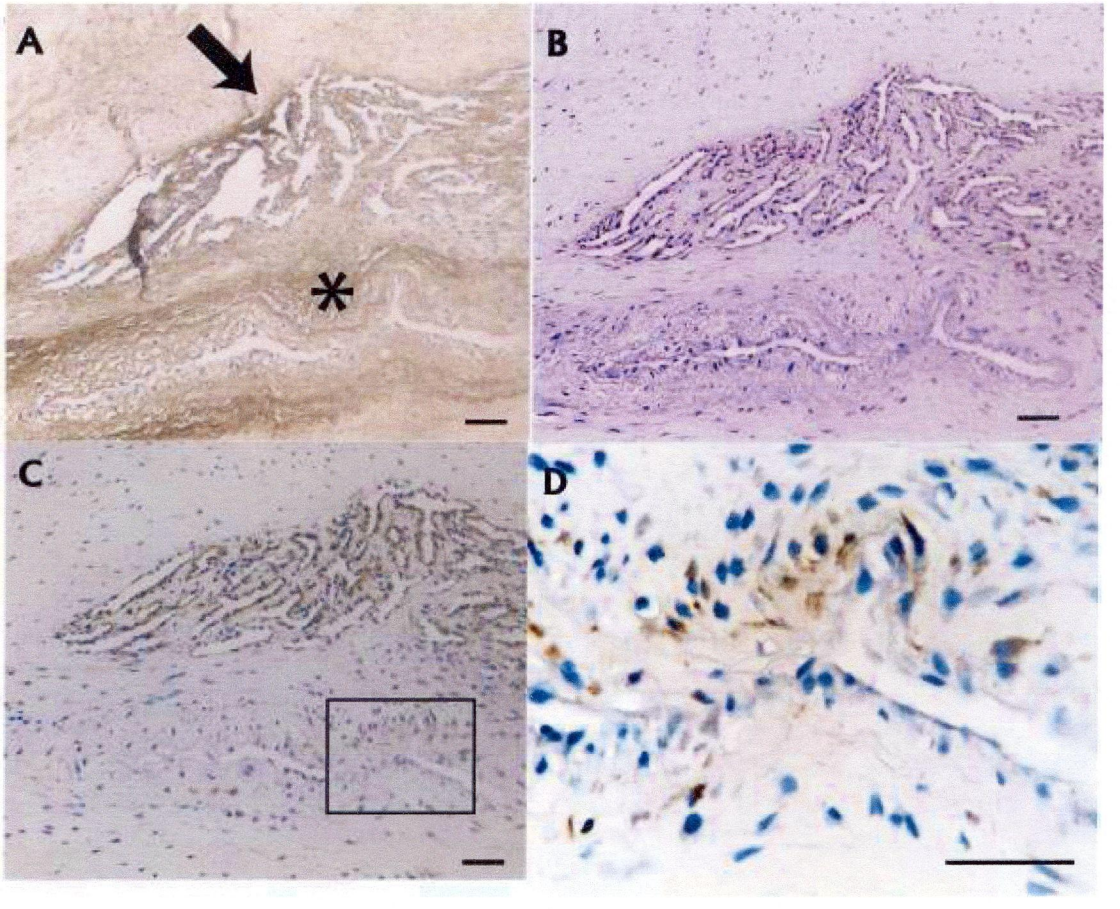

Figure 2.5 Association of versican with microvascular hyperplasia and myofibroblasts. (a) Versican in association with an abnormal microvascular bed (arrow), and remodeling tissue (asterisk). (b) CD31 confirms that the upper structure is vascular, whereas the lower structure is not. (c) Intensely labeling of smooth muscle actin (α-SMA) in the vascular structure indicating arterioles. α-SMA is also positive in scattered myofibroblasts in the adjacent remodeling area (rectangle). (d) Higher power view of rectangular area in (c) Scale bars = 50 μm.

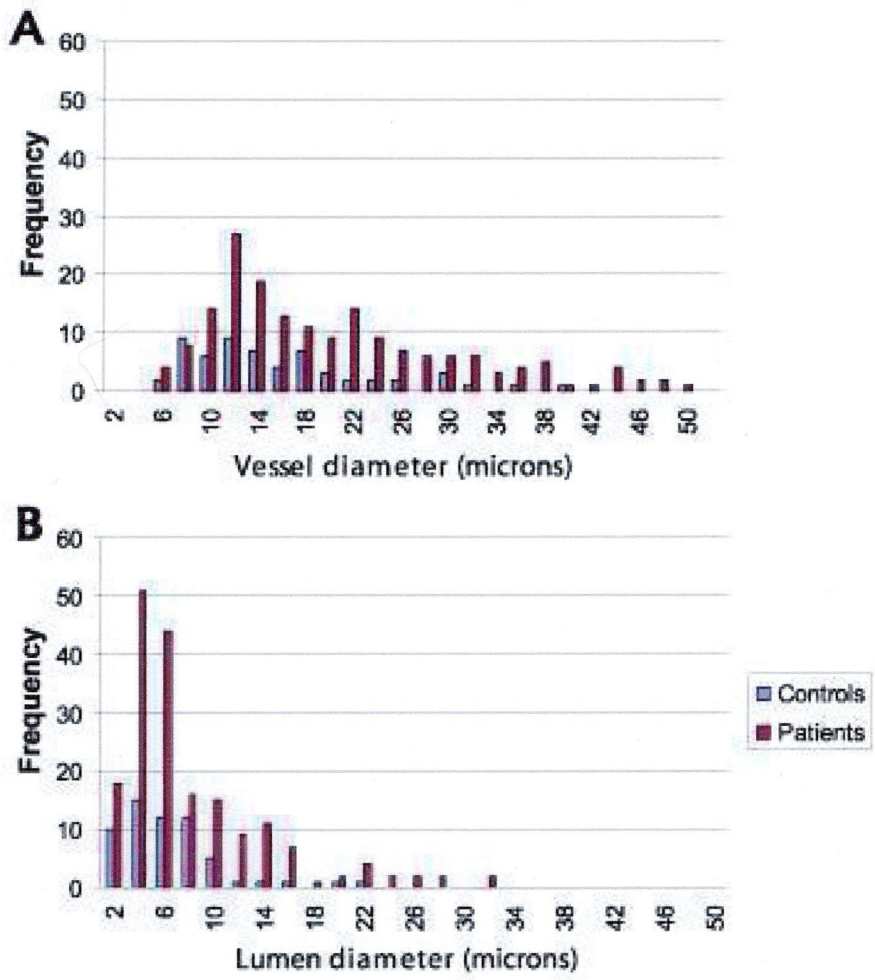

Figure 2.6. Quantitation of vessel diameters in the proximal 0.35mm^2 of the patellar tendon. Morphometric analysis demonstrates increased presence of microvessels in patellartendinosis.

vascular tissue has been noted previously in the supraspinatus tendon. [15] Fukuda and co-workers examined en-bloc sections of full thickness rotator cuff tears and reported chondroid metaplasia in the proximal remnant of the tendon, whereas the distal torn end was characterized by hypervascular granulation tissue [15]. This is consistent with a hypothesis that fibrocartilage metaplasia may initially develop as an adaptive response to

compression under the acromion, but that this adaptation weakens the tendon's ability to withstand tensile loads, thereby leading to microtears adjacent to the site of fibrocartilage formation. A similar process has been postulated for the tibialis posterior where it is compressed by the medial malleolus [31]. These data will therefore be of interest to those who have hypothesized that compression may play a role in the etiology of patellar tendinopathy [1, 17]. Fibrocartilage tends to develop within areas of tendon (distinct from the enthesis) that are subjected to compression or shear, for example at the deep surface of wrap-around tendons such as the supraspinatus or tibialis posterior. However, fibrocartilage metaplasia is also sometimes observed as a pathological dysregulation of cell phenotype, for example in scar tissue [6].

Versican was increased in association with microvessels in two overlapping patterns. First, and most commonly, there were often dense beds of capillaries and/or arterioles, and the entire region demonstrated excess staining for versican. Proliferating endothelial and smooth muscle cells could be identified in these regions. This pattern is consistent with versican's role in facilitating proliferation and migration in angiogenesis.[7] The second abnormality was the increased presence of versican in association with intimal and medial hyperplasia of arterioles. We have found preliminary evidence of vascular endothelial growth factor (VEGF) expression by endothelial cells in patellar tendinosis tissue (Chapter 4). In addition to causing endothelial cells to proliferate, VEGF has been reported to upregulate their expression of versican [7]. Taken together, these findings suggest a potential role of VEGF in causing or perpetuating the increased versican expression in Zone 2.

Proliferating microvessels were occasionally seen in proximity to versican-rich tissue populated by myofibroblasts (α-SMA positive fibroblasts). The presence of myofibroblasts is in keeping with prior studies of tendinosis [20] and of other healing soft tissues [39], and their association with versican confirms the importance of this proteoglycan in soft-tissue repair and remodeling [37]. The factors leading to increased versican expression in healing wounds may include hypoxia (leading to hypoxia-induced-factor-1α (HIF-1α) and VEGF expression), cytokines including TGFβ and PDGF [12, 13, 30], and compressive or tensile loads [16, 27, 29].

A third zone of versican expression was typically seen distal and/or superficial to Zones 1 and 2, and this was characterized by a relatively even elevation in versican levels in association with increased tenocyte density. This could be interpreted as evidence of adaptation. In animals and in humans, exercise can induce adaptive improvements in the material properties of the tendon, including increased stiffness and cross-sectional area [23, 43, 44]. Indeed, chondroitin sulphate content is increased in animals subjected to treadmill running vs. sedentary controls [2, 23, 43, 44]. Conversely, the increased versican in Zone 3 could represent a secondary effect due to diffusion of cytokines from adjacent areas.

In the current study, Western blot analysis demonstrated that versican content in patient samples consisted both of newly synthesized, high molecular weight forms, as well as lower weight catabolic products. The regulation of versican expression and turnover is complex and includes alternate splicing of mRNA from the versican gene to generate at

least three possible isoforms [22, 33]. Versican degradation involves proteolytic cleavage into distinct entities, and regulation of its catabolism by aggrecanases and matrix metalloproteases, and by their endogenous inhibitors [22, 33]. The processes of versican degradation and maturation were not the focus of the current study. Future studies should therefore examine these regulatory processes in relation to the development of tendinosis pathology.

In this study, we did not examine the distribution of other potentially relevant components of the extracellular matrix (e.g. aggrecan, biglycan, decorin, lumican, collagens, fibronectin, hyaluronan). In particular, aggrecan is likely to contribute to the regions of fibrocartilagenous metaplasia observed here [29]. Aggrecan mRNA levels were increased in Achilles tendinosis [10] and in the ruptured supraspinatus [26] whereas versican levels were unchanged compared to controls [10]. The discrepancy between findings at the protein and mRNA levels in these studies is consistent with a report from cartilage showing that versican protein can remain in the matrix long after mRNA levels have declined [28]. The complex regulation of versican and other matrix molecules in the development and progression of tendinosis clearly requires further research.

CONCLUSION

In summary, we found that versican is present at elevated levels in human patellar tendinopathy. Versican was enriched in distinct regions of the tendon tissue, specifically in the perivascular matrix of proliferating microvessels, within the media and intima of arterioles, in regions of fibroblast proliferation, and in areas of fibrocartilage. We

conclude that future studies should address both pathological and adaptive mechanisms underlying the upregulation of versican in the tendon proper and the vascular matrix to better understand the basis and consequences of the elevated protein levels detected in the present studies.

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CHAPTER 3

Elevated Mast Cell Numbers in Human Patellar Tendinosis: Correlation with Symptom Duration and Vascular Hyperplasia*

INTRODUCTION

Repetitive overuse tendon injury – tendinopathy – is a major burden to the health care system and a challenge to orthopaedic and sports medicine practitioners [14]. Clinical symptoms include pain, swelling, and impaired function [14, 28]. Color Doppler ultrasound reveals excessive vascular flow in tendinopathy and this finding has been associated with the presence of tendon pain [28, 38].

Despite the increased vascular flow and vascular hyperplasia which characterizes tendinosis, an acute cellular inflammation (neutrophils, macrophages, lymphocytes) is rarely reported in the chronic stages. This lack of typical acute inflammatory cells has led to increasing adoption of the term "tendinosis" as opposed to "tendonitis"[19]. Recently, Schubert et al. [31] compared the numbers of CD68+ macrophages, CD3+ T-lymphocytes, CD20+ B-lymphocytes, and granulocytes in 10 patients with Achilles tendinosis vs 10 patients with an acutely ruptured Achilles. In this study, tendinosis patients displayed increased numbers of macrophages and lymphocytes in association

* A version of this chapter is in press: Scott A, Lian Ø, Bahr R, Hart DA, Duronio V, Khan KM. Elevated mast cell numbers in human patellar tendinosis. British Journal of Sports Medicine

with hypervascular tissue, whereas patients with acutely ruptured tendon displayed greater numbers of granulocytes [31]. Thus, inflammatory and reparative cell types may play a role in the pathogenesis of tendinosis and the development of vascular hyperplasia in chronic tendon lesions.

Along with macrophages and lymphocytes, mast cell numbers are also known to be increased in a variety of chronic inflammatory or fibrotic disorders. In addition to their well known physiological role in triggering allergic reactions, mast cells release a variety of vasoactive, angiogenic and profibrotic substances that are either stored in granules or synthesized de novo in response to hypoxia, mechanical stimulation, or neurological and inflammatory mediators [16, 32, 36]. Mast cell products of potential relevance to overuse tendinopathies include histamine, prostaglandins, leukotrienes, growth factors and proteinases [12, 13]. Despite this, to our knowledge the distribution of mast cells has not been examined in tendinosis tissue. Therefore, in the current study we examined their distribution in both normal and chronically painful patellar tendons. Due to the anatomic proximity of vessels and mast cells in connective tissues [4, 36], we hypothesized that mast cells would be more prevalent in tendinosis than in control tendon tissue, and would be correlated with the vessel area fraction.

METHODS

Participants

Twenty one patients (18 men and 3 women) of mean age 30.4 (22 - 40) and 10 control subjects (7 men and 3 women) of mean age 28.2 (20 – 42) were included. Patients were

athletes who had had at least 3 months of patellar tendon pain and tenderness severe enough to prevent them from participating in sports. The mean symptom duration was 22 months (5-81 months). MRI confirmed high signal changes corresponding to the area of symptoms (infrapatellar pole). The control group consisted of patients treated with intramedullary nailing for tibial fractures with no current or previous knee pain. Exclusion criteria included; age less than 18 years, previous knee surgery, corticosteroid injections in or around the knee, knee trauma requiring medical attention, and rheumatic or degenerative knee conditions. All patients provided written, informed consent. The study was approved by the University and Hospital Research Ethics Committees.

Biopsy procedure

The surgical exposure was identical in the two groups with a 5 cm longitudinal midline or lateral parapatellar incision, splitting of the paratenon and exposure of the patellar tendon. The paratenon was split longitudinally, any pathologic paratenon tissue was removed and the tendon was fully exposed. In both groups, the biopsies were taken from the proximal bone-tendon junction. The tendon tissue was excised using a full thickness wedge-shaped incision, being widest at the patellar pole and narrowing distally. In the patient group, all abnormal tissue was removed. When abnormal tissue was not evident macroscopically, the excision was based on the location of MRI signal changes. Typically, the surgeon removed a wedge with a proximal base 1 cm wide and extending to an apex 2 to 3 cm distal from the patellar pole. In the control group, the biopsies were taken with a width of at least 5 mm and a length of at least 20 mm from the middle portion of the tendon starting at the bone-tendon junction. Immediately after the surgical procedure, biopsies were transferred to Zamboni's fixative where they were stored for 4 to 24 h, and then

washed in 0.1 M phosphate-buffered NaCl, pH 7.2, with 15% sucrose (weight/volume) (PBS) and 0.1% natriumazide. The biopsies were then stored in PBS at 4°C for a minimum of 48 h after which they were embedded in paraffin.

Light microscopic appearance

Five µm sections were routinely stained for H&E (general morphology) and Alcian Blue (sulphated glycosaminoglycans) and viewed at 100 to 630x magnification on a Zeiss Axioplan upright microscope. Areas of adipose or peritendinous tissue were avoided during analysis. Tendinosis was defined according to the features of the Bonar histopathological scale [5]. Briefly, this scale defines tendinosis according to the presence of four features; hypervascularity, collagen disorientation, tenocyte rounding and chondroid metaplasia, and increased glycosaminoglycans [5].

Labeling of cell types

The following mouse monoclonal antibodies were used to identify cell types: CD68 (Signet KP1 clone, Cedarlane Laboratories, Hornby, Canada) for macrophages, CD3 (DAKO, clone F7.2.38) for T-lymphocytes, and mast cell tryptase (DAKO, clone AA1) for mast cells [34, 35, 37]. Processing for mast cell tryptase was carried out using an autostainer (DAKO Diagnostics, Glostrup, Denmark). The sections were cleared in xylene (3 x 15 min), steamed in sodium citrate for 3 x 5 minutes, incubated at 37° with 0.1% trypsin in 0.1% calcium chloride pH 7.3 for 5 minutes, quenched for 15 minutes in 3% hydrogen peroxide, incubated in protein-free blocking solution (DAKO) for 15 min, then exposed to 100 µl of primary antibody diluted 1:50 in 0.1% bovine serum albumin in TBS for 1 hour. A secondary anti-mouse IgG antibody (DAKO) was used, followed

by incubation with alkaline phosphatase anti-alkaline phosphatase (APAAP) (DAKO) for 30 minutes. The signal was visualized with New Fuschin as the substrate (Sigma-Aldritch, Oakville, Canada). Identically fixed and processed tonsil with or without the mast cell tryptase antibody was used as positive or negative control, respectively. CD68 processing was identical to the mast cells protocol but without the trypsin pre-treatment. For T-lymphocytes, the CSA II detection system (DAKO) was used with 3,3'-diamino-benzidine as the chromogen (Vector Laboratories, Burlingame, U.S.A.). Human tonsil was used as a reference for all cell types. In tonsil, mast cells, lymphocytes and macrophages were labeled only in the positive controls.

Image analysis

Slides were examined with the patient/control identity code masked with black tape. For quantitation of mast cell density, the 10 most proximal 40x viewing fields were captured in a pre-determined raster pattern, and # positive cells per mm^2 calculated. Vessel area fraction was quantitated on H&E sections (10 most proximal 40x viewing fields) using image analysis software (Northern Eclipse). Vessels were manually outlined on-screen using a mouse, and the area occupied by vessels was expressed as a percentage of the slide area.

Data analysis

Results are presented as means with SD. Mast cell density was counted on two separate occasions and averaged (intra-rater reliability, r^2=0.75). Mast cell density in normal and patellar tendinosis tendons was compared using the non-parametric Mann-Whitney U test for independent samples. Correlation between mast cell density and vessel area fraction,

and between mast cell density and symptom duration, were conducted using Pearson's correlation analysis. All statistical tests were carried out using SPSS 14.0 (SPSS Inc., Chicago, U.S.A.)

RESULTS

Light microscopic appearance.

One control patient demonstrated tenocyte rounding in the proximal patellar tendon suggestive of chondroid metaplasia, but no other features of tendinosis pathology. All tendinosis patients demonstrated all four features of tendinosis pathology as defined by the Bonar histopathologic scale [5], namely vascular hyperplasia, collagen disorientation, tenocyte rounding and increased glycosaminoglycan.

Prevalence of mast cells in patient biopsies

CD3+ lymphocytes and CD68+ macrophages were present too infrequently either in patient or control tendon tissue to be practically quantifiable, despite being readily identified in tonsil. Conversely, there were a greater number of prominent mast cells in patient tendon biopsies. The mast cell density [3.35 (3.5) cells/mm^2] was three times greater in tendinosis tissue than in control [1.0 (1.5) cells/mm^2] (Figure 3.1). The mast cells were typically present in a perivascular distribution in areas of increased vessel density within the tendon proper (Figures 3.2 -3.4). In 5/22 cases of tendinosis, and 0/10 controls, mast cells were also occasionally observed within the tendon proper, i.e., in between longitudinal collagen bundles.

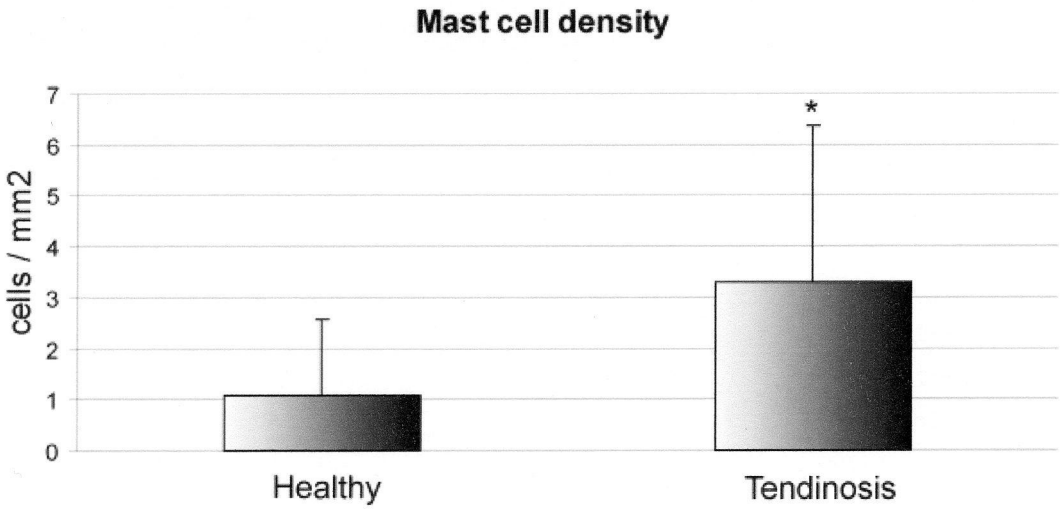

Figure 3.1 Mast cell density in normal and tendinosis patellar tendons. * denotes statistical significance, p=0.036. Scale bars denote SD.

Correlation of mast cells with tendon vascularity

Capillaries, arterioles and venules were present in both patient and control biopsies. Patient biopsies consistently revealed a greater number of vessels in the posterior, proximal substance of the tendon proper. However, the increase in vascular area [2.8% (3.2) in patients vs. 1.2% (2.2) in controls] was not statistically significant. There was a moderate but significant correlation between mast cells and vessel area fraction (r^2=0.49, $p<0.01$).

Correlation of mast cells with symptom duration

Mast cells were found to be most prominent in biopsies from those patients with a longer reported symptom duration. There was a moderate but significant correlation between symptom duration and mast cells in patients was r^2=0.52 ($p<0.05$).

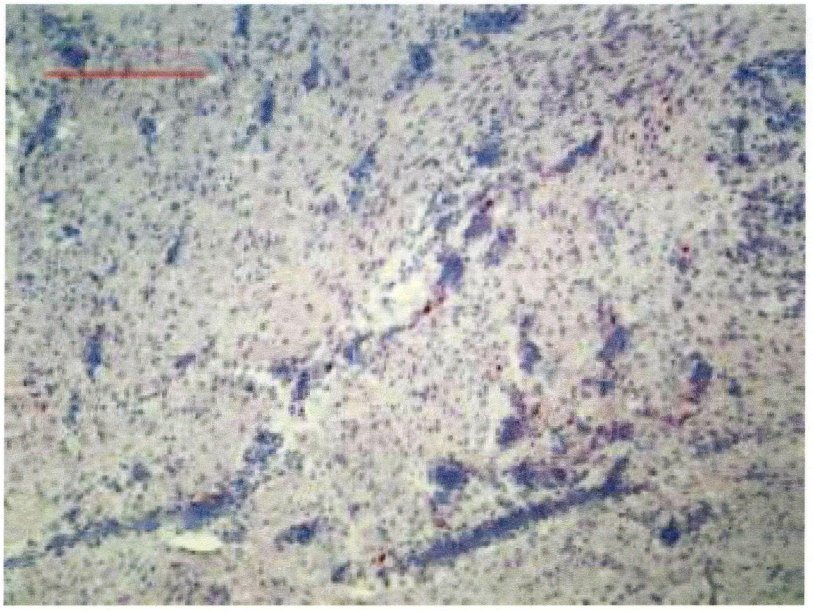

Figure 3.2 Patellar tendinosis biopsy demonstrating numerous mast cells stained red (New Fuschin), counterstained with Haematoxylin. Arrows denote examples of microvessels. Note numerous mast cells in association with microvessels. Original magnification x 100 (scale bar = 250 μm).

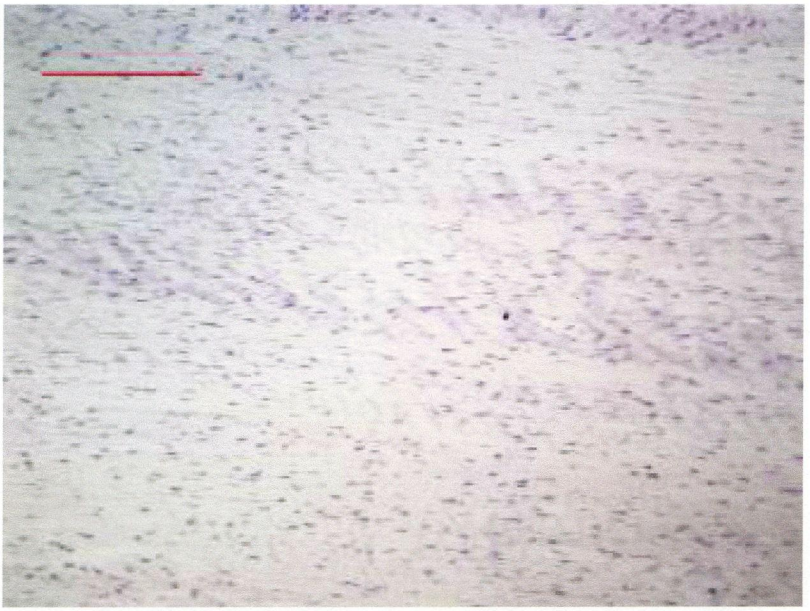

Figure 3.3 Normal tendon processed identically to tendinosis tendon shown above Note lack of microvascular structures and mast cells in comparison with tendinosis tendon. Original magnification x 100 (scale bar = 250 μm).

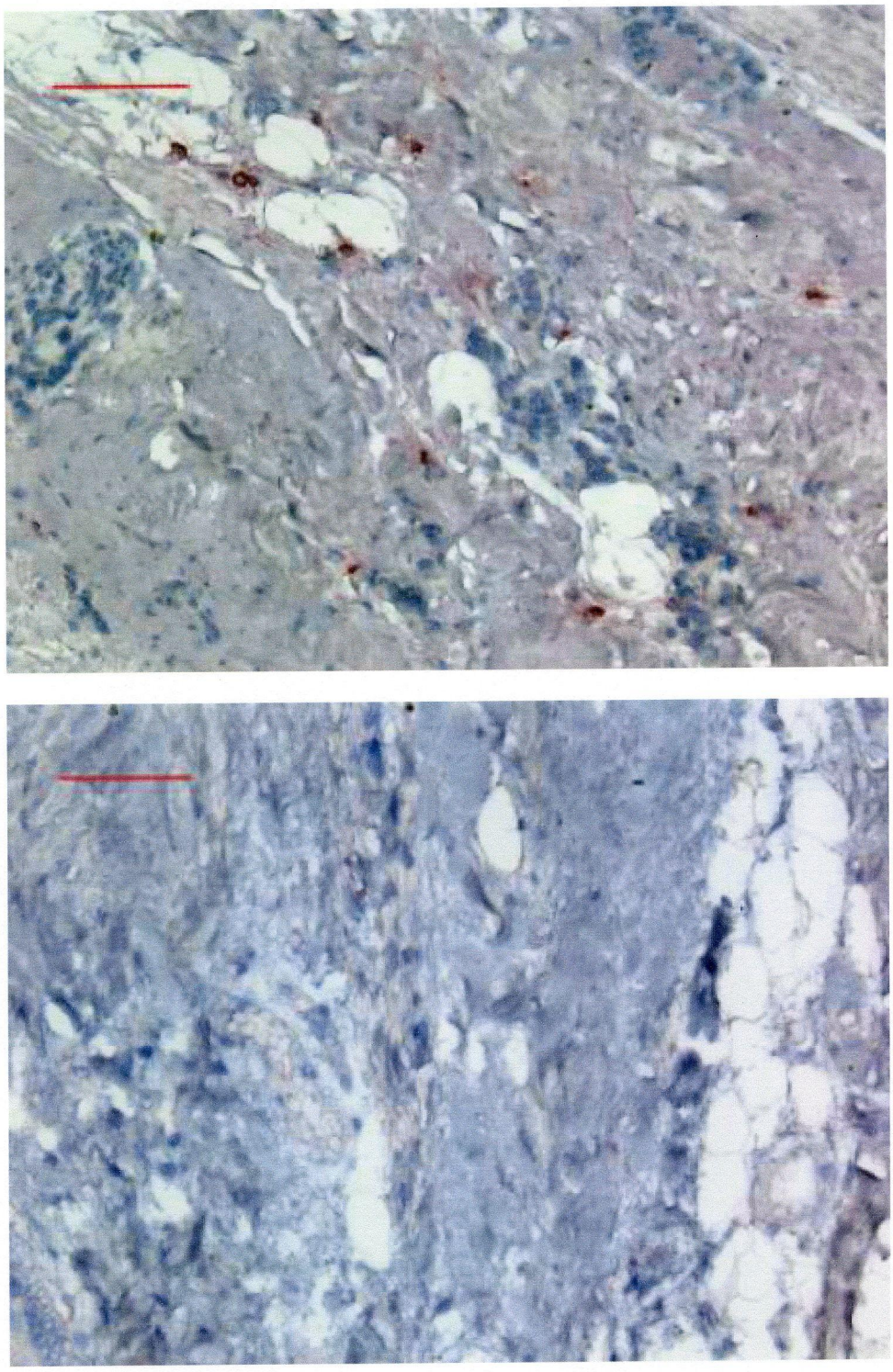

Figure 3.4 Tendinosis tendon processed with (upper) and without (lower) antibody to mast cell tryptase. Original magnification x 200 (scale bar = 100 μm).

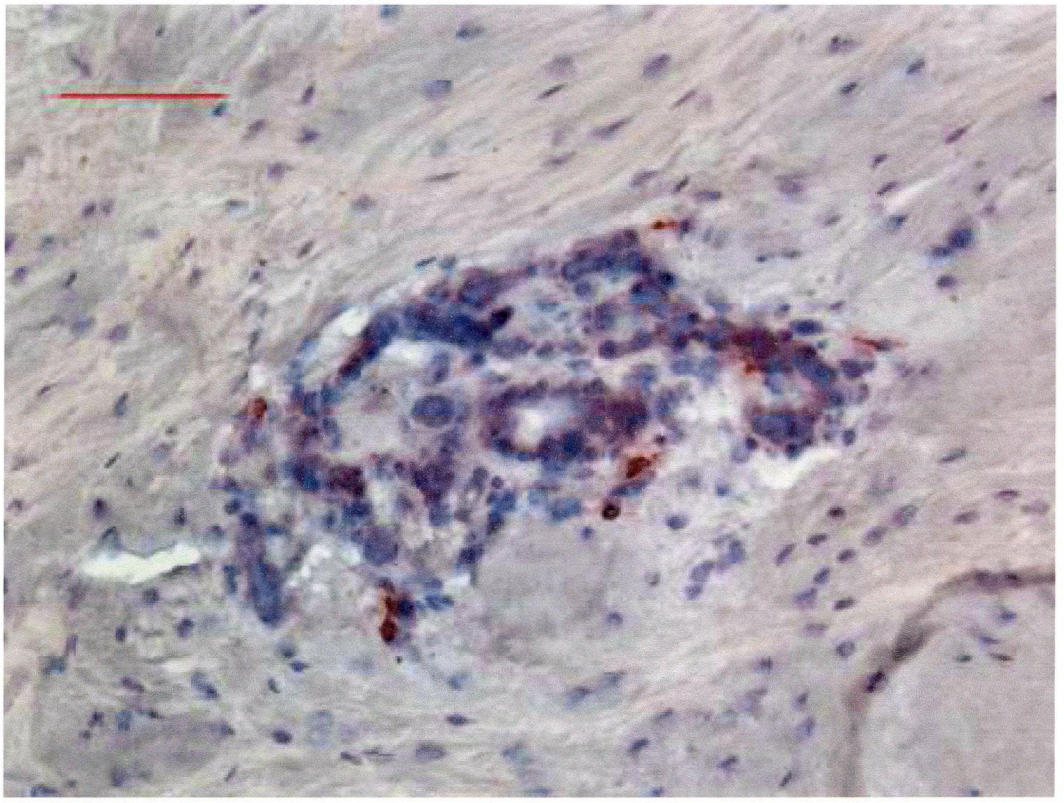

Figure 3.5 Positively labeled mast cells in association with a microvascular structure in patellar tendinosis biopsy.

DISCUSSION

In tendon obtained from symptomatic patellar tendinopathy, the mast cell density was three times higher than in control tendon and was positively correlated with symptom duration. In addition, mast cells were usually found in association with the tendon vascularity. In support of this observation, mast cell density was statistically correlated with the vascular area fraction (r^2=0.49). These findings suggest that the accumulation of mast cells in tendinosis is a feature of the chronic stage of tendon pathology and occurs in association with vascular hyperplasia.

Novel findings of tendinosis cellularity

We identified a relatively high number of mast cells within tendon -- the first direct evidence that this cell type is over-represented in tendinosis tissue compared with control tendon. Classic descriptions of tendinosis tissue obtained from patients undergoing surgery for overuse tendinopathy have not reported prominent mast cells [15, 17, 21, 22, 27]. Increased numbers of mast cells are common in many chronic inflammatory or fibrotic conditions; these include afflictions of joints, the GI tract, the liver, and the respiratory system. Mast cells proliferate at sites of injury and in turn release a range of pro-fibrotic factors including TGFβ, IL1 and IL4 [32]. Mast cell tryptase can also act directly on fibroblasts by triggering their proteinase-activated-receptors, thereby inducing a COX2 dependent proliferative and fibrotic response in fibroblasts [10]. Thus, the potential exists for previously undocumented interactions between mast cells and tenocytes.

Potential role of cellular inflammation in tendinosis

Achilles tendinopathies with symptom duration < 3 months have demonstrated favourable clinical responses to corticosteroids delivered by iontophoresis including a reduction in pain and morning stiffness, suggesting the existence of an acute or subacute inflammatory phase which was not captured in the present study [26]. In support of this clinical observation, several cellular and molecular features of tendinosis suggest the existence of a chronic inflammatory or fibrotic response including increased expression of TGFβ, increased levels of proteoglycan and collagen III , high intratendinous lactate levels, increased numbers of myofibroblasts, and increased fibroblast turnover

(apoptosis and proliferation) [1, 11, 18, 20, 23, 24, 29, 30]. In keeping with these prior studies, the present data demonstrate that increased mast cell number, a well-known component of soft tissues in the chronic inflammatory or fibrotic phase of injury, is indeed present in the patellar tendons of patients with symptoms of pain and swelling for > 5 months, and that the mast cell number may be highest in those patients with longer symptom durations.

In contrast to the current study, Schubert et al [31] demonstrated an increased density of CD68+ macrophages and CD3+ T-lymphocytes in chronically painful Achilles tendon compared to ruptured tendon, which demonstrated an increased density of granulocytes (i.e. neutrophils). This suggests that tendinosis may be characterized by an evolving population of migrating or locally proliferating cell types, with neutrophils predominant in the early phases, followed by macrophages and lymphocytes, and eventually mast cells and vascular cell types [32]. Indeed, only a few of the patients in the study by Schubert et al had symptom duration > 12 months, compared to the majority of patients in the current report. This may account for the discrepancy in terms of the number of observable macrophages and lymphocytes between the two studies. A further discrepancy between these two studies is that several of Schubert et al.'s patients had received corticosteroid injections, which can potentially result in necrosis and focal inflammation [33]. In contrast, no patient in the current study had received any corticosteroid injection in or around the patellar tendon.

Potential role of neurogenic inflammation in tendinosis

The mast cells observed in the current study were positive for mast cell tryptase, which is a potent angiogenic factor [4]. Mast cells are also capable of producing a potent neurotrophin, nerve growth factor [25], which is interesting given recent findings that in addition to angiogenesis, neural sprouting is a prominent feature of tendinosis [31]. Our cross-sectional study does not allow us to determine whether the prominence of mast cells is causally associated with the increased density of microvessels and accompanying sensory and autonomic innervation, which are strongly implicated in symptomatic tendinopathy [2, 3, 6-9]. Nonetheless, the findings tempt speculation that mast cells may play pro-angiogenic and/or neurotrophic roles in tendinopathies. Because the potential exists for multiple paracrine intereactions between mast cells, nerves, endothelial cells, and tenocytes, much work will be required to determine the possible roles of mast cells at different stages in the development and progression of tendinosis. One possible set of interactions is presented below (Figure 3.6).

CONCLUSION

In summary, we have quantitated the density of mast cells in human patellar tendinopathy tissue and found them to be significantly increased in comparison to healthy tendon. Further, we have found that elevated mast cell numbers in the patellar tendon are correlated with both the symptom duration and the vessel area fraction. These observations highlight the need for mechanistic and longitudinal studies to clarify the role of mast cells in tendinopathy.

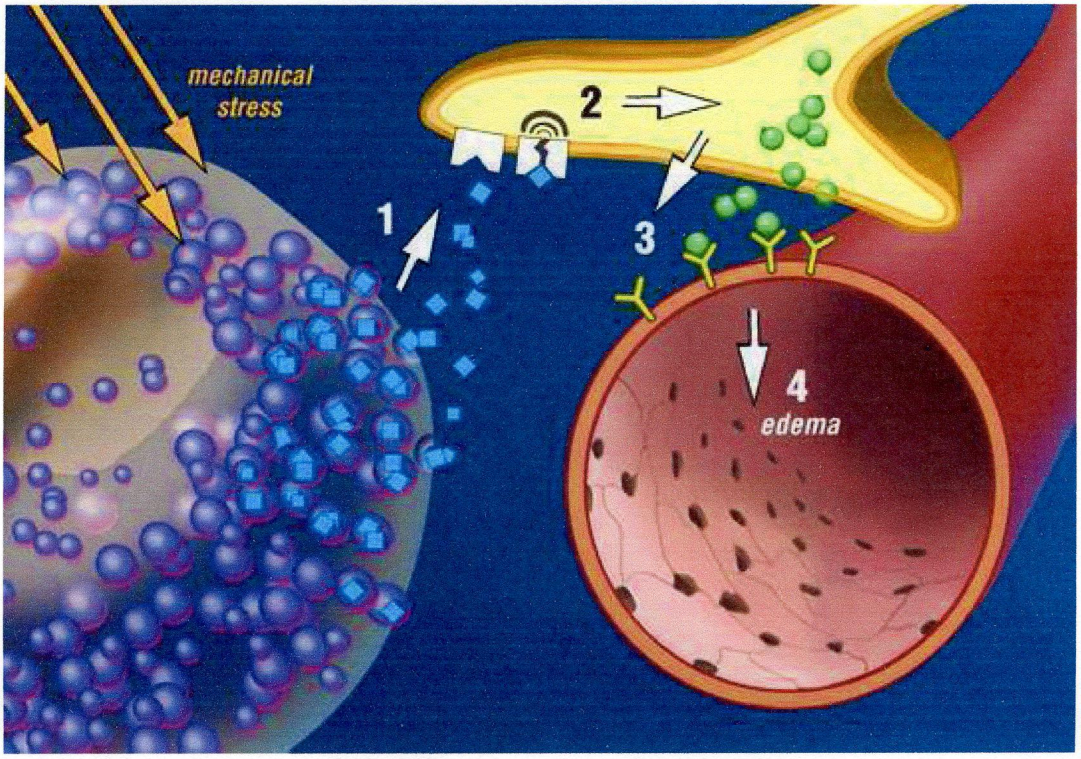

Figure 3.6 A schematic illustration proposing a potential role of mast cells in the development of tendinosis. 1: In response to mechanical stress (strain, compression, shear), mast cells degranulate and release mast cell tryptase, along with a variety of other vasoactive and angiogenic substances. 2: Mast cell tryptase has the capacity to activate receptors (e.g. proteinase-activated receptor 2) on sensory nerve endings. 3: Sensory nerves are capable of releasing SP, and this could lead to neurogenic edema via NK1 receptors.

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CHAPTER 4

VEGF Expression in Patellar Tendinopathy: A Preliminary Study*

INTRODUCTION

Tendon injuries may be chronic or acute in nature, and occur at a variety of common anatomic locations[24]. Acute tendon injuries include lacerations or ruptures, whereas chronic injuries (tendinopathies) are typically insidious in their onset. Achilles tendinopathy, patellar tendinopathy (jumper's knee), lateral epicondylalgia, and rotator cuff tendinopathy are major causes of chronic pain and disability both in general and sporting populations [5, 17, 23]. Although many of these tendinopathies resolve spontaneously[7] or with conservative management including eccentric training [4, 18, 25, 46], a proportion of injuries remain chronically symptomatic (painful and edematous) and require surgery [10, 42]. One report suggests chronic tendon injuries pass through several overlapping stages, including an initial tendinitis (Stage I), followed by tendinosis (Stage II), complete rupture (Stage III), and tendinosis plus other changes including fibrosis or calcification (Stage IV) [21]. Tendinosis is characterized histopathologically in the chronic stage by the disruption of fibroblast arrays with proliferation and apoptosis, and the proliferation of vessels and of nerves containing sensory, nociceptive, and autonomic elements [3, 11, 12, 20, 21, 38]. This histopathological picture has been

* A version of this chapter is conditionally accepted: Scott A, Lian Ø, Bahr R, Hart DA, Duronio V. VEGF expression in patellar tendinopathy: a preliminary study. Clinical Orthopaedics and Related Research

characterized as a "failed healing response" and has also been termed "angiofibroblastic tendinosis" to refer to the major finding of increased vascular and fibroblastic cellularity [28].

Modern imaging methods, color Doppler and power Doppler ultrasound, reveal vascular hyperplasia in and around painful tendons [35, 48]. Several authors report an association between the appearance of excessive vascular flow on power Doppler imaging and tendon pain and impaired function [13, 30]. Thus, both clinical and anatomic findings of regionally increased vascularity in tendinopathies have sparked interest in the possible role of angiogenesis in painful tendons [34, 35].

Vascular endothelial growth factor (VEGF), a potent angiogenic cytokine and signaling peptide with seven molecularly diverse isoforms[43], may play a role in this process. VEGF regulates many genes that drive the adaptive and angiogenic response to hypoxia or inflammation in pathologies such as cancer and soft tissue repair [43], promoting endothelial cell proliferation, migration, and survival, as well as the permeabilization of microvessels leading to localized edema [43].

VEGF is not highly expressed in adult tendon, but its expression is increased in several animal models of acute injury or mechanical loading, including an overuse model [31, 34]. VEGF mRNA levels are elevated in homogenized Achilles tendinosis biopsies [2] and could arise from tendon fibroblasts, endothelial cells, or extrinsic cell populations recruited to the site of the lesion. Further, VEGF mRNA can be detected in ruptured Achilles tendon, but not in normal adult tendon[33]. Some authors have suggested that

tendon rupture is preceded by degenerative changes including evidence of hypoxia [19]. Thus, mechanical overload, injury and inflammation, hypoxic conditions, or some combination of the above could lead to increased expression of VEGF in tendon [34].

The current study represents ongoing work by our group into the histopathology of tendinopathy; prior work with this set of tissue has been previously reported, and includes the findings of increased microvascular density, proliferation of endothelial and smooth muscle cells, and increased perivascular mast cell density in patient biopsies [22, 39, 40]. The purpose of this study was to answer the following questions; is VEGF expression in the patellar tendon more prevalent in men and women with patellar tendinopathy than in individuals with normal, pain-free patellar tendons; which cell populations express VEGF in normal and tendinopathic tendon; and is there a difference in symptom duration between VEGF+ and VEGF- tendons?

METHODS

The present study included biopsies from the patellar tendons of 32 patients (mean age, 30 years; range, 21-42 years), who were divided into two groups. One group consisted of 22 patellar tendinopathy patients (19 men and 3 women; mean age, 30.4 years; range, 22-40 years) with pain at the infrapatellar pole for at least 3 months severe enough to prevent them from participating in activities at the preinjury level. MRI confirmed high signal changes that corresponded to the area of pain (infrapatellar pole). The duration of current symptoms ranged from 5 to 81 months. These patients had been randomized to receive surgical treatment for tendon pain with an open procedure, as part of a clinical trial

comparing the effects of eccentric exercise and surgery [6]. The control group was comprised of 10 patients (seven men and three women; mean age, 28.2 years; range, 20-42 years) with normal patellar tendons confirmed by clinical examination, with no current or prior history of patellar tendon pain. These individuals were being treated with intramedullary nailing for tibial fractures. Exclusion criteria included: (1) age less than 18 years, (2) previous knee surgery, (3) corticosteroid injections in or around the knee, (4) knee trauma requiring medical attention, and (5) rheumatic or degenerative knee conditions. All patients provided written, informed consent. The study was approved by the University and Hospital Research Ethics Committees, and was conducted in accordance with the World Medical Association Declaration of Helsinki. Patient confidentiality was protected according to the U.S. Health Insurance Portability and Accountability Act.

The surgical technique and biopsy handling were identical in the two groups. Biopsies were taken from the proximal bone-tendon junction, and the tendon tissue was excised using a full thickness wedge-shaped incision that was widest at the patellar pole (1 cm) and narrower distally (2-3 cm in length). A suture was passed through the proximal end of the tendon to aid orientation. Immediately after the procedure, biopsies were transferred to Zamboni's fixative where they were stored for 4 to 24 hours, and then washed in 0.1 M phosphate-buffered NaCl, pH 7.2, with 15% sucrose (weight/volume) (PBS) and 0.1% natriumazide. The entire biopsies were then stored in PBS at 4°C for a minimum of 48 hours after which they were embedded in paraffin.

From each biopsy, we stained one 5 μm tissue section for hematoxylin and eosin (H&E) (general morphology) and one for Alcian blue (sulphated glycosaminoglycans) and viewed them at 100× to 630× magnification on a Zeiss Axioplan upright microscope (Carl Zeiss Canada Ltd, Toronto, Canada). The entire longitudinal tissue section was examined during analysis, avoiding areas of adipose or peritendinous tissue.
VEGF expression was examined using a well-characterized mouse monoclonal antibody raised against amino acids 1-140 of VEGF of human origin (Santa Cruz clone sc-7269).

Immunohistochemistry was carried out using an autostainer (DAKO Diagnostics, Glostrup, Denmark). The sections were cleared in xylene (3 × 15 minutes), quenched for 15 minutes in 3% hydrogen peroxide, incubated in protein-free blocking solution (DAKO) for 15 minutes, then exposed to 100 μL of primary antibody diluted 1:50 in 0.1% bovine serum albumin in TBS for 1 hour. A secondary anti-mouse IgG antibody (DAKO) was used, followed by incubation with APAAP (DAKO) for 30 minutes. The signal was visualized with New Fuschin as the substrate (Sigma-Aldritch, Oakville, Canada). Identically fixed and processed tonsil with or without the VEGF antibody was used as positive or negative control, respectively.

As previously reported [22, 39, 40] we used the following mouse monoclonal antibodies to identify cell types potentially expressing VEGF: CD68 (Signet KP1 clone, Cedarlane Laboratories, Hornby, Canada) for macrophages, CD3 (DAKO, clone F7.2.38) for T-lymphocytes, mast cell tryptase (DAKO, clone AA1) for mast cells, CD31 (DAKO, clone JC70A) for endothelial cells, α- smooth muscle actin (Biomeda 1A4) for perivascular

cells and myofibroblasts. For mast cell tryptase, sections were cleared in xylene (3 × 15 minutes), steamed in sodium citrate for 3 × 5 minutes, incubated at 37° with 0.1% trypsin in 0.1% calcium chloride pH 7.3 for 5 minutes, quenched for 15 minutes in 3% hydrogen peroxide, incubated in protein-free blocking solution (DAKO) for 15 minutes, then exposed to 100 µL of primary antibody diluted 1:50 in 0.1% bovine serum albumin in TBS for 1 hour. A secondary anti-mouse IgG antibody (DAKO) was used, followed by incubation with APAAP (DAKO) for 30 minutes. The signal was visualized with New Fuschin as the substrate. CD68 and α-SMA processing was identical to mast cells but without the trypsin pre-treatment. For T-lymphocytes and CD31, the CSA II detection system (DAKO) was used with 3,3'-diamino-benzidine as the chromogen (Vector Laboratories, Burlingame, U.S.A.). Identically fixed and processed tonsil with or without the primary antibody was used as positive or negative control, respectively. In tonsil, mast cells, lymphocytes, macrophages, and endothelial cells were labeled only in the positive controls.

Slides were examined with the patient/control identity code masked with black tape. Using a 40× objective lens, the entire tissue section was scanned and all areas of nonartifactual, cellular VEGF staining were captured at 1392 × 1045 pixels with a digital camera (Retiga Exi 1394, Qimaging Corp, Burnaby, Canada). To be considered non-artefactual, the staining had to be red, intracellular and absent in negative controls. Based on the number of fields with positive staining, each section was considered VEGF-positive or VEGF-negative. To be considered positive, specific cellular staining had to be

present in three or more captured viewing fields. This method ensured that sections with only one or two areas of staining were not considered positive.

We computed means and standard deviations (SD). We compared VEGF presence or absence between normal and tendinosis tissue using the Fisher exact probability test. We compared the mean symptom duration (in months) of tendinopathy patients with and without VEGF expression using an unpaired t-test. All statistical tests were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL).

RESULTS

Patellar tendon from control subjects was normal in all cases but one, which showed mild cell rounding. In tonsil, scattered VEGF positive leukocytes and endothelial cells were seen only in the positive controls (Figure 4.1).

VEGF was more often ($p = 0.03$) observed in tendinopathy samples than normals. VEGF expression could be detected in 8 of 22 patient samples, but was absent in all controls.

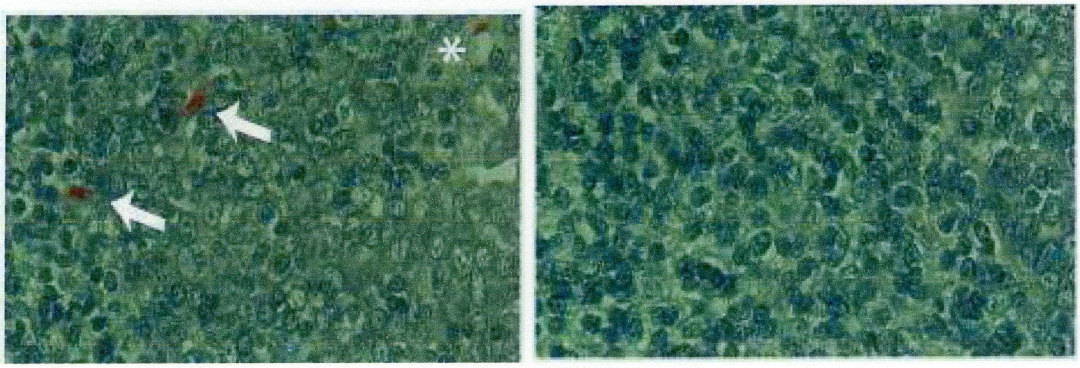

Figure 4.1 VEGF + and – control. Human tonsil was processed as described with (left) or without (right) primary antibody. Left panel – arrows indicate postively labeled cells, asterisk indicates VEGF + capillary-like structure. Right pane – absence of labeling when antibody omitted.

When present, VEGF was clearly localized to endothelial cells, rather than fibroblasts, perivascular cells, or inflammatory cell types (Figure 4.2).

The duration of symptoms in patients demonstrating VEGF expression was greater ($p = 0.015$) than that of patients without VEGF expression (12 ± 7.7 months versus $32.8 \pm$

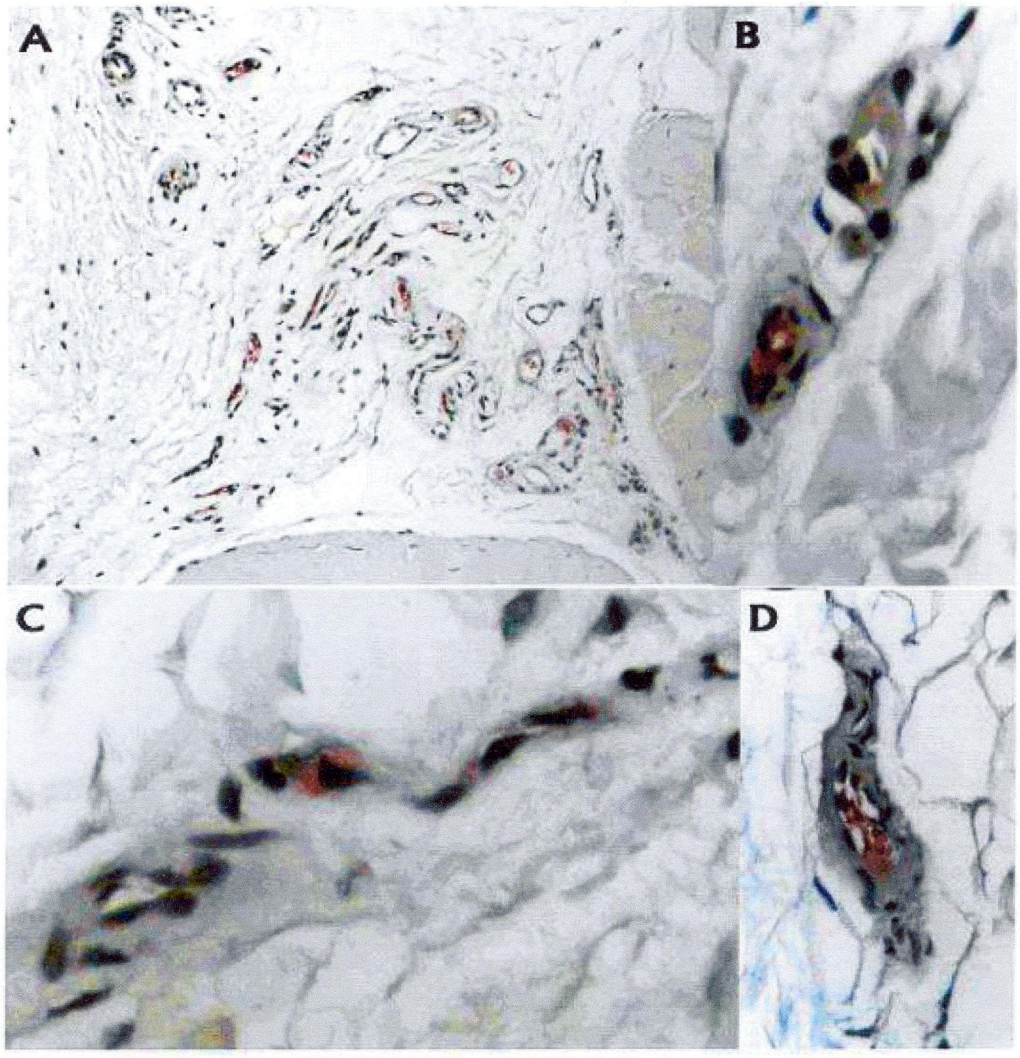

Figure 4.2 VEGF expression in patellar tendinopathy (a) VEGF is expressed in endothelial cells in an area of expanded endotendon (New Fuschin, original magnificatino x 200). (b-d) Higher-power views demonstrate endotendon endothelial cells with cytoplasmic VEGF staining. New Fuschin, original magnification x 600 (b,c) and x 400 (d).

23.5 months, respectively). The symptom duration range was 5 to 18 months for VEGF-positive patients, and 6 to 81 months for VEGF-negative patients (Table 4.1).

Table 4.1 Clinical characterstics of patellar tendinosis patients

Patient #	Age	Gender	VISA score	Symptom duration	Presence of VEGF
1	38	M	27	50	neg
2	32	M	53	5	pos
3	31	M	-	-	neg
4	37	M	41	6	pos
5	23	M	-	-	neg
6	22	M	-	-	neg
7	35	M	38	6	neg
8	37	M	31	24	pos
9	32	M	43	81	neg
10	29	M	-	-	pos
11	34	M	-	-	neg
12	22	M	-	-	neg
13	27	F	15	6	pos
14	40	F	47	30	neg
15	22	M	17	33	neg
16	22	M	20	6	neg
17	38	M	35	13	pos
18	23	M	-	-	pos
19	37	M	4	6	neg
20	32	M	13	18	pos
21	27	F	12	20	neg
22	21	M	50	24	neg

Neg = negative; Pos = postive; - = data unavaiable

DISCUSSION

The aims of this study were to determine whether VEGF expression in the patellar tendon is more prevalent in individuals with patellar tendinopathy compared to those with asymptomatic patellar tendons, to clarify the cell populations responsible for VEGF production in tendon, and to determine if patients with VEGF-positive or VEGF-negative patellar tendons differed in terms of symptom duration. Eight of 22 patellar tendinopathy patients demonstrated VEGF expression compared to 0/10 asymptomatic tendons ($p = 0.03$). VEGF expression was localized to endothelial cells rather than fibroblasts, mast cells or inflammatory cell types. Patients demonstrating VEGF expression in the patellar tendon had shorter symptom duration than those with no VEGF expression. Thus, the results are consistent with the hypothesis that VEGF may contribute to a temporally regulated angiogenic process as part of the injury response in patellar tendinopathy [31, 34].

A limitation of this study is that VEGF expression was examined in a cross-sectional cohort of surgically treated tendinopathy patients with symptom duration greater than 3 months. Studies of the temporal expression of factors regulated by wound healing have generally been conducted using acute injury models [27]. These studies have demonstrated VEGF expression peaks early in the healing process, eg, in the first week after an acute injury [8, 44]. In our study VEGF expression may have reached maximal levels at an earlier time point in the course of tendinosis development. Despite this, the varying degrees of chronicity in the current patient sample revealed a significant distinction between VEGF-positive and VEGF-negative patients in terms of the duration

of symptoms. There was no detectable difference in age, gender distribution or VISA score betweeen patients with VEGF-positive and VEGF-negative tendons.

A further limitation is that we did not measure other factors that may be involved in angiogenesis, including other isoforms of VEGF, as well as a host of other substances with well-known or emerging roles in angiogenesis [36]. VEGF is the most potent angiogenic peptide in humans, and its activity is largely effected by activation of the tyrosine kinase VEGF receptor 2, which stimulates endothelial cell proliferation, survival, migration and tube formation [43].

The occurrence of VEGF expression was apparently influenced by the duration of symptoms. Normal adult tendons demonstrate a relatively low level of vascularity due to their low metabolic rate, as well as their mechanical requirements [34, 41]. Following injury, the metabolic requirements increase and correspondingly tendons undergo an angiogenic response characterized by the expression of VEGF and IGF-I, both of which have been suggested to contribute to the growth of vessels into the healing tendon [15, 33]. One study in an animal model of supraspinatus tendinopathy demonstrated up-regulation of VEGF expression after 8 weeks of overuse [31].

An important finding in the current study is that VEGF expression was absent in patients with the most chronic symptoms, yet these patients still demonstrated angiofibroblastic tendinosis with morphological evidence of increased vascularity. During the later stages of neovascularization and vessel matruation, VEGF expression declines and vessel

survival and stability is promoted by the presence of perivascular and vascular smooth muscle containing cells (reviewed by Goh et. al. [14]). Whether smooth muscle-containing cells play a role in the chronicity and survival of neovessels in tendons will be a key point to examine in future, as many neovessels appear to persist despite the absence of VEGF in the chronic stage. The persistent presence of vessels in tendinopathies has been identified as an important aspect of the "failed healing response" in tendons [28].

In addition to promoting the proliferation, migration and survival of endothelial cells, following an acute injury VEGF contributes to the increased permeability of immature vessels by influencing cell-cell contacts in the vessel walls, thus contributing to soft tissue edema [1, 43]. With regard to the role of VEGF in modulating vascular function during overuse injuries, the events are less clear. A recently proposed theoretical model suggests chronic tendon loading leads to mechanical trauma of the tissue with ensuing microruptures of the tendon microvasculature [34]. These microvascular ruptures initiate a VEGF-mediated vascular remodeling and edema which, over time, can become pathological [34]. According to this conceptual model, VEGF expression is nearly completely repressed in healthy adult tendon due to the presence of endogenous inhibitors, but is reexpressed following mechanical overload. Our data are in accordance with such a model, as we detected VEGF expression within the endothelial cell layer only in tendinopathy patients and always in association with other well-known features of angiofibroblastic tendinosis. However, the actual consequences of increased VEGF expression on endothelial function in tendinopathic tendon remain to be determined.

VEGF expression is a recognized element of the inflammatory response to soft tissue injury, being acutely induced in endothelial cells in the wound environment by hypoxia or by other cytokines and growth factors [29]. Alternatively, it has been proposed VEGF expression can be induced directly in tenocytes by mechanical loading via mechanotransduction pathways [32]. In support of the possible relevance of this alternate mechanism of VEGF up-regulation in tendinopathy, the VEGF gene, along with its upstream transcriptional regulator HIF-1α, is expressed in cultured rat tendon cells in response to cyclic stretch at high (1 Hz) but not low (0.5 Hz) frequencies [32]. However, our data do not offer support for the hypothesis of tenocyte-derived VEGF expression, as VEGF expression was absent in tenocytes in both controls and patients.

Tendinosis lesions are characterized by increased numbers of fibroblasts and vascular cells (endothelial and smooth muscle cells) and abnormal extracellular matrix [9, 21, 47]. An increased prominence of tenocytes demonstrating that PCNA has been reported in patellar and supraspinatus tendinopathies, demonstrating proliferation of intrinsic cellularity may be an ongoing phenomenon in chronic injuries [26, 37]. VEGF, along with many other peptides, promotes the proliferation of fibroblasts and endothelial cells [16]. VEGF treatment of tendon autografts led to an increase in the number of endothelial cells, and increased numbers of fibroblastic cells [45]. Therefore, it is possible VEGF contributes in some part to the hypercellularity that characterizes tendinosis lesions. It is likely any proliferative effect of VEGF would occur in concert with other growth factors and cytokines present in the lesion [27].

CONCLUSION

In conclusion, our data are consistent with the concepts of Kraushaar and Nirschl [21], who postulated the majority of tendinopathies in athletes are due to angiofibroblastic tendinosis, rather than tendonitis. The current study provides evidence that a potent angiogenic peptide, VEGF, may contribute to the vascular hyperplasia which is a cardinal feature of chronic tendinosis.

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CHAPTER 5

VGluT2 Expression In Painful Achilles And Patellar Tendinosis: Evidence Of Local Glutamate Release By Tenocytes*

INTRODUCTION

Glutamate is a pervasive amino acid with a well known role in excitatory synaptic transmission in the central nervous system (CNS), where it is considered responsible for transmitting most fast synaptic potentials [12, 36]. The transmission of cellular signals among neurons by glutamate requires a number of genes including dedicated glutamate receptors, receptor interacting proteins, plasma membrane transporters, and vesicular transporters [32]. There are two main classes of glutamate receptors, including metabotropic receptors (signaling via IP_3, diacylglycerol, and cyclic AMP) and ionotropic receptors (capable of altering permeability to specific cations). Ionotropic receptors are further classed as N-methyl-D-aspartate (NMDA) receptors, DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA) receptors, and kainate (KA) receptors [32]. In addition to receptors, glutamate transporters in the plasma membrane (GLAST, GLT-1, EAAC1, EAAT4, -5) or in vesicles (VGluT1-3) play key roles in the regulation of extracellular glutamate concentrations [18].

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* A version of this chapter is in press: Scott A, Alfredson H, Forsgren S. VGluT2 expression in painful Achilles and patellar tendinosis: evidence of local glutamate release by tenocytes. Journal of Orthopaedic Research 2008, 26(5):685-92 Reprtinted with permission of Wiley-Liss, Inc, a subsidiary of John Wiley & Sons, Inc.

It has become increasingly evident that genes encoding elements of the glutamate signaling machinery are also expressed by non-neuronal cells in a variety of peripheral tissues, and that glutamate functions as a regulatory cytokine with autocrine and paracrine functions in diverse physiologic processes such as bone turnover, insulin and growth hormone secretion, platelet formation and function, and keratinocyte development and differentiation [21, 36, 43]. In addition to diverse roles in physiologic cell signaling, glutamate also plays known or emerging roles in a range of pathologies including inflammation and soft tissue repair [9, 13, 17, 20, 25]. During acute inflammation of the knee, glutamate is released into the knee joint from peripheral glutamatergic nerves, resulting in a nitric oxide-dependent increase in local blood flow [25]. Furthermore, glutamate receptor antagonism can reduce nociception and sensitization in response to a variety of inflammatory stimuli [39].

Painful overuse tendon injuries are a common medical problem seen by general and sports medicine practitioners, with a cumulative incidence of 52% in middle or long-distance runners (Achilles tendinopathy) and a prevalence of 32% to 44% with current symptoms in elite jumping athletes (patellar tendinopathy) [24, 27]. The pathology considered to be responsible for many cases of chronic tendon overuse pain is often classified as "tendinosis." Tendinosis is characterized morphologically by collagen fragmentation and disarray, tenocyte abnormalities (including apoptosis and necrosis, increased proliferation and excessive glycosoaminoglycan production) and by an increase in local intra- and peritendinous blood flow [11, 42]. In this condition, high levels of glutamate have been measured from within tendinosis lesions using microdialysis in

patients with Achilles, patellar and extensor carpi radialis brevis tendinosis [2-4]. The observations thus suggest that glutamate may contribute to the symptoms of chronic tendon pain [2-4]. Glutamate has also recently been suggested by Murrell and coworkers to be present within tendinosis lesions at levels sufficiently high to induce tenocyte apoptosis [33], a pathological feature of some advanced tendinosis lesions [26, 50].

Beyond the high levels of glutamate detected by microdialysis in painful tendons, nothing is known regarding the glutamate signaling machinery which is present in normal or pathological human tendons, although we previously reported that NMDA receptors are present in nerve fascicles in the human Achilles and patellar tendons [1, 2]. More recent studies conducted on rat tendons have documented the presence of mRNA in tenocytes for several key elements of glutamate signaling machinery, including receptors (metabotropic glutamate receptors mGluR5 and mGluR6), glutamate receptor-interacting proteins (GRIP1 and GRIP2) and plasma membrane transporters (EAAT4)[33, 34]. To our knowledge, there is no information on elements of the signal transduction pathway required for vesicular release of glutamate in human tendons, namely the presence of vesicular glutamate transporters, either at the protein or mRNA levels. This lack of knowledge is a drawback when considering the fact that high extracellular glutamate levels occur in tendinosis. In contrast to plasma membrane transporters which play a role in signal termination by internalization of glutamate, VGluTs are required for the transport of glutamate into secretory vesicles, and may therefore be considered as indirect markers of potential sources of free glutamate. VGluT1 and 2 are widely expressed in the central nervous system and considered definitive markers for excitatory glutamatergic

synapses, whereas VGluT3 has a more restricted pattern of central nervous system expression [31].

Therefore, the purpose of the current study was to determine whether VGluT1 or -2 are present in human tendons. Further, our purpose was to determine whether their expression levels or pattern of distribution would be influenced by tendinosis pathology. We hypothesized that at least one or more of the two main VGluT isoforms examined would be present to a greater degree in tendinosis, due to the higher levels of extracellular glutamate associated with this condition.

METHODS

Patients and tendon sampling

The current study included samples from 29 individuals and 30 patellar or Achilles tendons. There were 14 tendinosis patients (1 patellar, 13 Achilles) and 15 subjects with no history of current or past tendon pain (8 patellar and 7 Achilles). There were 19 men and 10 women, average age 40.7 years (range 18 – 54), with no significant difference in age between patient and control groups. Both Achilles and patellar tendons were included, as it has been reported that there is no significant difference in tendon pathology between the two sites, i.e. the morphology seen in patellar tendinosis closely resembles that seen in Achilles tendinosis [28]. All tendinosis patients displayed tenderness and pain during loading, and exhibited tendon changes verified by ultrasonography (localized widening of the tendon, irregular structure, and focal

hypoechoic areas) or MRI (increased signal intensity and localized widening). All subjects were otherwise healthy and on no medications.

In the case of tendinosis patients, samples (2-3 cubic millimeters in size) were obtained from the area of the tendon corresponding to structural changes observed on ultrasound or during surgery. Samples were transported on ice in sterile conditions to the laboratory. Some samples were fixed for 24-48 hours in 4% formaldehyde buffered in 0.1 M phosphate buffer at 4° C, followed by embedding in OCT (Miles Laboratories, Naperville, IL, USA) and freezing. Other samples were directly snap frozen in isopentane chilled in liquid nitrogen. All samples were then stored at -80°.

The study protocol was approved by the Committee of Ethics at the Faculty of Medicine and Odontology, Umeå University, and the Regional Ethical Review Board in Umeå, and the experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Morphological analysis

The presence of any significant artifacts was examined in longitudinal haematoxylin-eosin sections from normal and painful tendons using previously published morphological features of tendinosis [8]. All samples included were free from significant artifact, and the tendinosis samples demonstrated, to varying degrees, typical features of the pathology including tenocyte hypercellularity, pyknotic or necrotic nuclei, tenocyte rounding, vascular hyperplasia, and collagen fibre disarray.

Immunofluorescence for VGluT1 and -2

Immunohistochemical staining was carried out on biopsies from all patients using goat polyclonal antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), namely sc26026 and sc46569 for VGluT1 and -2 respectively. Control staining included omission of the primary antibody, replacement of primary antibody with normal goat serum, or pre-absorption of the antibody with the corresponding blocking peptide at 150-200 μg/ml (sc26026P for VGluT1 and sc46569P for VGluT2). Both fixed and unfixed samples were included, as fixation appeared to have a minimal influence on the strength or distribution of observed reactions for VGluT1 or 2. Specimens of fixed and unfixed human colitis tissue were used as a reference tissue [45]. After air drying for 30 minutes at room temperature, 7μm thick crysections were treated with acid potassium permanganate to reduce fixation-induced autofluorescence (in fixed samples), then washed in PBS and placed in 1% Triton in PBS for 20 minutes, rinsed in PBS and blocked for 15 minutes with normal 5% Donkey serum. Primary antibodies were applied overnight at 4° at 1:100 dilution, rinsed in PBS, then incubated with the secondary antibody (1:100 donkey anti-goat conjugated to flourescein isothiocyanate (FITC), Jackson ImmunoResearch Europe, Newmarket, UK) for 30 minutes in the dark. Sections were washed and coverslipped with Vectashield hardset mounting medium (Vector Laboratories, Burlingame, CA, USA) and viewed with a fluorescence microscope (Axioskop2, Carl Zeiss, Stockholm, Sweden) to determine the location and intensity of specific reactions. All sections, including those with and without peptide block, were viewed independently and then together by two observers (AS and SF) to achieve consensus regarding the extent and specificity of reactions. Parallel sections of some

biopsies were stained identically on separate occasions to ensure repeatability of the procedure, and each round of staining included both normal and tendinosis tendons to ensure validity of comparisons.

In Situ Hybridization for VGluT2

In situ hybridization was carried out on a subset of tendon samples (n=3) to examine whether the mRNA would be localized in a similar or different pattern to the protein. A digoxigenin (DIG)-hyperlabeled oligonucleotide probe (ssDNA) for detection of human VGluT2 mRNA was used on sections from frozen tendinosis specimens. The probe sequence (CCTTG TACAA ATTCC TCTTT CTTTT CCCAA CCACT AGGCC AACCT CCA) was complementary to nucleotides 2066 - 2133 located within the coding sequence of human VGluT2 (GeneDetect, Auckland, New Zealand). In situ hybridization was performed according to an established protocol [38], using an alkaline phosphatase (AP)-labeled anti-digoxinin antibody (GeneDetect, Auckland, New Zealand) for detection,. Series of 10 mm thick cryosections were air-dried at room temperature (RT) for 30 min, then fixed in sterile 4 % paraformaldehyde in 0.1 M PBS for 60 min at RT. The slides were then washed with 2x saline sodium citrate (SSC) for 2x10 min. The sections were thereafter incubated in 0.2 M HCl for 8 min at room temperature to inhibit endogenous alkaline phosphatase activity. After this, the sections were acetylated by incubation of slides for 15 min at RT in a mixture of 195 ml DEPC- H_2O, 2.7 ml tiethanolamide, 0.355 ml HCl, and 0.5 ml acetic anhydride. Slides were then again rinsed in 2xSSC. After that, 25-50 ng of the single strand DNA probe was put in 15 ml of hybridization solution in a 1.5 ml Eppendorf tube, denaturated for 5 min in 80°C and then

put on ice. The probe-containing hybridization solution (500 ml formamide, 200 ml 20xSSC, 50 ml of 20x Denhardt's solution, 50 ml herring sperm DNA (10 mg/ml) heat-denatured, 25 ml bakers yeast RNA (10 mg/ml), 175 ml dextran sulfate (50%)) was then applied to each section and incubated at 56°C overnight. The slides were then washed for 2x10 min at RT in 2xSSC and for 5 min at room temperature in STE-buffer (STE-buffer: 500 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA) And incubated in 100 ml RNase A (40 mg/ml in STE) for 30 min at 37°C. After this, the slides were washed for 20 min at 56°C in 2xSSC, 50% formamide (25 mL 100% and 25 ml 2xSSC buffer), then placed for 2x5 min at RT in 1xSSC, and for 2x5 min at room temperature in 0.5xSSC. Then the slides were washed for 5 min in buffer 1 (100 mM Tris-HCl [pH 7.5] + 150 mM NaCl) and incubated in buffer 1 containing 4% normal horse serum for 60 min at RT in a humid chamber. Sections were then incubated in 100 mL of the AP-labeled anti-DIG antibody (diluted 1:500 in buffer 1 with 4 % normal horse serum) for 60 min at room temperature in a humid chamber. The slides were then washed for 2x10 min in buffer 1, and for 2x5 min in buffer 2 (100 mM Tris-HCl [pH 9.5] + 100 mM NaCl + 50 mM MgCl2). After this, the enzyme (AP) substrate solution (20 ml NBT/BCIP in 1 ml buffer 2 with 10 ml levamisole) was sterile filtered (22 μm) and added to the sections, and slides were incubated upside down in the dark at 4°C overnight. Color reaction was stopped by placing the slides in buffer 3 (10 mM Tris-HCl [pH 8.0] + 1 mM EDTA). Slides were then dehydrated and counter-stained in 0.5% methyl green and mounted in Pertex microscopy mounting medium. The corresponding sense digoxinin-hyperlabeled single strand DNA probes were used as negative controls. As positive control, a β-actin probe (GD5000-OP) was used (GeneDetect, Auckland, New Zealand).

Statistical analysis

The extent of immunofluorescence was graded semi-quantitatively on blinded sections from 0 (absent expression) to 3 (intense and widespread expression) concerning specific tenocyte immunofluorescence, and a Mann-Whitney U test was conducted on the resultant data (n=30) with statistical significance predetermined at p=0.05.

RESULTS

Immunofluorescence

Specific VGluT1 immunofluorescence could not be observed in either tendon or colon. By contrast, VGluT2 immunofluorescence was observed in both tendon and colon. VGluT2 immunoreactions were not present in tissue exposed to the preabsorbed antibody processed in parallel at the same concentration (Figure 5.1). In tendon, VGluT2 expression was observed in tenocytes and not in other structures (nerves or blood vessels). In colon specimens, VGluT2 expression was detected in the myenteric plexus, as previously described [45].

VGluT2 immunoreaction was detected in 38% of normal tendons and in 71% of painful tendons (Table 5.1). Accordingly, semiquantitative grading revealed a significantly greater expression of VGluT2 in tenocytes from tendinosis patients than in those of controls (p=0.005, Figure 5.2). The expression of VGluT2 in tendinosis tendons was thus both more widespread (i.e. large numbers of tendons showed were immunoreactiv tenocytes) and more intense than in normal tendons (Figure 5.2, 5.3). There was no

Table 5.1 Summary of patient characteristics and VgluT2 immunoreactions

Patient	Tendon	Group	Fixation	Age	Gender	VGluT2 rxn
1	Patellar	Tendinosis	N	32	m	3
2	Achilles	Tendinosis	Y	43	m	0
3	Achilles	Tendinosis	N	51	m	1
4	Achilles	Tendinosis	Y	39	m	2
5	Achilles	Tendinosis	Y	54	m	2
6	Achilles	Tendinosis	Y	54	f	1
7	Achilles	Tendinosis	Y	50	f	0
8	Achilles	Tendinosis	N	46	m	3
			Y			3
8	Achilles	Healthy	N	46		0
9	Achilles	Tendinosis	N	48	m	0
10	Achilles	Tendinosis	N	51	f	3
11	Achilles	Tendinosis	Y	47	f	3
12	Achilles	Tendinosis	N	18	f	3
13	Achilles	Tendinosis	N	52	f	0
14	Achilles	Tendinosis	Y	37	m	2
15	Patellar	Healthy	Y	20	f	2
16	Patellar	Healthy	N	30	m	0
17	Patellar	Healthy	N	28	f	0
18	Patellar	Healthy	N	28	m	0
19	Patellar	Healthy	Y	28	m	0
20	Patellar	Healthy	Y	25	m	0
21	Patellar	Healthy	N	47	m	0
22	Patellar	Healthy	Y	45	m	0
23	Achilles	Healthy	Y	39	m	1
24	Achilles	Healthy	N	47	m	0
25	Achilles	Healthy	Y	47	m	1
26	Achilles	Healthy	N	44	f	1
27	Achilles	Healthy	N	47	f	1
28	Achilles	Healthy	N	36	m	3
29	Achilles	Healthy	Y	41	m	0

apparent difference in the extent of expression among men or women, nor any apparent relationship with age.

VGluT2 immunoreactivitity in tenocytes was often seen in a punctuate cytoplasmic pattern (Figure 5.1). Reactivity was most intense in abnormally appearing tenocytes, including those showing rounded (as opposed to spindle shaped) or crimped (i.e. wavy)

appearances (Figure 5.4). VGluT2 immunoreactivity in control tendons showed a similar pattern, but was often less intense and less widespread (Figure 5.4).

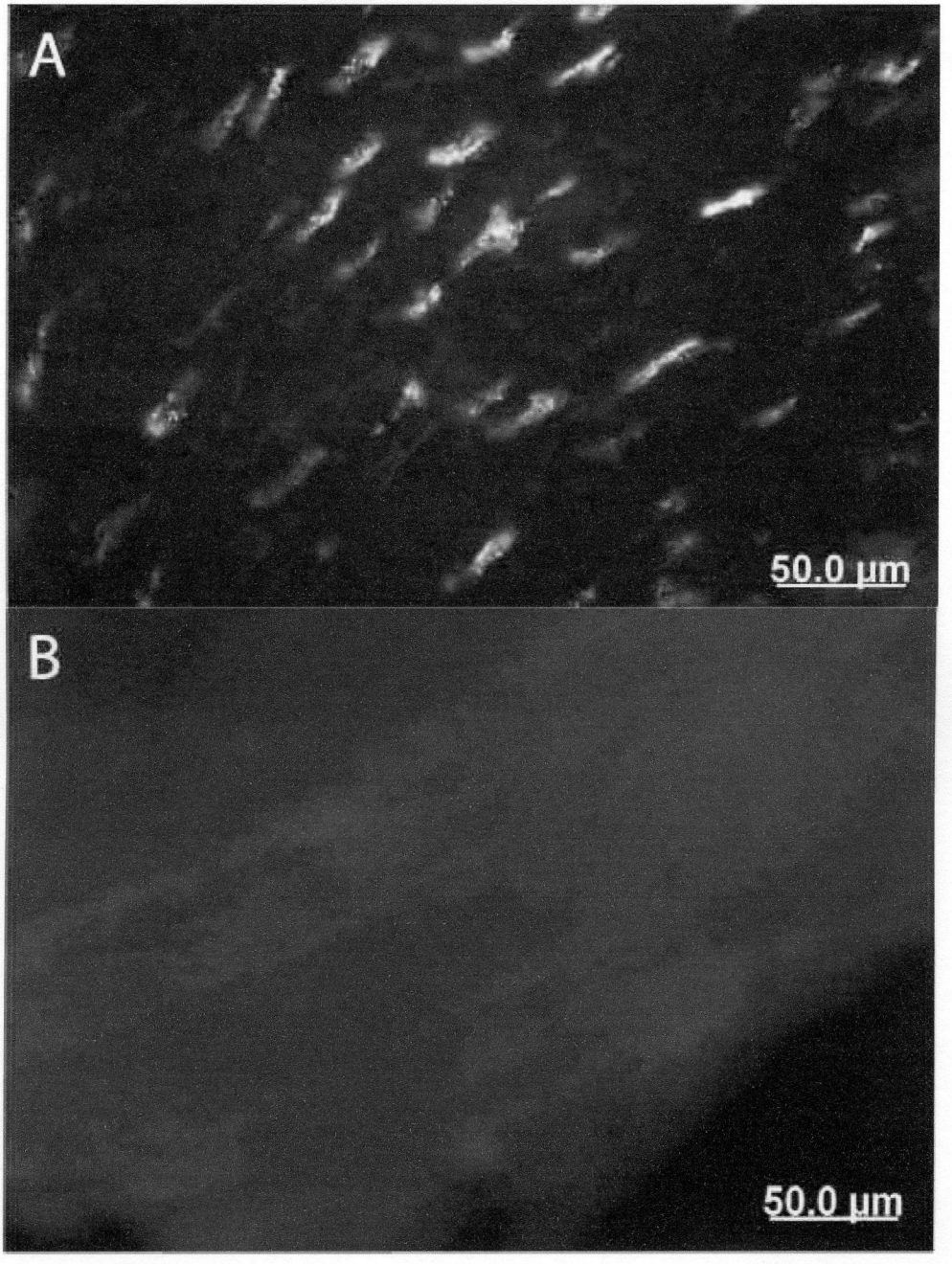

Figure 5.1 VGluT2 immunofluorescence in tendinosis tendon. (A) Numerous tenocytes demonstrating positive immunoreactions (original magnification, x400). (B) Adjacent section demonstrating no reactions with preabsorbed antibody.

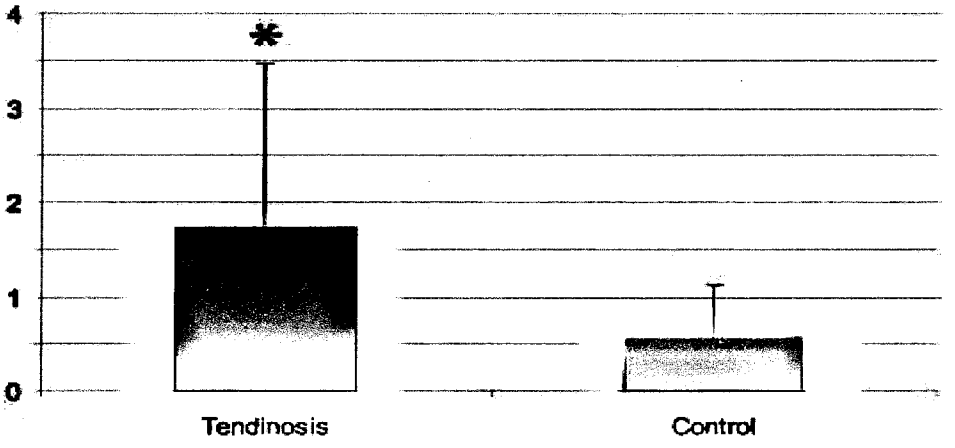

Figure 5. 2. Results of VGluT2 immunofluorescence in tenocytes using semiquantitative grading. * significantly different, p=0.005 (Mann-Whitney U). Error bars represent standard deviation. 0= no immunoreaction, 3= intense and widespread immunoreaction.

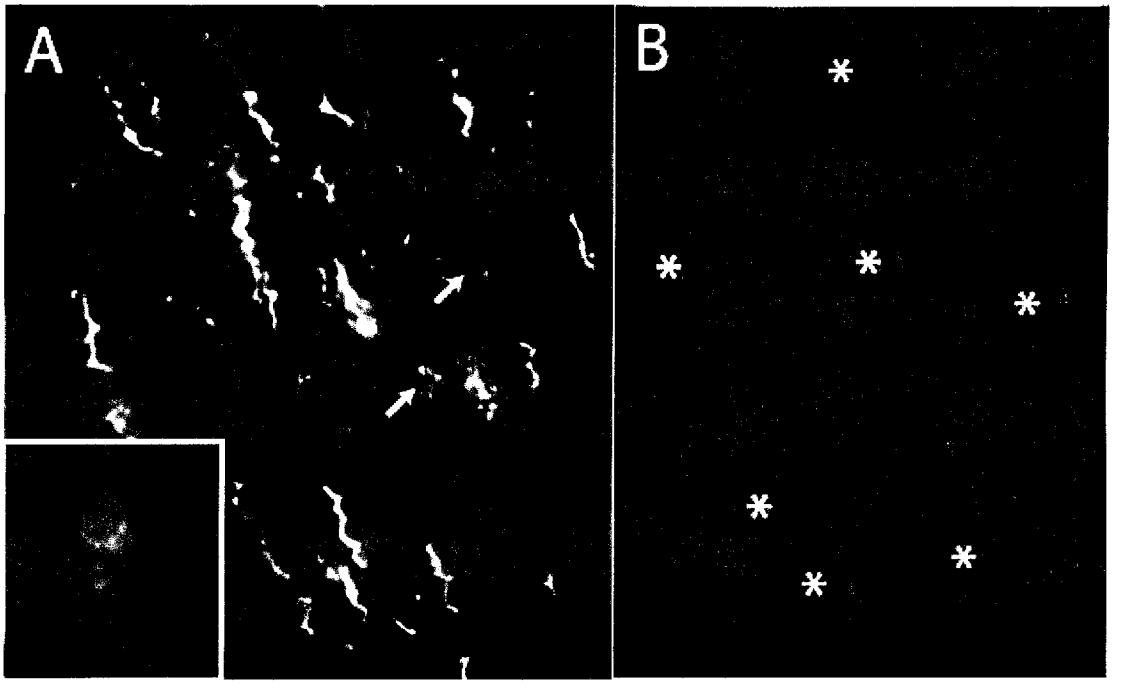

Figure 5.3. Marked VGluT2 immunofluorescence in abnormal tenocytes. (a) Many tenocytes with an exaggerated crimp (waviness) are present in regions of tendinosis samples. Rounded tenocytes (arrows) are also present in the same field. Inset: Higher power view of a rounded tenocyte, demonstrating evident cytoplasmic localization of VGluT2. (b) Tenocytes in healthy tendon, demonstrating faint punctuate reactions (asterisks).

VGluT2 mRNA in tenocytes

VGluT2 mRNA expression was observed in the majority of tenocytes in both normal and abnormal regions of tendon, suggesting a more sensitive detection of VGluT2 mRNA compared to VGluT2 immunofluorescence (Figure 5.4). Nevertheless, the expression levels were somewhat variable within and between specimens, being most marked in tenocytes with abnormal appearance (rounded, broadened or crimped). Expression was not seen in any nerve or blood vessel structures.

Parallel in situ hybridization with the sense (control) probe verified that the observed AP end-reaction product resulted from the specific hybridization of the DNA probe to its complementary RNA sequence rather than due to a non-specific interaction (Figure 5.4B, inset). The β-actin probe labeled both tenocytes and vascular cells.

DISCUSSION

The current study documents the presence of vesicular glutamate transporters (VGluT2) in a novel cell type – the tendon fibroblast (tenocyte). This is completely new information for human tendons. However, along with other elements of glutamate signaling machinery which have recently been reported in rat tenocytes (glutamate receptors, glutamate receptor interacting proteins, and plasma membrane transporters) [33, 34], the current report concerning VGluT2 mRNA and protein supports a hypothesis that a peripheral glutamatergic system may be present in tendons. Moreover, the expression of VGluT2 at the protein level was most prominent in patients with chronic tendon pain ($p=0.005$), which strengthens previous suggestions that glutamate may be involved in the pathology of tendinosis [1, 2].

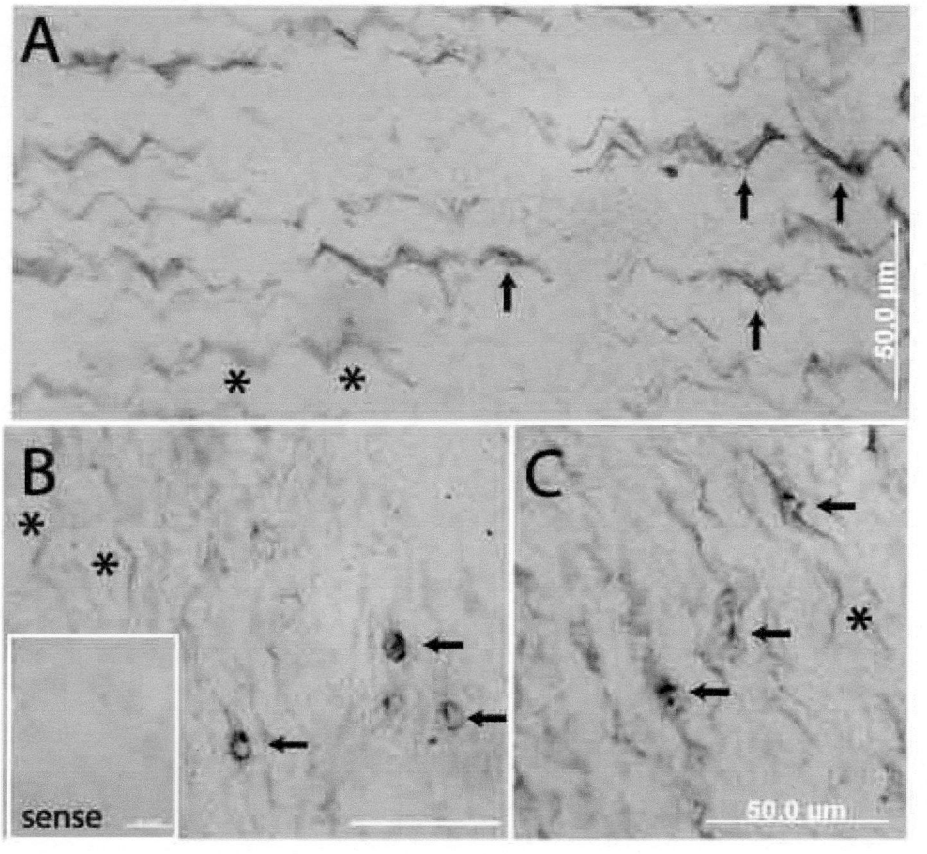

Figure 5.4. VGluT2 in situ hybridization. (a) VGluT2 expression shows variability, with some tenocytes showing marked expression (arrows) and others only faint or absent (asterisks). (b,c) Abnormal (rounded or broadened) tenocytes demonstrating prominent expression of VGluT2, with closely adjacent tenocytes demonstrating minimal or no expression (asterisks). Inset – sense control in a section adjacent to (b) demonstrates only background reactions.

Non-neuronal expression of a glutamatergic marker in tendons

In the central nervous system, VGluT1 and 2 are expressed specifically at excitatory, glutamatergic synapses and play key roles in synaptogenesis as well as in memory and learning [12, 32]. In the peripheral nervous system, the expression of vesicular glutamate transporters has been documented at a variety of locations including nerve ganglia in the colonic mucosa, the extrinsic and intrinsic innervation of the rat esophagus, in nerve

terminals contacting neuroepithelial bodies in the lung, and in free nerve endings in the palatine mucosa [5, 10, 45]. Expression has also recently been revealed in mechanosensitive nerve-associated structures such as Merkel cells and muscle spindles, implying that glutamate signaling in these locations may play a role in modulating touch sensitivity and muscle tone [14, 49]. It is therefore of interest to note that in the present study, VGluT1 or 2 expression was not observed in nerve elements. During inflammation of joint structures (i.e. the rat knee synovium), glutamate has been suggested to be locally released by glutamatergic nerves, as the increase in glutamate could be blocked by blockade of peripheral nerves with lidocaine or dorsal rhizotomy [25]. Conversely, the current study suggests that free glutamate within painful tendons probably derives from locally located cells (the tenocytes), particularly in tendinosis tendons.

Evidence for peripheral glutamatergic cell signaling

VGluTs have been proposed as molecular markers that may define the existence of a peripheral, locally acting glutamatergic signaling system with autocrine and paracrine functions. In the pineal gland, VGluT1 and 2 are expressed in synaptic-like-microvessels, and functional glutamate signaling (via metabotropic receptors) was shown to be involved in an important pineal gland function, the regulation of melatonin synthesis [36]. VGluT1 and 2 are also expressed in glucagon-containing secretory granules in α cells in the Islets of Langherhans, where neighbouring β cells express functional AMPA-type receptors [15]. Even more established is a physiologic role of glutamate signaling in the regulation of bone turnover, a process regulated in part by

mechanotransduction (the cellular response to mechanical force). In bone, VGluT1 is expressed by mature osteoclasts and is directly responsible for their ability to release glutamate via transcytotic vesicles [35]. Furthermore, VGluT1 knock-out mice develop an osteoporotic phenotype, strengthening the suggestion that glutamate plays a key role in suppressing bone resorption [35]. Another bone cell type, osteoblasts, do not express VGluTs [35] but demonstrate all classes of glutamate receptors as well as plasma membrane transporters (reviewed in [7]). The expression levels of several ionotropic and metabotropic glutamate receptors on osteoblasts was shown to be reduced by mechanical loading of bone segments *in vitro* [44]. Likewise, in osteocytes and osteoblasts, GLAST expression appears to be reduced in areas of load-induced bone formation *in vivo* [30]. Thus, paracrine glutamate signaling amongst osteoclasts, osteoblasts and osteocytes is heavily implicated in the process of mechanotransduction in bone. Preliminary evidence also points to a requirement for glutamate signaling in chondrocyte mechanotransduction [40, 48].

Potential role of glutamate in tendon mechanotransduction

Like bone and cartilage, tendon is a tissue in which the content and structure of the extracellular matrix is dynamically regulated in response to altered loading conditions. Mechanotransduction in tendon is relatively understudied, however. Tenocytes form elaborate gap-junction-linked arrays and, in response to mechanical load, release a variety of cytokines which are thought to regulate the activation of calcium-dependent signal pathways leading to proliferation and increased turnover and production of the collagen-proteoglycan extracellular matrix [46]. Like chondrocytes and osteoblasts,

human tenocytes express both voltage gated calcium channels as well as the mechanosensitive tandem pore domain potassium channel TREK-1 [29]. Considering that the hyperpolarization response to mechanical stimulation in chondrocytes requires NMDA receptor activation [40], the current study supports the hypothesis that tendons may exhibit a similar dependence on glutamate signaling for certain mechanotransduction signaling events. Thus, the regulation of extracellular glutamate concentrations by tenocytes via VGluT2 and via plasma membrane transporters requires further study.

Role of glutamate in tendinopathy?

Current evidence from physiologic studies in humans and animals suggests that activation of AMPA and NMDA receptors can result in peripheral nociception [9, 13, 22, 39]. We have previously shown that peripheral nerves in human tendons demonstrate the existence of NMDA receptors, which have also been observed in the nerve fibres of skin [6]. A suggestion which is more directly supported by the current observations is that glutamate could modulate tenocyte behaviour within the tendinosis lesion. Glutamate is a well known toxic stimulus when present in excess, leading both to necrosis or apoptosis (or both) in a variety of cell types including neurons, fibroblasts, and chondrocytes [33, 37, 47]. Murrell and coworkers have demonstrated that primary tenocyte cell cultures undergo a significant increase in the rate of cell death when exposed to 500μM glutamate for 24 hours. A strength in the study by Murrell is that the glutamate concentration to which tenocytes were exposed is on the same scale as has been measured in tendinpathy patients with microdialysis (250 μM) [4, 33]. Advanced tendinosis lesions (ruptured supraspinatus tendon or long-standing severe jumper's knee) also demonstrate increased

numbers of apoptotic and necrotic tenocytes [26, 42, 50]. Therefore, increased glutamate levels may contribute to cell death and subsequent local tissue degeneration in tendinopathy. Large numbers of apoptotic tenocytes have been reported in equine tendinosis lesions, but relatively few in the standard rat laboratory model, making it a challenge to study potential roles of glutamate and its relation to apoptosis in tendon overuse injury [19, 41]. Glutamate toxicity and cell death are also thought to play a role in disorders such as Alzheimer's disease [16].

Although tenocyte death occurs in some tendinosis lesions, chronically painful tendons are in general hypercellular mainly due to increased numbers of tenocytes, with a variety of other reparative cell types also found [23]. Thus, another highly relevant feature of tendinosis pathology which glutamate could influence is cellular proliferation. Glutamate NMDA and AMPA antagonists inhibit cellular proliferation in a variety of studied cell cultures, including human colon adenocarcinoma, astrocytoma, breast and lung carcinoma, and neuroblastoma [26]. Furthermore, the addition of 500μM glutamate to synovial fibroblasts resulted in a doubling of BrdU incorporation (indicative of increased cellular proliferation) [17]. Interestingly, this stimulation of fibroblast proliferation by glutamate only occurred in fibroblast cultures that had been derived from arthritic animals (collagen-induced rat arthritis model), suggesting that the influence of glutamate on cell behavior involves as-yet unspecified interactions with other aspects of the pathology. Nonetheless, elevated glutamate levels in tendinosis lesions could potentially contribute to the simultaneous presence of increased apoptosis, necrosis, and proliferation which is observed in tendinosis [42]. Glutamate may also modulate pro-apoptotic or

proliferative effects of other conditions thought to be present in tendon such as hypoxia, oxidative stress, cytokines or growth factors.

CONCLUSION

In conclusion, the current study demonstrates the existence of VGluT2 in human tendon. The expression of VGluT2 protein was upregulated in association with tendinosis, suggesting that locally derived glutamate may play roles in the pathology or in an attempted (i.e. failed) repair response. Local regulation of glutamate levels may influence key tenocyte functions modulated in response to injury and/or mechanical loading, including proliferation, apoptosis and extracellular matrix metabolism. Further studies of the role of glutamate signaling in tendon may shed light on the processes of mechanotransduction and overuse injury.

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CHAPTER 6

Tenocyte Responses to Mechanical Loading In Vivo: A Role for Local IGF-I Signaling in Early Tendinosis*

INTRODUCTION

Tendinosis (formerly known as tendinitis) is a common problem among athletes and workers [16, 30] and constitutes a high proportion of referrals to rheumatologists [38]. Tendinosis can be disabling and frequently results in lost productivity, reduced physical activity and early retirement from sport or labour [13, 47, 51].

Despite the prevalence and recalcitrant nature of tendinosis, its pathogenesis remains poorly understood, as few studies have examined its earliest development [20]. Biopsies obtained at end stage disease – from patients undergoing surgery for longstanding tendon pain – typically reveal variable tenocyte density, increased hyaluronan and chondroitin sulfate content, increased collagen turnover with decreased type I collagen, and neurovascular proliferation [26, 36, 40, 41]. Other commonly observed pathological changes include adipose, fibrocartilagenous, and bony metaplasia [10, 28, 33, 39]. Cross-sectional data in asymptomatic tendons suggests that tenocyte rounding, proliferation and

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increased glycosaminoglycan production may precede collagen tearing and neurovascular ingrowth [14].

An appropriate animal model of early tendon injury is needed to better understand the pathogenesis of the disease. To date, tendon pathology has generally been induced via acute injuries such as laceration, crush, collagenase, or injection of inflammatory substances [17, 19, 27, 50]. Although these studies provide important data, they may have limited clinical generalizability as overuse tendon injury could involve quite different mechanisms from acute injury [4]. Soslowsky et al. developed a rat model of overuse tendinosis in the supraspinatus tendon [48, 49]. They reported histological and biomechanical deficits after 12 weeks of downhill running. The supraspinatus tendon was significantly thickened, and demonstrated regions of hypercellularity and collagen disarray, and reduced modulus and ultimate tensile stress [49]. This model may be useful in studying early mechanisms of overuse tendinosis. In particular, it has recently been suggested that overuse may lead to apoptosis of tenocytes, predisposing to development of tendinosis and eventual rupture [57], but the hypothesis has not been tested *in vivo*.

Apoptosis, or programmed cell death, can be modulated by many cytokines and growth factors; in cultured tenocytes IGF-I exerts a potent proliferative and pro-survival effect [45]. IGF-I is also considered to be responsible in part for the proliferative response of cultured tenocytes to *in vitro* loading [52] and the stimulation of collagen synthesis in response to *in vivo* loading [35]. IGF-I may further be involved in regulating chondrogenesis, a process involved in fibrocartilagenous metaplasia in tendons [32].

IGF-I could therefore play either adaptive or pathological roles in the response of tenocytes to increased or altered loading. IGF-I signals mainly through the IGF-I receptor, a membrane-anchored tyrosine kinase whose downstream activation of the ERK-1/2 signal cascade is largely mediated by recruitment and phosphorylation of an adaptor protein, IRS-1 [53].

Therefore, we aimed to assess the extent of tenocyte proliferation and apoptosis, as well as local IGF-I expression and signaling, in an overuse tendinosis model. We hypothesized that early tendinosis would be associated with increased tenocyte proliferation and apoptosis, and with autocrine IGF-I signaling events (IRS-1 and ERK 1/2 phosphorylation).

METHODS

Thirty-two male Sprague-Dawley rats were randomly divided into controls (standard cage care) and runners (standard cage care plus treadmill protocol).

Treadmill running

Runners were subjected to a published treadmill protocol [49]. Two standard human treadmills were fitted with custom lane dividers and adjusted to an 11 degree downhill grade. Rats were acclimatized to the treadmills by gradually increasing their exposure over a 2 week period. Rats that could not consistently run within the lane during the 2 week acclimatization were removed from the study. Following the training and selection period, rats ran for 1 hour per day at 1km/hr. From weeks 8 to 16, a five minute rest

break was inserted half way through the run. At each time point (4, 8, 12 and 16 weeks), 5 running and 3 control rats were sacrificed by carbon dioxide inhalation and cervical dislocation. Three rats were prematurely removed from the study (one sprain, two sudden death).

Tissue processing

At each time point, for light microscopy and immunohistological examination, bilateral whole tendons (n=6 runners and n=4 control) were removed with the supraspinatus muscle still attached and fixed in fresh 4% paraformaldehyde for 16-24 hrs at 4°C, then subsequently dehydrated, paraffin embedded, and sectioned longitudinally. For transmission electron microscopy (n=4 runners and n=2 control tendons per time point), tendons were rapidly dissected, cut into 1 mm^3 pieces and fixed in 2.5% glutaraldehyde in 1M sodium cacodylate buffer for 24 hrs, and processed for transmission electron microscopy (TEM) as previously carried out in the same facility [54].

Immunohistochemistry

With the exception of the F7-26 antibody for the apoptosis assay, all antibodies were conjugated with biotinylated anti-mouse or anti-rabbit secondaries, followed by a commercially available amplification system (CSA II, Dako) with 3,3'-diamino-benzidine as the chromogen. Commercially available antibodies used were used to localize CD90 (Pharmingen; HIS51 – 1:1000), proliferating cell nuclear antigen (PCNA; Santa Cruz; F-2 -- 1:5,000), IGF-I (Upstate; Sm1.2 -- 1:1000), phosphorylated IRS-1 (Upstate; 07-247 -- 1:100), and phosphorylated ERK-1/2 (Cell Signaling; 20G11 -- 1:500).

Histological grading of tendinosis severity

Sections were stained with H&E for morphology, Alcian blue at pH 2.5 for negatively charged glycosaminoglycans with fast nuclear red counterstain, and picrosirius red. The extent of tendinosis was assessed using the light microscope by a blinded examiner (JC) using a modified Bonar scale [14]. The assessor assigned a score of 0 – 4 for each of 5 categories; tenocyte morphology, tenocyte proliferation, collagen organization, glycosaminoglycans, and neovascularization. Using this scale, a completely normal tendon would score 0, whereas a tendon with the maximum score in all categories would score 20. The Spearman's correlation coefficient for test-retest reliability was r^2=0.81.

Cell death and proliferation

Apoptosis was examined using the mouse monoclonal F7-26 antibody against single-stranded DNA breaks (Chemicon), with biotinylated anti-mouse secondaries and an avidin-FITC visualization system as described previously [46]. Hypoxic rat supraspinatus tendon explants cultured in serum-free DMEM in an anaerobic chamber demonstrated numerous apoptotic tenocytes in keeping with prior studies [45], and were used as positive controls to validate this assay. Proliferation index was expressed as the percentage of cells with positive PCNA nuclear staining, divided by the total number of cells counted (Haematoxylin nuclear counterstain), starting distally and progressing proximally counting every cell until at least 100 were counted. Segments of normal rat skin were used as positive PCNA controls, demonstrating positive nuclear staining in the basal layer of the epidermis.

Assessment of IGF-I expression and activity

In pilot studies, tendinosis was found primarily adjacent to the osseotendinous junction. Therefore, this region was defined *a priori* as the region of interest and was used to compare control and running tendons for assessment of IGF-I expression and activity (IRS-1 and ERK-1/2 phosphorylation) and proliferation index. The identity of slides was masked with black tape, and the region of interest was captured at 1392 x 1045 pixels using a digital camera (Retiga Exi 1394, Qimaging Corp, Burnaby, Canada) attached to a Zeiss Axioplan microscope, with constant illumination and exposure (20ms). The resultant scans were flat-field corrected in Image Pro Plus (Media Cybernetics Inc., Silver Spring, MD) and the number of pixels with positive 3,3'-diamino-benzidine end-product was quantitated.

Statistical analysis

2 x 4 ANOVAs were used to detect a main effect for each of the dependent variables (IGF-I, ERK 1/2, IRS 1, proliferation index), with group allocation (runners vs cage controls) and time as the between-groups factors. Correlations between IGF-I expression with each of proliferation index, ERK-1/2 and IRS-1 phosphorylation were examined using Pearson's one-tailed correlation. Error bars represent standard deviation, with the exception of cellularity (Figure 3b; bars denote standard error). For clarity of presentation, the results of IGF-I, ERK 1/2 and IRS-1 quantitation are depicted as the absolute difference between the means of controls and downhill runners at each time point.

RESULTS

Morphological changes with downhill running

Four of the diagnostic features of tendinosis – fibroblastic alterations (hyper or hypocellularity, and/or chondrocytic metaplasia), increased GAG staining, collagen disorganization or disarray, and hypervascularity were increasingly prominent among rats that had run downhill for longer periods (Table 1). These findings were concentrated proximal to the osteotendinous junction of the rat supraspinatus tendon. The Bonar score

Table 6.1. Tendon morphology with duration of downhill running.

	4 weeks		8 weeks		12 weeks		16 weeks	
Overuse	-	+	-	+	-	+	-	+
Tenocyte morphology	0.4 (0.5)	0.8 (0.5)	0.5 (0.3)	0.8 (0.8)	0.3 (0.6)	1.5 (0.6)	0.3 (0.6)	1.8 (0.8)
Tenocyte proliferation	0.4 (0.5)	0.4 (0.6)	0.3 (0.3)	0.7 (0.5)	0.3 (0.6)	1.5 (0.6)	0.3 (0.6)	1.2 (0.5)
Glycos-aminoglycans	0 (0)	0.2 (0.5)	0 (0)	0.2 (0.4)	0 (0)	0.5 (1.0)	0.3 (0.6)	1.0 (1.0)
Collagen fragmentation	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.8 (0.5)	0 (0)	1.0 (0.7)
Vascularity	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.5 (0.6)	0 (0)	0.2 (0.5)
TOTAL	0.8 (1.0)	1.4 (0.9)	0.8 (0.3)	1.7 (1.0)	0.6 (1.2)	4.8 (1.3)	0.9 (0)	5.2 (1.9)

Values represent the Bonar Score [modified from [14]] expressed as mean (SD).

of severity of tendinosis in the overuse group was significantly increased compared to controls at weeks 12 and 16 ($p<0.01$; Figures 6.1, 6.2). In the runners, the Bonar scores at 12 and 16 weeks were significantly higher than at 4 and 8 weeks ($p<0.01$). Affected tenocytes were noticably rounded by 12 and 16 weeks, but retained their expression of CD90 at all time points in both controls and tendinosis animals. The area of involvement comprised 1 or less than 1 40x viewing field in 4 and 8 week runners, expanding proximally in 12 and 16 week animals up to 350 μm from the point of transection and throughout the apparent width of the tendon proper. No change was observed in the supraspinatus tendon of control animals from 4 to 16 weeks. Extrinsic cellular invasion (e.g. inflammatory or peritendon cells) was not identified either by light or electron microscopy.

Tenocyte death and proliferation

Apoptosis assays were negative for supraspinatus overuse and control tissues at all time points, despite consistent detection of apoptotic tenocytes in positive controls (hypoxic tendon explants). TEM images also confirmed the absence of apoptosis, and a minimal presence of necrotic cells.

In contrast to the absence of apoptosis, treadmill running resulted in an increase in mitotic figures (Figure 6.3). The proliferation index of the runners increased progressively over time (Figure 6.3). The average cellularity of the supraspinatus did not increase significantly with running, but displayed more variability with increasing durations of running (Figure 6.3).

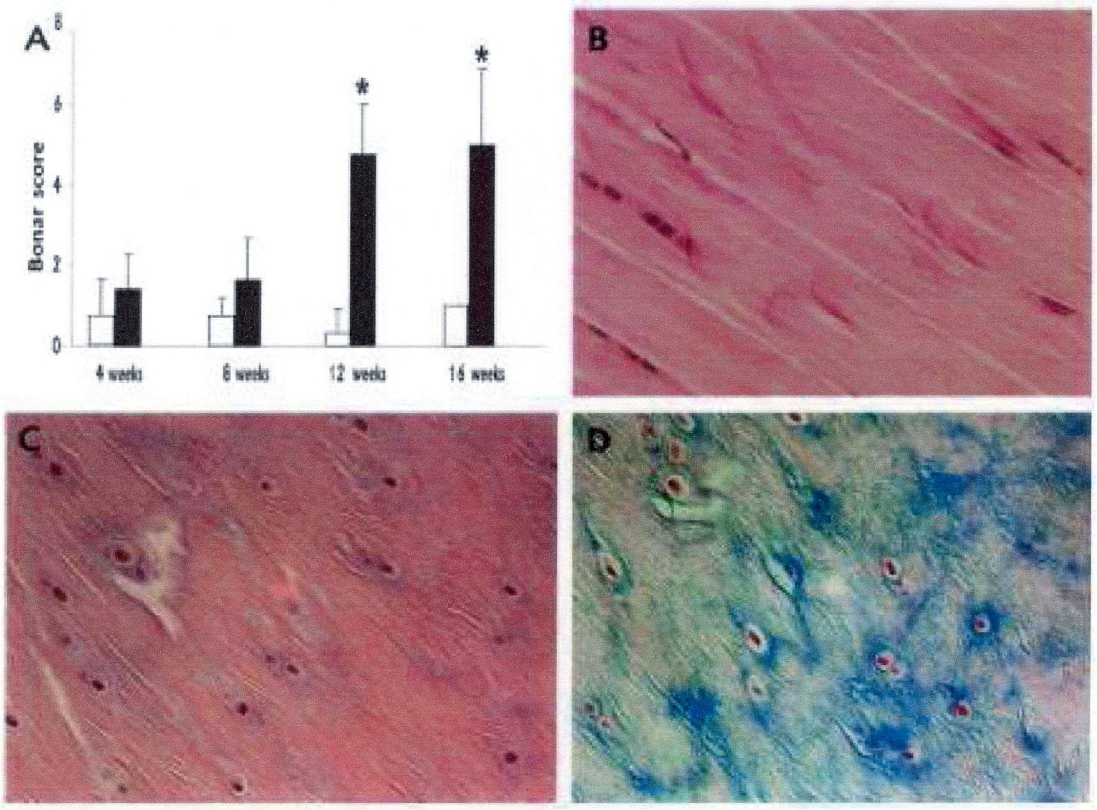

Figure 6.1. Development of tendinosis in response to increasing durations of overuse in male Sprague-Dawley rats. (a) Modified Bonar scale showing significant tendinosis after 12 and 16 weeks of downhill running. See Table 1 for raw data from individual categories (tenocyte morphology and proliferation, glycosaminoglycans (GAGs), collagen fragmentation, and vascularity). Open bars represent controls; solid bars represent downhill runners. Values are the mean and SD. * $P < 0.01$ versus controls at 12 and 16 weeks and versus downhill runners at 4 and 8 weeks. (b) Normal appearance of hematoxylin and eosin (H&E)–stained control rat supraspinatus, demonstrating longitudinal, slender tenocytes and tightly packed collagen. (c) Appearance of tendinosis resulting from 16 weeks of downhill running (H&E staining). Note rounded tenocytes, regions of abnormally staining matrix, and collagen degeneration originating at a tenocyte. (d) Alcian blue–stained section (adjacent to section shown in c), demonstrating increased levels of GAGs (normal tendon in this region has no stainable GAGs). (Original magnification x 600.)

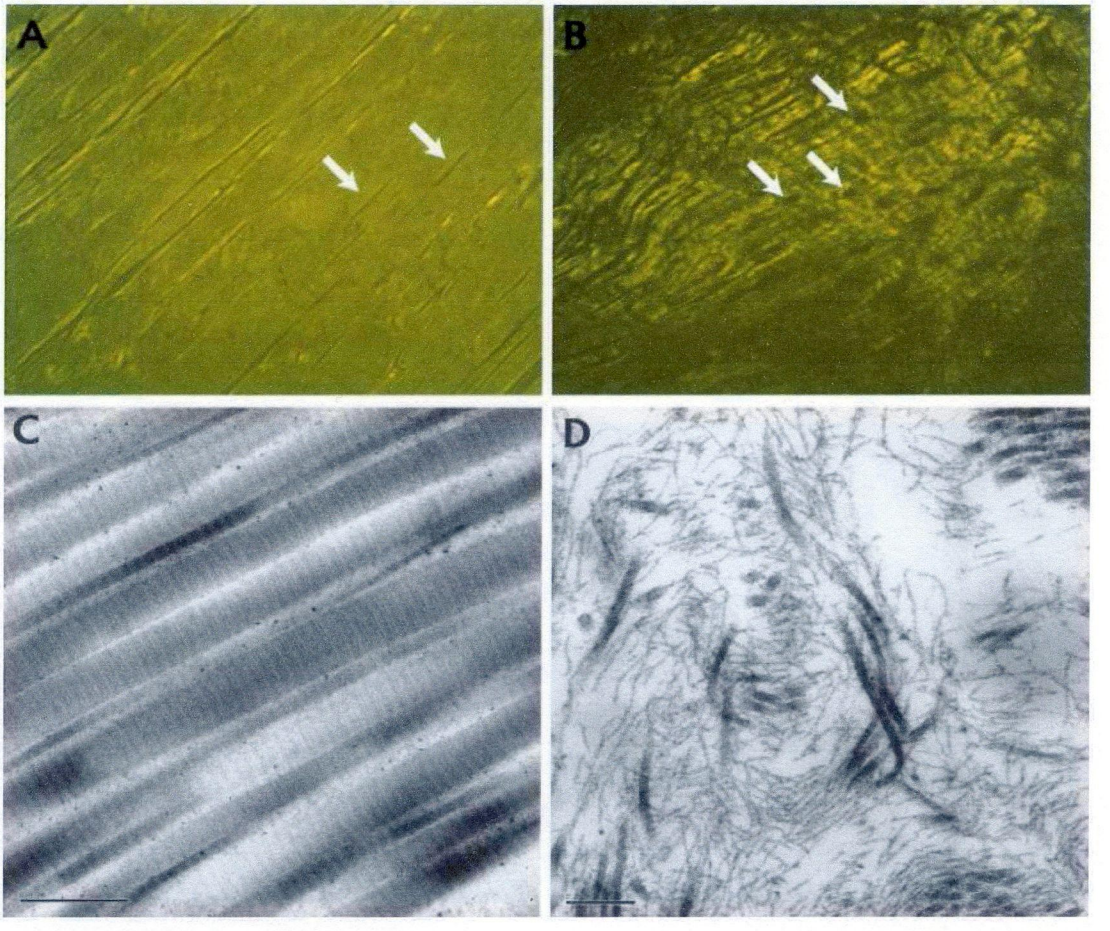

Figure 6.2. Collagen morphology in tendinosis. (a) and (b) Polarized light microscopy, showing collagen birefringence in picrosirius red–stained supraspinatus. (a) Normal rat supraspinatus, demonstrating tightly packed collagen bundles. Arrows indicate slender spaces occupied by tenocytes. (Original magnification x 400.) (b) Overuse-injured supraspinatus (at 16 weeks), demonstrating separation of collagen fibers and uneven intensity of birefringence. Arrows indicate rounded holes containing tenocytes. (Original magnification x 400.) (c) Transmission electron microscopy of normal tendon, demonstrating tightly packed fibrillar collagen with typical banding pattern. (d) Overuse-injured supraspinatus, demonstrating regions of thin, disarrayed collagen and fine, irregular fibrillar material. Bar in c = 0.5 μm; bar in d = 0.2 μm.

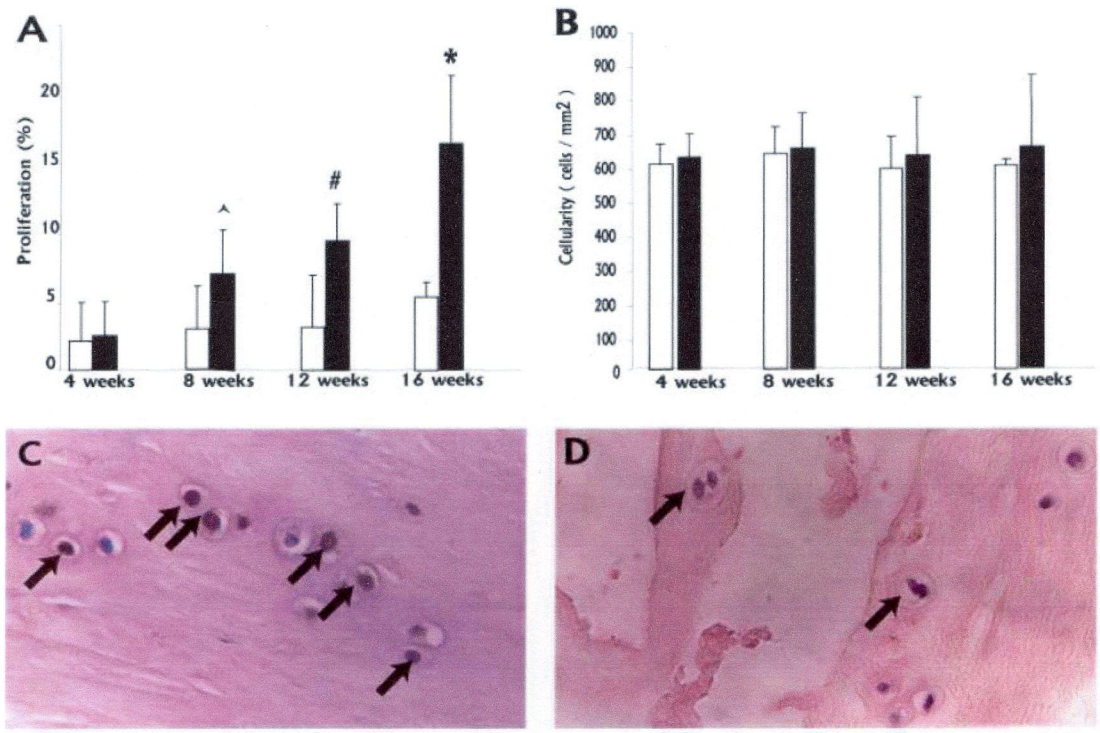

Figure 6.3. Tenocyte proliferation in tendinosis. (a) Association of elevated proliferation index with development of tendinosis. Open bars represent controls; solid bars represent downhill runners. Values are the mean and SD. * P = 0.016; # P = 0.052; ^ P = 0.054, versus controls. (b) No detectable change in cell density with progressive overuse. However, note increasing variability with time. Values are the mean and SE. (c) Association of proliferating tenocytes with regional hypercellularity and proliferating cell nuclear antigen–positive nuclei (arrows). (Hematoxylin counterstained; original magnification x 600.) (d) Presence of mitotic tenocytes (arrows) visible in overuse-injured tendon but not in control tendon. Each arrow indicates a single cell. (Original magnification x 600.)

Ultrastructure of rounded tenocytes

TEM images demonstrated that some rounded tenocytes had a chondrocytic appearance (c.f. 32), often sitting in a lacuna and buffered from the dense, banded type I collagen by an amorphous, less electron dense matrix (Figure 6.4). These cells were often in regions of collagen with thin or frayed fibrils and haphazard alignment (e.g. collagen in Figure 6.2D was closely adjacent to tenocytes shown in Figure 6.4A and B).

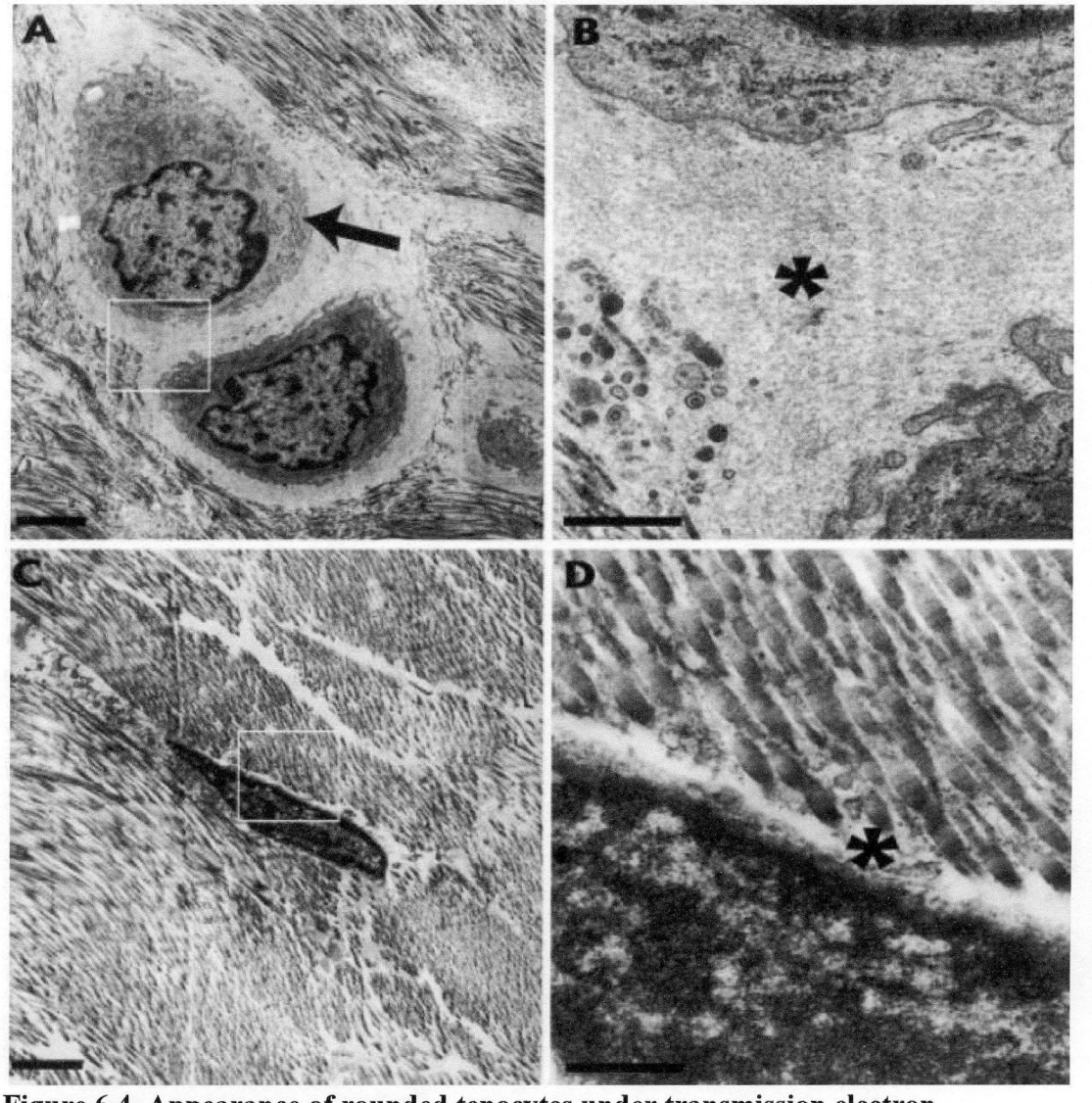

Figure 6.4. Appearance of rounded tenocytes under transmission electron microscopy, demonstrating differing tenocyte ultrastructure in overuseinjured tendon (a and b) and in control tendon (c and d). Boxed areas in (a) and (c) are shown at 5-fold higher magnification in (b) and (d), respectively. (a) A pair of rounded tenocytes from the distal overuse-injured tendon, with prominent cytoplasm and well-developed pericellular matrix.Surrounding matrix consists of type I collagen fibrils in oblique section. Arrow indicates abundant, dilated rough endoplasmic reticulum. (b) Asterisk indicates thickened pericellular matrix. (c) Typical slender tenocyte in oblique section with minimal cytoplasm and dense nucleus. (d) Asterisk denotes lack of pericellular space. Original magnification X 5,800; bar = 2 μm in (a); x 37,000 (bar = 0.5 μm) in (c).

Assessment of IGF-I expression

IGF-I was occasionally detected in sections of control tendon with a faint cytoplasmic or perinuclear distribution. In the distal supraspinatus of the runners, immunostaining of IGF-I was significantly increased at 12 and 16 weeks ($p<0.05$; Figure 6.5). Qualitatively, staining was often concentrated in the cytoplasm and perinuclear regions (arrowheads, Figure 5c), and was increased both in terms of the number of positive cells and the intensity of staining. Because IGF-I activity is modulated by the local presence of IGF binding proteins, it was important to confirm that IGF-I signaling was occurring in IGF-I-positive tenocytes by assessing phosphorylation of its direct downstream targets in adjacent sections. IRS-1 phosphorylation in the runners' supraspinatus was significantly greater than in controls at 16 weeks ($p<0.05$). Both the amount and the location of IRS-1 phosphorylation correlated with IGF-I ($r^2=0.503$, $p=0.001$) and PCNA expression ($r^2=0.4864$, $p=0.003$), suggesting that the IGF-I was potentially inducing an autocrine signaling response leading to tenocyte proliferation.

ERK 1/2 activation

ERK-1/2 activation was qualitatively more prominent in rounded, IGF-I+ tenocytes of the runners' distal supraspinatus but the increase was not statistically significant ($F=1.8$, $p=0.192$). ERK activation was strongly correlated with proliferation index ($r^2=0.6075$, $p<0.001$), but only moderately correlated with IGF-I ($r^2=0.3621$, $p<0.001$), suggesting the possibility of additional, currently unidentified stimulators of ERK activation in the rat supraspinatus tendon.

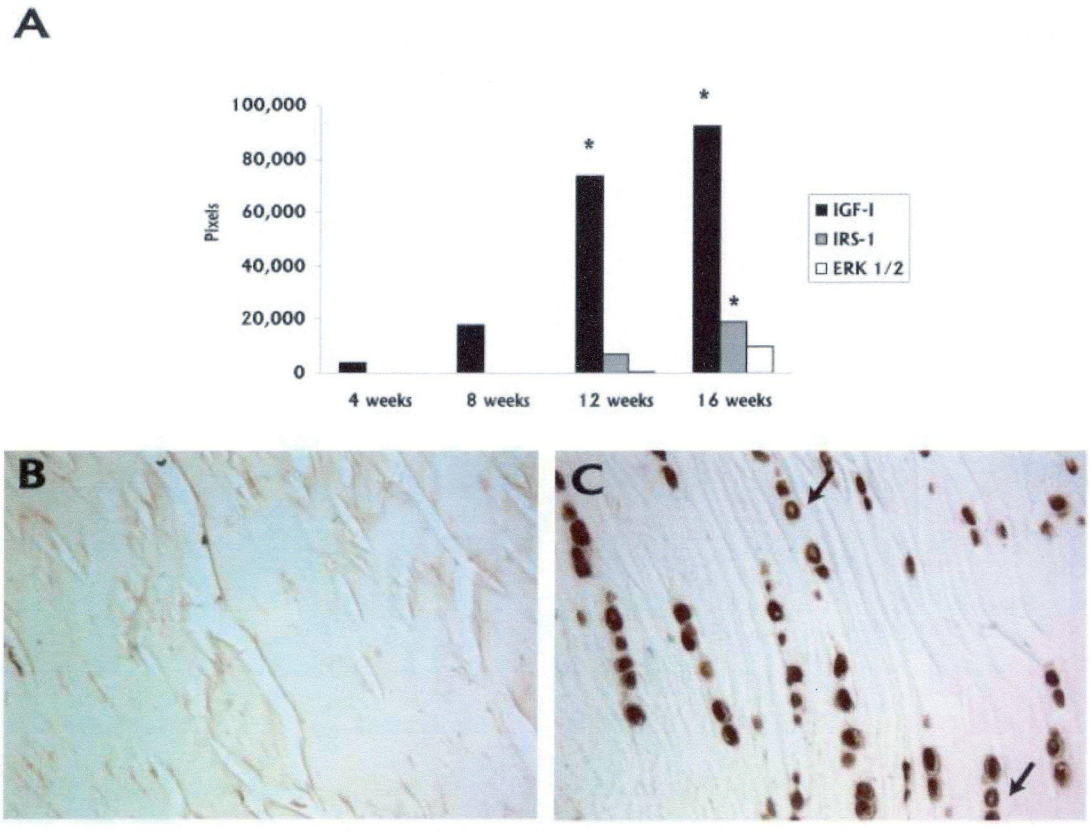

Figure 6.5. Increase in insulin-like growth factor 1 (IGF-1) expression with progressive tendon overuse. Data are presented as absolute increases over controls at the corresponding time points. Analysis of variance was performed as described in Materials and Methods. (a) IGF-1 is significantly increased at 12 and 16 weeks (* $P < 0.05$). Insulin receptor substrate 1 (IRS-1) phosphorylation is increased at 16 weeks (* $P < 0.05$). The increase in ERK-1/2 was not significant. (b) Control tendon, demonstrating typically minimal or absent IGF-1 staining. (c) Overuse-injured tendon, demonstrating increased numbers of IGF-1–positive cells and increased intensity of staining. Arrows indicate examples of perinuclear and cytoplasmic staining. (Original magnification x 400.)

DISCUSSION

This study supports an emerging role for IGF-I during the development of overuse tendinosis. In internally located tenocytes of the supraspinatus tendon, IGF-I expression was correlated with the phosphorylation of downstream targets (IRS-1 and ERK-1/2) and

with cellular proliferation, and was associated with altered collagen morphology and GAG accumulation.

The coarse resolution of time points (separated by 4 weeks) prevents firm conclusions regarding the exact timing of the observed changes. In a recent study, IGF-I mRNA was upregulated in the rat plantaris tendon following only 8 days of increased loading via synergist ablation [35]. Mechano-growth factor (MGF), a splice variant of IGF-I, as well IGF binding proteins (IGFBP-4 and -5) were also modulated by this model of increased mechanical load, suggesting a coordinated regulation of multiple elements of the local IGF system in response to tendon loading. The increase in IGF-I immunostaining observed in the current study reflected the development of altered tendon morphology, both being minimally detected at 4 and 8 weeks, and significantly increased at 12 and 16 weeks.

IGF-I upregulation and the development of tendinosis were not associated with an observable extrinsic inflammatory response, suggesting that mechanical loading of tenocytes -- either tensile, compressive or shearing -- may have been the direct stimulus for the observed tenocyte changes. This model supports the proposal of Benjamin and co-workers, who undertook detailed histopathological studies of tendon biopsy and cadaver material [33] and concluded that proliferation and clustering of fibrocartilage cells at the humeral epicondylar entheses could result from increased mechanical loading at the site of stress concentration. If infiltration by cytokines or inflammatory cells from the peritendon or bursae were responsible for tendinosis, one might expect tendinosis to

be maximal directly adjacent to the peritendon. Instead, the changes in the current study were concentrated in the supraspinatus tendon proper, often throughout its apparent width. A similar, non-inflammatory upregulation of IGF-I in mechanically loaded tendon appears to hold true in the tendon midsubstance [35, 58].

In the current study, the progressive development of tendinosis was associated with an elevated proliferation index and the appearance of mitotic figures. With increasing running, cellular density displayed wider variability but the mean remained fairly constant. This suggests that the matrix expansion associated with increased tendon loading [35, 49] may be sufficient to offset the increased cell number. Increased tenocyte number and matrix expansion are both prominent features of chronic tendinosis [26], and the recent detection of Ki 67+ tenocytes in tendinosis biopsies suggests that local tenocyte proliferation contributes to this process [44]. TGFβ, PDGFRβ and IGF-I are all reported to be upregulated in tendinosis biopsies, even in the chronic stage (months after loading has been discontinued) [21, 22, 42]. Although the current study suggests that the stimulus for tenocyte proliferation may be driven locally by load-induced proliferation, in chronic stages other factors such as hypoxia or transformation to a fibrotic phenotype may play a role in persistent growth factor upregulation and proliferation [4, 31, 43, 55].

ERK-1/2 activation is a final common pathway for many anabolic stimuli [12]. In cultured human osteoblasts, ERK-1/2 blockade prevented shear-induced proliferation and matrix synthesis [29]. Tenocytes appear to share elements of a load-sensing mechanism similar to osteoblasts and osteocytes; stretch-activated potassium and calcium channels,

internal calcium release, interstitial adenosine triphosphate release, and gap-junction signaling all play a role in the proliferative response to membrane deformation, substrate deformation, or fluid shear [7, 11, 24, 25, 29]. In elongated tenocytes, proliferation and collagen synthesis in response to ex vivo loading of chicken flexor tendon could be blocked by a gap junction inhibitor [8]. The current study highlights the need to examine cell-cell and cell-matrix interactions in the early stages of tendinosis (e.g. tenocyte expression and activation of integrins, cadherins, connexins).

Normally the rat supraspinatus enthesis is defined by a narrow transition zone (less than 100 μm, on decalcified preparations; unpublished obsevations) of chondrocytic cells and GAG-rich matrix interposed between bone and tendon, which acts to minimize stress concentration at the interface between soft and bony tissue [9]. The normal rat supraspinatus tendon also has a wedge-shaped fibrocartilage on the deep (compressed) surface, typically two to three cell layers thick. The apparent expansion of the chondrocytic phenotype proximally (up to 350 μm) and superficially (throughout the tendon width) into areas normally occupied by typical, spindle-shaped tenocytes suggests the possibility of early fibrocartilaginous metaplasia, similar to that seen in recent human biopsy studies [9]. However, our morphological findings need to be confirmed by examining specific components of the matrix in overuse supraspinatus, and by examining enthesis components specifically rather than confining observations to the tendon proper.

In the current study, regions of cell death were not seen, but occasionally necrotic tenocytes were identified on TEM. Apoptosis has been suggested to play a primary role

in the process of tendon overuse injury in a newly proposed model of tendinosis [57]. The present study argues *against* apoptosis in the primary stages of tendinosis. Load-driven cellular responses including increased glycosaminoglycans appear to predominate in early tendinosis rather than cell death *per se*, which may better explain the hypertrophic (thickened) and hypoechoic nature of many tendinosis lesions. Apoptosis might play a secondary role in more advanced stages of injury such as fibrosis or scar remodeling following macro- or microscopic rupture.

Neovascularization has gained increasing attention in rheumatology and in human tendon studies [1, 2, 15, 34]. Colour and power Doppler can demonstrate hyperemia in painful tendons [56] and therapy with sclerosing agents show promise [23]. In this downhill rat running model, neovascularization was not consistently observed in tendons, even at 12 and 16 weeks. There are at least 3 possible explanations for this finding.

First, the rat model may not adequately model human tendon overuse injuries, due to differences in the regional blood supply between species and tendons. Larger tendons might more readily become hypoxic in their deep regions due to compression and ischemia during loading or following vascular microtrauma [18].

Secondly, the rat supraspinatus overuse model may not be intensive enough to create a substantial injury. Indeed, the pathology appeared to plateau from 12 to 16 weeks, rather than continuing to progress to larger microtears. Nonetheless, the features of the pathology induced by this model (tendon thickening, reduced tensile strength, collagen

disarray, etc) [49] suggest that the exercise stimulus was intense enough to create several cardinal features of tendinosis. Neovascularization may be a feature of more advanced tendinosis, which would be consistent with findings in a cross-sectional study of very early human tendon pathology [14].

Finally, this animal model may not be ideal for inducing neovascularization. The Backman rabbit model of Achilles tendinosis differs from the Soslowsky model in some important respects; it induces a substantial paratenonitis with tendinosis of the midsubstance, including gross thickening and hyperemia, and may be more amenable to biomechanical analysis [5, 6, 37]. On the other hand, it is associated with greater laboratory costs and one group reported difficulties in reproducing the original findings in mature rabbits [3].

Although this study provides novel data regarding the possible role of IGF-I in early tendinosis in a rat model, we acknowledge the limitation that we restricted our observations to the tenocytes and their surrounding matrix. We did not perform any biomechanical testing or gross measures of the tendon tissue, any non-destructive imaging modalities, or kinematic analysis of the gait cycle which might have generated additional, relevant data.

CONCLUSION

In summary, *in vivo* tendon loading [49] produced a non-inflammatory pathology morphologically consistent with that observed in early tendinosis in humans. A novel

aspect of this study was that IGF-I appeared to modulate tenocyte responses to early stage tendon overuse injury. Further, our data suggest that at least in this rat model, tendon cell morphological changes were not accompanied by apoptosis and neovascularization. Whether apoptosis and neovascularization would arise with running of longer duration or greater intensity requires further study. Potential future clinical implications of this study may arise from a better understanding of the pathogenesis of tendinosis. Also, future studies should focus on specific pathways involved in matrix synthesis and degradation, and determine whether similar tenocyte regulatory events are occurring in clinically relevant human tendons.

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CHAPTER 7

IGF-I Activates PKB and Prevents Anoxic Apoptosis in Achilles Tendon Cells*

INTRODUCTION

Achilles tendon injury is an important clinical problem in both athletes and non-athletes [22, 25]. The histopathology of ruptured tendons shows evidence of pre-existing pathology that predisposes to rupture [32]. Of various patterns of pathology including hypoxic, mucoid, fatty, and calcific tendinopathies, hypoxic tendinopathy was the most common in a series of 891 tendon ruptures [21]. Although the histology of hypoxic tendinopathy is well described (including regions of hypocellularity, pyknotic nuclei, necrosis, mitochondrial abnormalities, and collagen thinning and disarray) [21],the underlying cellular processes are poorly understood.

Apoptosis, or programmed cell death, is associated with chronic tendon pathology [42, 43]. In patients with supraspinatus rupture and with tennis elbow, apoptosis has been reported based on TUNEL staining, DNA laddering and transmission electron microscopy [18, 43]. Other features of apoptosis that distinguish it from classic cell death (necrosis) include the activation of pro-death proteases – caspases – and the exposure of phosphatidylserine on the outer plasma membrane that serves as a signal for phagocytosis by macrophages [4, 16, 23]. Although apoptosis plays important

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homeostatic and physiologic roles (for example during regression of embryonic structures) it also plays negative roles in degenerative conditions of bone, cartilage, muscle and nerve. In general, the cell's decision to survive or to commit to apoptosis appears to result from a balance of pro-survival and death-inducing signals [6].

The events leading to tendon cell death or survival are beginning to be explored [42, 43] but the influences of hypoxia and growth factors on tendon cell viability have not been examined. In other cell types, hypoxia and anoxia induce necrosis, or apoptosis via transcription pathways involving p53 and p38 [11, 36]. Opposing such damage following injury is IGF-I a prosurvival factor released by platelets, macrophages, and fibroblasts [17, 33-35]. By binding to the IGF-I receptor, IGF-I enacts a number of pro-survival signaling events, including activation of protein kinase B (PKB) which prevents cell death by phosphorylating various cytoplasmic and nuclear targets [40]. The effects of IGF-I on tendon cells are of special interest given its promise as a factor that can accelerate biochemical and biomechanical recovery after tendon injury [1-3, 7, 12, 24, 26].

Given that reduced oxygen may play a role in acute or chronic Achilles tendinopathies [10, 19, 30] and that IGF-I has documented anabolic and regenerative effects in tendon [1-3, 7, 12, 24] we evaluated the influence of anoxia and IGF-I on death and survival signal pathways in Achilles tendon cells. The specific objectives were to determine (i) the extent and type of cell death induced by anoxia, and (ii) the ability of IGF-I to inhibit anoxia-induced cell death and to activate the PI3K / PKB pathway.

METHODS

Reagents

Type I collagenase (clostridiopeptidase A), IGF-I, and FITC were purchased from Sigma (St Louis, MO). Heat-inactivated fetal bovine serum was purchased from Invitrogen (Carlsbad, CA). Annexin-V conjugated to biotin was obtained from Pharmingen (San Diego, CA). Anaerobic chambers, pouches and indicator strips were supplied by BD (Gaspak, Franklin Lakes, NJ). A primary rabbit polyclonal antibody detecting the phosphorylated form of PKB was from Stressgen (Victoria, Canada). P85 antibody was obtained from Cell Signaling Technologies (Beverly, MA).

Achilles tendon cell culture

Porcine Achilles tendon tissue, harvested aseptically from animals being euthanized for university teaching purposes, was placed immediately into sterile PBS. Adherent muscle and peritendinous tissue was removed. The sample was then washed repeatedly in sterile PBS, cut into 0.5 mm pieces with a sterile blade and forceps and digested for 24-48 hours in DMEM containing 0.5mg/ml collagenase, 10% fetal bovine serum, (v/v) 20 mM HEPES, pH 7.4, 2% (v/v) penicillin-streptomycin, and 0.1% (v/v) gentamycin on an agitator at 37°C. The tendon fibroblasts released by this procedure were plated, along with any undigested collagenous tissue, on plasma-treated polystyrene tissue culture dishes. Tendon cell cultures were maintained in DMEM with 10% (v/v) fetal bovine serum in a 5% CO_2 incubator at 37°C. Cells were used in experiments up to the fifth passage.

To examine the effects of anoxia, tendon cells were seeded at 500,000 cells per 60mm plate and allowed to adhere for 48-72 hours, at which point the FBS concentration was reduced to 1%; cells were then maintained as usual in the 5% CO_2 incubator (normoxia) or placed at 37°C in an anaerobic chamber (GasPak system) for 24-96 hours (anoxia). Anoxia was confirmed by using methylene-blue indicator strips which become colorless when oxygen is absent. All experiments were performed in triplicate.

Cell viability assay

After indicated timepoints of normoxia or anoxia, cells were harvested by trypsinization and scraping, washed in PBS, fixed for 1h in ice-cold 70% ethanol, then placed for 30 minutes in 50μg/ml propidium iodide (PI) in PBS with 0.1% glucose and 100μg/ml RNaseA. A histogram representing DNA content was generated from each sample using the FL3 channel on a flow cytometer (Coulter Epics XL-MCL); the percentage of apoptotic cells (with sub-diploid DNA) was quantified using the same gate for all samples.

Apoptosis assay

To analyze the mechanism of tendon cell death, normoxic and hypoxic (anoxic) cells were harvested and washed as above, then resuspended in 500 μl of 10mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4 to which 5μl of biotinylated annexin V was added. After 15 minutes incubation in the dark, 5μl of avidin-FITC (50μg/ml) and 5μl of PI (10mg/ml) were added. After 30 minutes, the samples were analyzed on a flow cytometer simultaneously on the FL1 and FL3 channels after appropriate compensation

with single-stained and unstained tendon cells. Each channel was gated, yielding a scatter plot with four quadrants representing cells that were viable, in early or late apoptosis, or necrosis [42].

Caspase activation assay

Caspase activation was examined in live and dying cells using a flourometric assay kit according to the manufacturer's instructions (Immunochemistry). Briefly, a fluorescent probe (FAM-VAD-FMK FLICA) was added to anoxic or normoxic tendon cells harvested as above. The probe diffuses freely across the plasma membrane and reacts broadly with active enzymes of the caspase family by binding covalently to a reactive cysteine residue on the active caspase [39]. After several washes in PBS, the cells were analyzed by flow cytometry and on a fluorescent microscope (Zeiss Axioplan) after a 10 minute incubation with Hoechst 33342, a nuclear dye which is more readily taken up by apoptotic cells [8].

Western blot analysis for PKB activation

Tendon cells were harvested and 500,000 cells were allowed to adhere in 60mm plates overnight. To render cells quiescent prior to IGF-I stimulation, serum was withdrawn for 12 hours, then IGF-I was added to the medium for varying concentrations and durations. Cells were released from the plate by scraping, centrifuged and lysed in ice-cold solubilization buffer containing 50mM Tris-HCL, 1% Triton X-100, 10% glycerol, 100mM NaCl, 25mM EDTA, 10mM NaF, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin.

Lysates were sonicated on ice for 10 seconds, centrifuged at 14,000 rpm for 10 min, boiled in SDS and beta-mercaptoethanol, then loaded onto a 9% SDS-PAGE separating gel. Gels were calibrated using prestained SDS-PAGE low molecular weight standards. Proteins were transferred electrophoretically to nitrocellulose membranes, blocked for 1 h in Tris-buffered saline (TBS) containing 3% skim milk, then incubated overnight with the primary anti-PKB antibody at room temperature. After three washes with tris buffered saline and 0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h. Bands were visualized by using enhanced chemiluminescence. Equal loading was confirmed by reprobing the membranes with antibodies recognizing the p85 subunit of PI 3-kinase.

Statistical analysis

All values in the text and figures are expressed as mean ± S.D.. Statistical comparison between experimental group and control was performed using a repeat measures ANOVA. The confidence limit was predetermined at an α level of 0.05.

RESULTS

Oxygen withdrawal induces apoptosis of Achilles tendon cells

Lack of oxygen resulted in a progressive increase in the rate of cell death. However, the rate of cell death did not reach substantial levels until after 48 hours (Figure 7.1). Morphologically, the majority of dying cells demonstrated features of apoptosis (shrunken cytoplasm, membrane blebbing) rather than necrosis (swollen cytoplasm, membrane disruption). In cells kept under anoxic conditions but in the presence of added

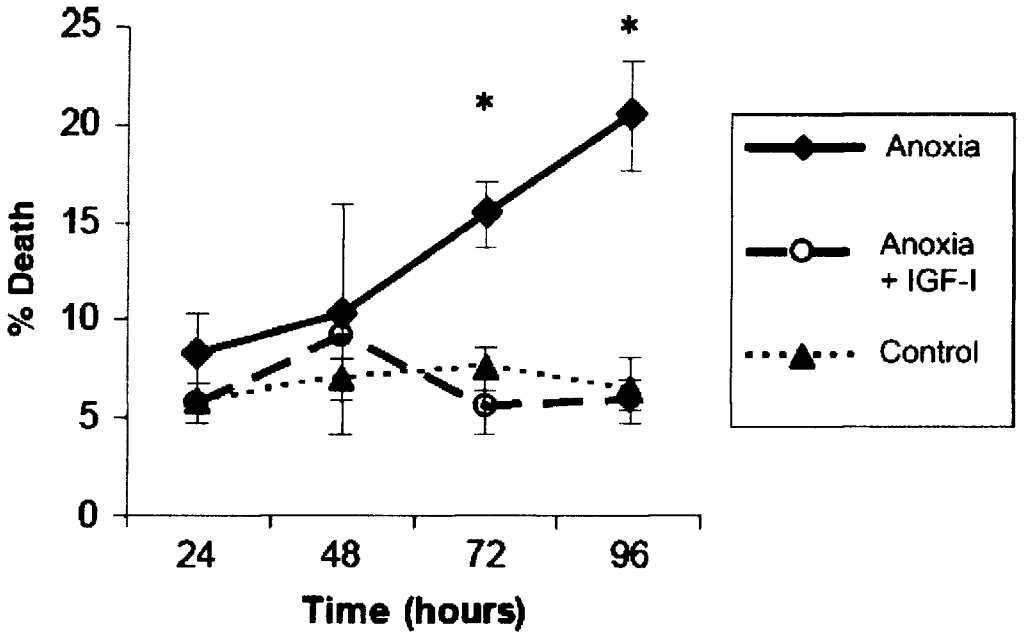

Figure 7.1 Pro-survival effect of IGF-I under anoxic conditions. Cells were mintained under normoxic or anoxic conditions for the indicated time periods and cell death was indicated by the number of cells having subdiploid DNA content. Incubation with IGF-I restored the level of tendon cell death to control levels at all time periods examined. *$p < 0.01$.

IGF-1, there was no evidence of an increase in cell death over the time period examined (up to 96 hours). Flow cytometry of cells double labeled with Annexin-V and PI confirmed that the predominant type of cell death induced by anoxia was apoptosis (Figure 7.2A&B). Among the dead cells (PI positive), the majority were Annexin positive (apoptotic), with few being Annexin negative (necrotic).

Caspase activation in anoxic tendon cells

As some authors refer to a "caspase-independent" form of programmed cell death ("aponecrosis")[4], we undertook experiments to confirm that cell death in these

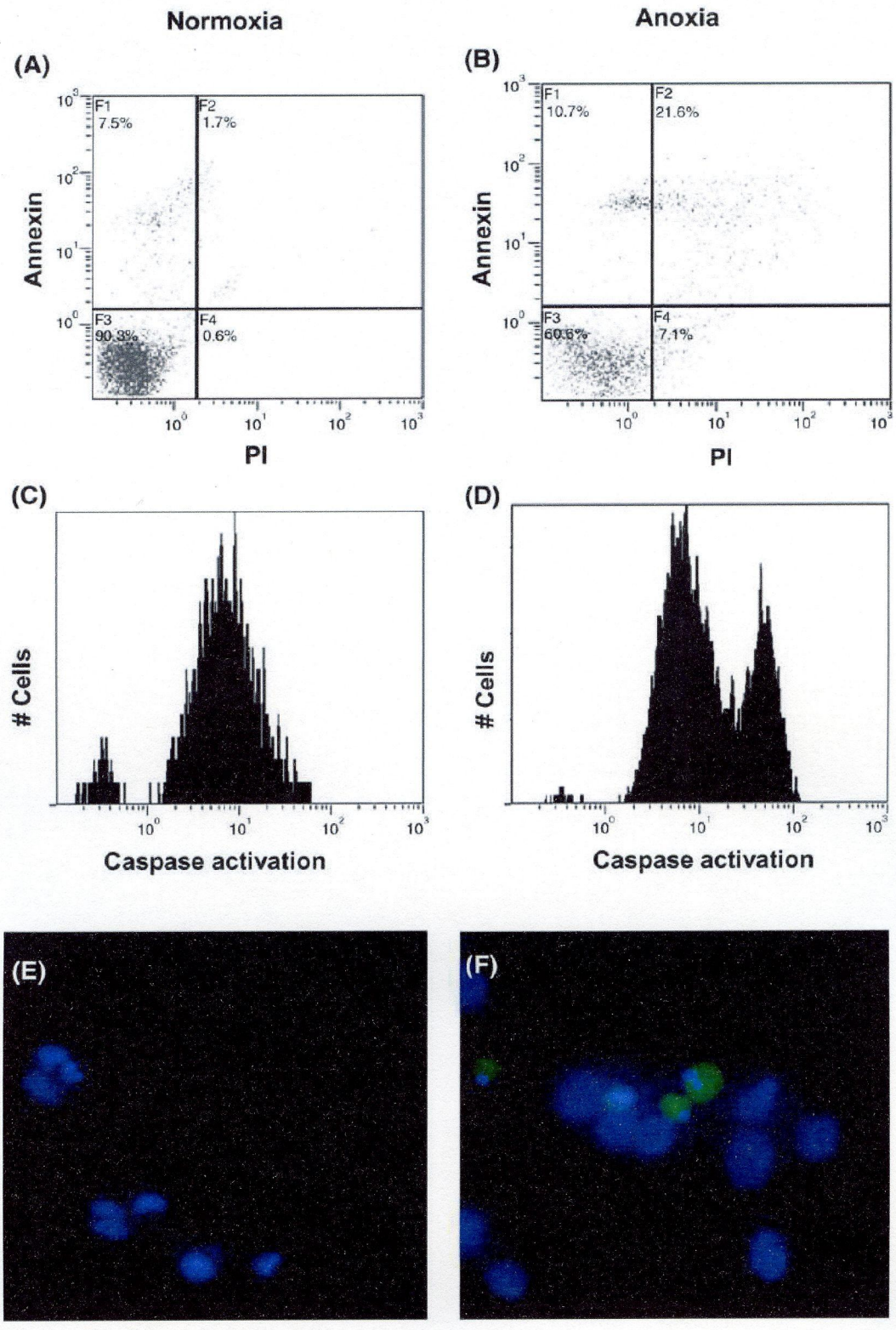

Figure 7.2 Legend next page

experiments was accompanied by caspase activation. Exposure of tendon cells to anoxia increased caspase activity 3- to 4-fold compared to cells treated identically but kept at normal oxygen tension, as evidenced by flow cytometry (Figure 7.2C&D). The finding of caspase activation was further validated by labeling anoxic tendon cells (72 hours) simultaneously with the same fluorometric caspase probe and with Hoechst 33342. Tendon cells that demonstrated green fluorescence in the cytoplasm, indicating active caspases, frequently showed increased uptake of Hoechst and fragmented nuclear morphology (Figure 7.2F).

IGF-I prevents anoxic cell death

Achilles tendon cells in which IGF-I was added at the same time that oxygen was withdrawn demonstrated levels of cell death equivalent to control values up to 96 hours. The dose-response effect of IGF-I on cell survival was tested using concentrations of IGF-1 up to 500ng/ml (Figure 7.3). A maximal survival effect was observed at a concentration of 250 ng/ml.

Figure 7.2 (Previous page) Anoxia-induced tendon cell death is predominantly apoptotic. Cells were kept at normoxia (a, c, e) or anoxia (b, d, f) for 72 h. (A, B) Cells were trypsinized and double-labeled with PI and Annexin V. The bottom left quadrant represents viable cells that are negative for both probes. The top two quadrants represent early (left) and late (right) apoptotic cells, whereas cells at the bottom right are necrotic (PI+, Annexin-). (c, d) Tenocytes were incubated with a fluorescent probe recognizing active caspases. In each panel, the largest peak represents background fluorescence from unstained cells. The right-most peak in the anoxic tencoytes (d) is due to positive labeling of tendon cells containing active caspases. (e, f) Tendon cells with active caspases (green) were common in the 72 h anoxic cultures. These same cells frequently demonstrated increased uptake of Hoechst 33342 (blue) and fragmented nuclei, indicating that they were undergoing apoptosis.

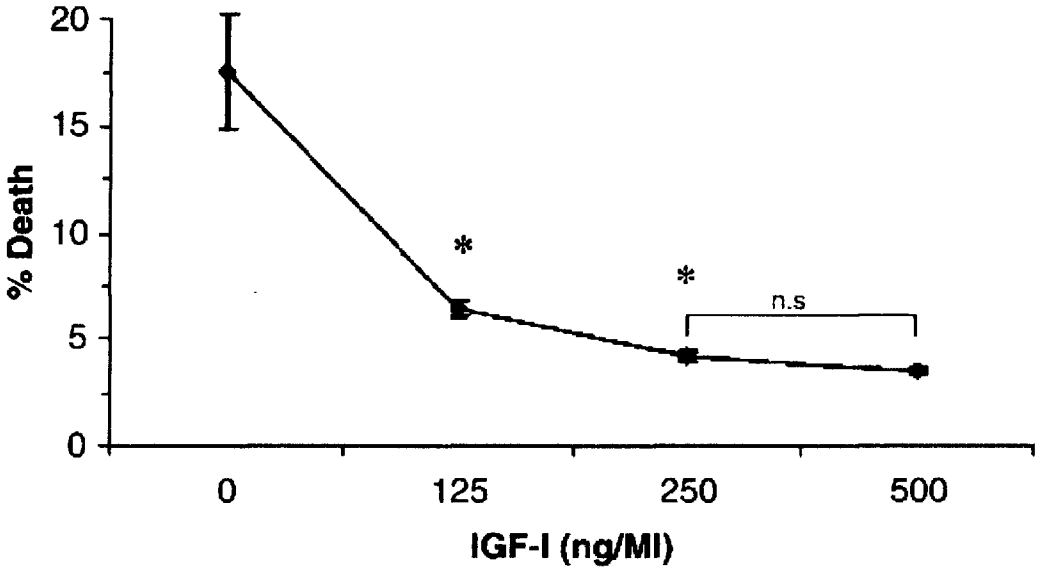

Figure 7.3 Dose–response of IGF-I protection from apoptosis during anoxia (72 h). * indicates significant difference from controls and from each other, $p < 0.001$. NS: not significantly different from one another.

IGF-I activates PKB through a PI3K dependent manner

To investigate one of the likely mechanisms of anti-apoptotic signaling by IGF-I in tendon cells, a phospho-specific antibody was employed that recognizes PKB when phosphorylated at the Ser473 activation site. Immunoblotting revealed a rapid increase in phosphorylation of PKB, which was elevated by 2', peaked at 5 minutes and had nearly disappeared by 20 minutes (Figure 4). The phosphorylated form of PKB was not detectable in cells that were pre-incubated with LY294002, a known PI3K inhibitor. LY294002 (25μm) also caused substantial cell death when added to normoxic, quiescent cell cultures indicating that ongoing survival of Achilles tendon cells is dependent on the PI3K – PKB pathway (data not shown).

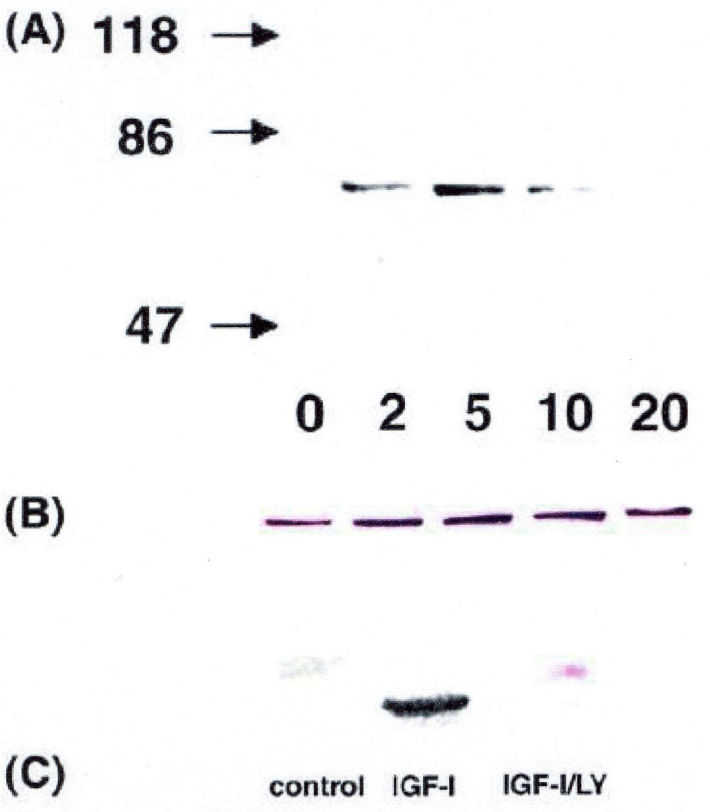

Figure 7.4 Activation of PKB by IGF-I. Time course is shown in minutes (from 0 to 20). (a) Phosphorylation at Ser473 showed a rapid onset and decay. (b) The same membrane probed with anti-p85 demonstrating equal loading. (c) Inhibitory effect of LY294200 (25 μm) on PKB activation, as detected by Ser473 phosphorylation.

DISCUSSION

In response to a lack of oxygen, Achilles tendon cells undergo either apoptosis or necrosis, with apoptosis representing the predominant mode of death. Apoptosis has not previously been well characterized in tendon cells. This study showed that it occurs in a manner that is characteristic of apoptosis in other cell types that includes exposure of phosphatidylserine on the outer plasma membrane, caspase activation, and DNA fragmentation [4].

We examined the effects of complete anoxia on tendon cells rather than gradations of PO_2, in order to induce a large effect that would allow us to determine whether oxygen withdrawal would induce apoptosis. In young rats, the mean PO_2 of tendons was 78.6 mmHg [31], but the PO_2 of aged, injured, or hypovascular regions of Achilles and other tendons are not known. Because oxygen supply is known to be a key determinant of a successful healing response [38], future studies to determine actual PO_2 in regions of tendinosis before or after rupture are required. Further experiments should also examine the effects of different hypoxic conditions with gradations of PO_2.

Although tendon cells express a functional aerobic metabolism [9], they are relatively insensitive to oxygen withdrawal compared to other cell types. Tendon explants maintained in 0% O_2 in 10% fetal bovine serum for 48 hours demonstrated no decline in cell proliferation or proteoglycan production, but collagen production was inhibited; this was in contrast to synovial explants, in which proliferation was significantly reduced, along with proteoglycan and collagen production [28]. Likewise, cultured human embryonic Achilles tendon cells whose oxygen was reduced from 20% to 2.7% did not experience a reduction in proliferation, whereas fibroblasts derived from other tissues did [41]. Furthermore, the magnitude of cell death we report here for tendon cells is several times lower than that reported in similar experiments with various cell types [5, 15, 27, 36].

The relative insensitivity of tendon fibroblasts to hypoxia raises the question of why the relatively low percentage of cells undergoing apoptosis at 72 hours (15-20%) were less

resistant than the cells which survived for this duration. From other cell types, hypoxia has been found to irreversibly damage some cells, while surviving cells from the same initial population can exhibit adaptive changes which increase their resistance to hypoxia and other stressful agents [14]. Such investigations have yet to be carried out in tendon cells, and may be relevant in understanding their resistance to cell death as shown in the current study.

Chapters 2, 3 and 4 provide preliminary evidence that angiogenesis may be occurring in tendon overuse injuries, which raises the possibility that cells within injured tendons may experience some degree of hypoxia. Hypoxia could lead not only to cell death, but could also lead to the transcription of hypoxia-responsive genes such as VEGF. As well, hypoxia can influence cell phenotype, promoting fibrocartilaginous change, calcification and bony metaplasia depending on the extent of hypoxia[13]. These speculations must be tested empirically as the actual conditions within injured tendons are likely to be very complex, with numerous interacting conditions and factors (including cytokines, mechanical loading, pH, and soluble or matrix-bound substances) determining the outcome.

Chronic hypoxia, one of many stresses that can trigger apoptosis, appears to operate through a pathway that is distinct from most stress-inducing agents [29]. Under acute anoxic conditions, the level of hypoxia-inducible factor (HIF-1α) - the "cellular sensor" of oxygen levels - rises, and activates transcription of genes involved in the response to hypoxia, including those encoding TGF-beta and VEGF. If oxygen is chronically low,

HIF-1α binds and stabilizes p53, a tumor-suppressor transcription factor capable of inducing cell-cycle arrest or apoptosis. P53 is known to be elevated in experimentally ruptured tendon [20]. Although the current study demonstrates that hypoxia induces tenocyte apoptosis, the involvement of p53 or other cell signaling pathways has not yet been examined.

In vivo, tendons undergo mechanical strain, and static tension held over several minutes reduces tendon oxygen levels [37]. It is unlikely that tension-induced hypoxia would induce apoptosis in tendons, as periods of complete hypoxia (anoxia) > 24 hours were required to induce substantial amounts of apoptosis in the current study. Nonetheless, if tenocytes were exposed to both hypoxia and mechanical loading simultaneously, the combined stresses may be more damaging than when applied in isolation as in the current study.

CONCLUSION

In summary, our studies demonstrate that IGF-I activated the PKB pathway in tendon cells in a PI3-kinase dependent manner, and that IGF-I prevented anoxic cell death of Achilles tendon cells. These findings extend studies conducted using other cell types, in which IGF-I exerts a protective effect from various cellular stresses. Whether other growth factors exert similar pro-survival effects in tendon cells is not yet known. We conclude that future studies should investigate the relative importance of downstream IGF-I signaling, and develop improved methods of achieving prolonged IGF-I delivery to areas of tendon pathology.

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CHAPTER 8

General discussion, conclusions and future directions

INTRODUCTION

The current thesis represents research that was conducted with two sets of human tendon biopsies including both patellar (Papers I - III) and Achilles and patellar biopsies (Paper IV), as well as a laboratory model of tendon overuse injury (Paper V) and exposure of primary tendon cell cultures to hypoxia and IGF-I (Paper VI). I aimed to further the understanding of tendon overuse pathology, identify new aspects of tenocyte cell biology, and improve knowledge of laboratory models of tendon injury. In this concluding chapter, I briefly summarise the major findings before discussing how those studies have contributed to the evolution of understanding in this field.

SUMMARY OF MAJOR FINDINGS

Paper I – Versican

Expansion of the extracellular matrix and vasculature in tendinosis are poorly studied processes, despite the fact that they are associated with clinical signs and symptoms [38, 56, 113]. The present study characterized the extent and distribution of the large aggregating proteoglycan versican in patients with patellar tendinosis. Tendon was obtained from patellar tendinopathy patients undergoing debridement surgery of the

patellar tendon, and from the equivalent part of the tendon of controls undergoing intramedullary tibial nailing. Pathology was confirmed by MR imaging [56]. Versican content was investigated by Western blotting and immunohistochemistry. Microvessel thickness and density were determined using computer-assisted image analysis. Markers for smooth muscle (α-SMA), endothelial cells (CD31) and proliferating cells (Ki67) were examined immunohistochemically.

Findings:

Versican content was enriched in the perivascular matrix of proliferating microvessels, within the media and intima of arterioles, as well as in regions of fibroblast proliferation and fibrocartilage metaplasia. Western blot analysis revealed elevated versican content in the proximal patellar tendon of tendinosis patients, and much of this was in the high molecular weight form, suggesting that it was newly synthesized. Microvessel density was higher in tendinosis tissue compared to control tissue. Microvessels in tendinosis biopsies demonstrated active proliferation (Ki67 nuclei) within versican-rich areas of tendon, whereas Ki67 labeling was absent in control tendon microvessels. These findings suggest the presence of an ongoing angiogenic process in patients with chronic patellar tendinopathy, and this possibility is discussed further below.

Paper II – Mast cells

The identity of cells within tendinosis lesions remains poorly characterized. Most of the cellularity is typically attributed to vascular cells and fibroblastic cells, although sprouting sensory and autonomic nerves within tendinosis tissue have also recently gained attention [2, 9, 25, 27, 61]. Due to close anatomic associations between mast

cells, vessels and nerves in other connective tissues, it has been postulated that mast cells may mediate the development of tendon hypervascularity on the one hand, or neurogenic inflammation and edema on the other [45, 46]. However, actual data on the distribution of mast cells in normal tendon and tendinosis tendon was not previously available. The same biopsy tissue from Paper I was assessed immunohistochemically by evaluating the distribution of mast cells, as well as markers for T-lymphocytes and macrophages. The vessel area fraction was quantitated using computer-assisted digital image analysis.

Findings:

The prevalence of mast cells per mm^2 [3.3 (3.0)] was greater in tendinosis tissue than in controls [1.1 (1.5)]. In tendinosis patients, mast cell density was moderately correlated with the vessel area fraction (r^2=0.49) and with symptom duration (r^2=0.52). Macrophages and lymphocytes were present too infrequently to be practically quantifiable in either control or patient tissue. In other tissues, mast cells are known to play a physiologically important role in angiogenesis, in neurogenic inflammation, as well as in regulating blood flow in response to physiologic stimuli such as mechanical load or hypoxia [19, 34, 66, 107]. The finding of increased mast cell density in patellar tendinosis opens up new avenues of research into the role of mast cells in the pathophysiology and potentially the treatment of tendinosis (discussed below).

Paper III – VEGF preliminary study

Vascular function and angiogenesis are regulated by vascular endothelial growth factor-A (VEGF), the key cytokine involved in endothelial cell proliferation and migration following injury or in response to hypoxia [1, 39, 44, 80, 82, 110]. Given the above

findings which suggested an angiogenic process in patellar tendinosis, this preliminary study addressed the following three questions; (i) is VEGF expression in the patellar tendon more prevalent in men and women with patellar tendinopathy than in individuals with normal, pain-free patellar tendons; (ii) which cell populations express VEGF in normal and tendinopathic tendon; and (iii) is there a difference in symptom duration between patients with VEGF+ and VEGF- tendons?

Findings:

In the same biopsy set as Paper I and II, VEGF expression was absent from control tendons, but was present in a subset of tendinosis patients (8/22). VEGF was expressed in the intimal layer of tendon vessels, but was absent in other cell types, including mast cells. Patients demonstrating VEGF expression in the patellar tendon had a shorter symptom duration (12 ± 7.8 months) than patients with no detectable VEGF (32.8 ± 23.5 months). This study suggests that VEGF expression by endothelial cells may contribute to the increased microvessel density in tendinosis (discussed below).

Paper IV – Vesicular glutamate transporter 2

This chapter represents the results of work conducted in collaboration with Håkan Alfredson and Sture Forsgren in Umeå, Sweden. This group has previously measured high intratendinous levels of glutamate in patients with tendinosis, suggesting potential roles of glutamate in the modulation of pain, vascular function, and/or degenerative changes including apoptosis of tenocytes [2, 3]. However, the origin of free glutamate found in tendon tissue had not been examined. Surgical biopsies of pain-free normal tendons and tendinosis tendons (Achilles and patellar) were examined

immunohistochemically using antibodies against vesicular glutamate transporters (VGluT1 and VGluT2), as indirect markers of glutamate release. In situ hybridization for VGluT2 mRNA was also conducted.

Findings:

Specific immunoreactions for VGluT2, but not VGluT1, could be consistently detected in tenocytes. The level of immunoreaction for VGluT2 was significantly higher in tendinosis tendons compared to normal tendon ($p < 0.05$). In situ hybridization of VGluT2 demonstrated that mRNA was localized in a similar pattern as the protein, with marked expression by certain tenocytes, particularly those showing abnormal appearances. Reactivity for VGluT2 was absent from nerves and vessel structures in both normal and painful tendons. Unexpectedly, the observations suggest that free glutamate may be locally produced and released by tenocytes, rather than by peripheral glutamatergic neurons. Excessive free glutamate may impact a variety of autocrine and paracrine functions important in the development of tendinosis, including tenocyte proliferation and apoptosis, extracellular matrix metabolism, nociception and blood flow [20, 48, 55, 67, 72, 97].

Paper V – IGF-I

The objective of this paper was to investigate tenocyte regulatory events during the development of overuse supraspinatus tendinosis in rats using a published overuse-tendinopathy protocol. Tendons were harvested after 4, 8, 12 and 16 weeks of downhill running and processed for brightfield, polarized light, or transmission electron microscopy. The development of tendinosis was assessed semi-quantitatively using a

modified Bonar histopathological scale. Apoptosis and proliferation were examined using antibodies against fragmented DNA or against PCNA, respectively. IGF-I expression was evaluated by computer-assisted quantification of immunohistochemical reaction. Local IGF-I signaling was probed using phospho-specific IRS-1 and ERK-1/2 antibodies.

Findings:

Morphological changes were present in the supraspinastus tendon after 12 weeks of downhill running characterized by tenocyte rounding and proliferation, as well as by glycosaminoglycan accumulation and collagen fragmentation or degeneration. The proliferation index was elevated in tenocytes, many of which took on a rounded or chondrocytic appearance, and correlated with increased local IGF-I expression and phosphorylation of IRS-1 and ERK-1/2. In this animal model, local stimulation of tenocytes appeared to be the predominant feature, whereas there was no evidence of cellular inflammation or apoptosis. The data suggest a role for IGF-I in the load-induced tenocyte responses to periods of increased mechanical loading. In addition, the data have implications for the application of this model for future studies of tendon overuse injury.

Paper VI – Combined effects of hypoxia and IGF-I on primary tenocytes

Hypoxia and apoptosis are both implicated in chronic tendon pathology [53, 58, 85, 88], however the influence of hypoxia on the viability of tendon cells is not known. The objectives of this study were to investigate the effect of oxygen withdrawal on the viability of porcine Achilles tendon cells (ATCs), and to examine the ability of IGF-I to prevent ATC death. Cultured ATCs were enclosed in an anaerobic chamber. The

mechanism of cell death (apoptosis vs necrosis) was examined by flow cytometry of ATCs double labeled with Annexin-V and propidium iodide. Caspase activity was determined by a fluorometric assay, and nuclear morphology was examined by Hoechst staining.

Findings:

The cell death induced by hypoxia was time-dependent, and was characterized by phosphatidylserine exposure on the outer membrane, caspase activation and DNA fragmentation. Death was inhibited by the addition of IGF-I in a dose-dependent manner. The ability of IGF-I to activate the pro-survival PKB pathway in ATCs was inhibited by LY294002, indicating the importance of PI3K in the response of ATCs to IGF-I. These data suggest that cell death induced by lack of oxygen is predominantly apoptotic and can be prevented by pro-survival IGF-I signaling. This mechanism indicates the existence of a previously unexplored, autocrine, pro-survival effect of IGF-I in tendon.

TENDINOPATHY - CONTRIBUTION OF THE CURRENT STUDIES TO EVOLUTION OF CONCEPTS AND TREATMENTS

The goal of the current research was to better understand the pathology underlying overuse tendinopathy, in order to help guide the development of new treatments. Treatments that are currently available for overuse tendinopathy vary greatly in their efficacy and in their underlying mechanisms. Many treatments are aimed at suppressing the activity of inflammatory cells. The current thesis supports previous work [35, 56, 63, 81, 91] which demonstrated that a traditional inflammatory response mediated by macrophages, neutrophils or lymphocytes is in fact absent in the chronic stage of patellar

tendinosis, therefore many anti-inflammatory strategies may have little biological rationale for patients with patellar tendinopathy of > 5 month duration (5 months being the shortest symptom duration in the current series). This is in contrast (but not in contradiction) to studies that show a beneficial effect of antinflammatory strategies (i.e. corticosteroid iontophoresis or topical NSAIDs) in tendinopathy patients with short-term symptoms of < 3 months [68, 73].

The current studies of human tendinosis biopsies found evidence of increased versican content, increased microvessel density and proliferation of endothelial cells in patellar tendinosis, increased mast cell density, and preliminary evidence of VEGF expression by tendon endothelial cells in patellar tendinosis. These findings are all typically associated with soft tissue repair, and appear to share a common association with angiogenesis. It has recently been shown that sensory and nociceptive nerves accompany the microvessels in tendinosis [25, 26] – furthermore, in addition to their close anatomic association in tendinosis lesions, vessels and nerves may respond to similar signals in repairing tissue (such as VEGF and NGF) which both result in sprouting of axons and of capillaries [86, 92, 105]. A new strategy of treatment for tendinopathy involves surgically ablating or targeting with sclerosing injections the abnormal tendon vasculature (and its accompanying nerves) under colour Doppler ultrasound guidance [7, 50, 77]. Thus, the current thesis is consistent with an "angiogenic model" of tendinopathy (Figure 8.1).

A limitation of the evidence arising from the current studies of human biopsy tissue is that the studies are descriptive. It is not possible to conclude that blocking (or promoting)

the proliferation of vessels would have any influence on symptoms or on tendon vascularity or innervation. Mechanistic studies with a suitable animal model will be required to examine this question, and are currently in the pilot stages.

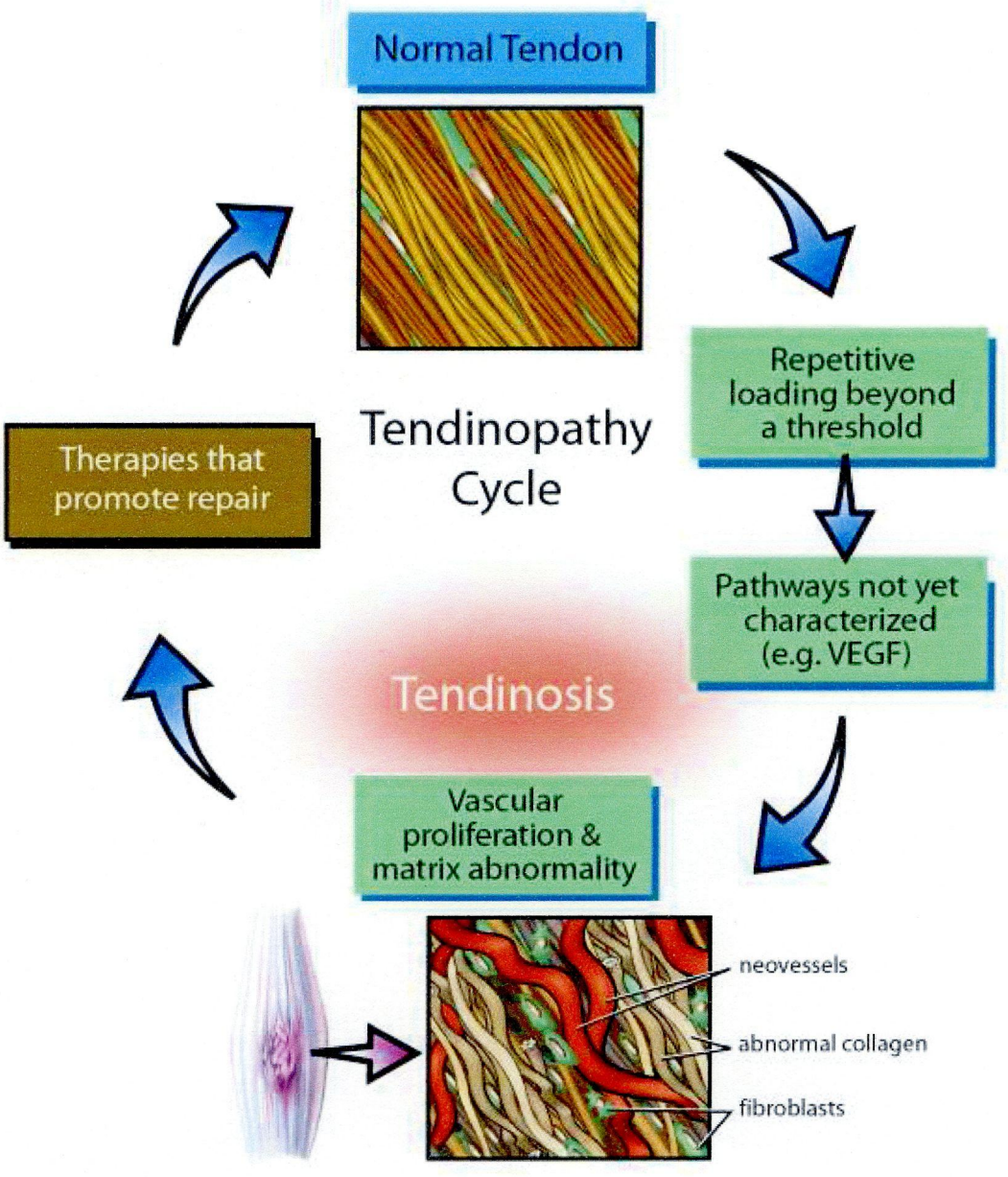

Figure 8.1 Angiogenesis model of tendinosis

STAGES IN THE DEVELOPMENT OF TENDINOSIS

Understanding how pathology develops over time is important in fully understanding and developing treatment strategies for patients at various points in the injury-repair process. As described in the introductory chapter, the morphology of tendinosis is strikingly similar to human scar tissue (a.k.a. fibrosis) observed in a variety of soft tissues. Scar tissue is rich in fibrin and Type III collagen, and is the end-result of the body's generalized inflammation-repair response, having the same basic elements throughout the musculoskeletal system [33, 71]. In acutely injured soft tissues, the triggering event is typically activation of platelets when they contact the endothelial basement membrane in the area of injury [95]. Platelet activation is rapidly followed by deposition of fibrin and fibronectin. This initial stabilization of the injured area is followed by the restoration of the blood supply via angiogenesis. In this context of soft tissue injury, VEGF is known to be expressed by numerous cell types in the repair phase, including neutrophils, macrophages, mast cells, and endothelial cells [32, 36, 101]. Versican expression is also increased during the repair and remodeling phases and remains elevated in the extracellular matrix in regions of fibroblast and vascular cell proliferation [18, 42, 90, 96]. Versican's major role is to create an extracellular matrix conducive to cellular proliferation and migration which are essential to tissue repair [10, 108, 109], and it is expressed by endothelial cells during in vitro angiogenesis [21]. When taken along with earlier data that show an accumulation of fibrin, fibronectin, collagen III, and TGFβ in tendinosis tendons [12, 35, 62], the current thesis adds circumstantial evidence to the case for viewing tendinosis as the common end-point of an earlier inflammation-repair response.

Why was VEGF expression only observed in a subset of patients (8/22) with shorter symptom duration? Furthermore, why is it that many regions of tendinosis which also demonstrated increased microvessel density did not display evidence of VEGF expression? In the initial stages of angiogenesis (endothelial cell proliferation and migration), VEGF plays key roles; indeed, the survival of vessels is initially VEGF-dependent. As angiogenesis progresses, the role of VEGF declines -- particularly with the recruitment of smooth muscle containing cells, which have a stabilizing and pro-survival effect on the endothelium [75]. The current work allows one to pose the hypotheses that VEGF expression may be temporally regulated in the development of tendinopathy and that it may peak at an earlier time point than that observed in the current study of patients with chronic tendinopathy. Furthermore, blocking VEGF expression in the chronic stage may have little influence on the pathology or symptoms. Studying biopsy material from tendinopathy patients with more acute symptoms and using more sensitive techniques may shed light on a potential role of VEGF and may help clariy the different stages of tendinosis.

POTENTIAL ROLES OF MAST CELLS IN TENDINOPATHY

Surprisingly, mast cells were present in substantial numbers in patellar tendinosis, particularly in patients with the most prolonged symptom duration. Although a role for mast cells in neurogenic inflammation has been postulated for several years [45, 46], data on their distribution was not previously available. The current thesis supports the need for further research as there are many mast cell inhibitory drugs already available which

could immediately be tested for their influence on symptoms such as blood flow, edema, fibrosis and pain.

Mast cells play a well-known physiologic role in allergic responses and in hypoxia- or vibration-induced erythema. A more recently discovered function is that mast cells (whose number is increased in many soft tissue pathologies at the chronic, fibrotic stage) release substances such as TGFβ, IL-4, IL-8, PDGF, and mast cell tryptase, which have been hypothesized to reinforce the scar tissue phenotype and promote or enhance angiogenesis [31, 76, 95]. Mast cells are known to express the VEGFR2 receptor [49] and to migrate in a tyrosine-kinase dependent manner in response to picomolar concentrations of VEGF [41]. This explanation (recruitment and participation of mast cells in angiogenesis) is, however, seemingly at odds with the current findings in which mast cells were positively correlated with symptom duration – an opposite trend to that seen with VEGF. Clearly, further work is required to sort out the timing, regulation and significance of mast cell density in tendinosis.

ROLES OF GLUTAMATE, HYPOXIA AND APOPTOSIS IN TENDINOSIS

Indirect evidence for local glutamate production in tendon was found in the expression of VGluT2 by tenocytes. This finding is in line with recent evidence that tenocytes can produce a variety of substances previously thought to be primarily produced by neurons, including acetylcholine, epinephrine and substance P [9, 24-28]. Tenocytes also demonstrate receptors for these neural substances, including glutamate receptors (mGluR5 and -6, NMDAR) and receptor interacting proteins (GRIP1 and -2) [70]. The

physiologic and functional implications of glutamate signaling in tenocytes have not yet been explored, thus the current thesis contributes data that supports this new direction for research in tendon biology.

It has been hypothesized that glutamate, as an excitatory neurotransmitter, may be responsible for pain originating from tendinosis tissue [8]. This notion was based on the fact that tendinosis patients displayed higher microdialysis levels of glutamate in the painful area of the tendon than control subjects, and that NMDAR+ nerve fascicles were demonstrated in tendon [2-4, 8]. However, a later study by the same group demonstrated that glutamate levels remained elevated in successfully treated tendons (i.e. from patients having undergone an eccentric training program with resolution of symptoms and reintroduction of sporting activities) [6]. This suggests that the role of glutamate in tendon may not relate primarily to pain. In other tissues and cell types, NMDAR receptors are involved both in mechanotransduction, and in the regulation of blood flow [22, 30, 37, 47, 54, 59, 93, 102].

Interesting data on the potential roles of glutamate in tenocyte biology have emerged from gene array experiments conducted with the same rat model that was employed in the current thesis [70]. These arrays identified elements of the glutamate signaling machinery whose expression levels were subsequently shown by RT-PCR to be upregulated in tenocytes in response to 4 weeks of downhill running. When glutamate was applied to tenocyte cell cultures in similar concentrations to those measured in patients via microdialysis, an increased rate of tenocyte apoptosis was found as a result

[70]. This led the authors to suggest that increased glutamate levels in the rat supraspinatus tendon may contribute to apoptosis of tenocytes and degeneration of the tendon, predisposing it to injury. Apoptosis could not be observed in the current set of rat studies, despite our consistent ability to detect apoptotic cells in control rat tissues (hypoxic tendon or mechanically loaded tendon explants) [94]. In fact, the rat supraspinatus tendons were essentially normal in appearance after 4 weeks of downhill exercise, with the only consistent histological change being rounding and proliferation of tenocytes. This rounding and proliferation does suggest that many genes were likely upregulated in response to mechanical loading [15], and this may include elements of the glutamate signaling pathway which have been implicated in cellular proliferation [47]. However, the coarse resolution of time points (i.e. separated by 4 weeks) makes it impossible to conclude that apoptosis did not occur to some extent in the rat supraspinatus tendon during or after the exercise sessions. A further difficulty in relating the finding of apoptosis in human tendinosis to the Soslowsky rat model is that in the former, apoptosis was located in the hypervascular zone of injury ("Zone 2"), whereas in the latter, this zone was not a prominent feature of the model.

In the final paper (Chapter 7), tenocyte cell cultures were found to be remarkably resistant to hypoxia, being able to withstand 24-48 hours of oxygen withdrawal with no observable effect on cell viability. The anaerobic chamber provided a readily available tool with which to test the hypothesis that hypoxia may be responsible for apoptosis in tendinosis. With more sophisticated tools, the levels of oxygen could be titrated to specific values. The actual oxygen levels within tendinosis lesions are not known, although recent studies using laser Doppler flowmetry have suggested that tendon venous

oxygen saturation is equivalent in normal and tendinosis Achilles tendons -- at least at the superficial depths capable of being measured with this technique, and taking into account the potential influence of skin perfusion [57]. The presence of VEGF in tendinosis lesions does suggest that hypoxia may be present despite the increased blood flow[5] (as in, for example, tumours or healing wounds) but other stimuli including mechanical load or cytokines and growth factors could equally be responsible for inducing VEGF expression in tendon endothelial cells.

Other factors which could regulate apoptosis levels in tendinosis

Although it is difficult to compare reports which have used different apoptosis assays, it appears that the extent of apoptosis in human rotator cuff tendons [104, 112] may be greater than that observed in patellar tendinosis [60]. Disturbed glenohumeral mechanics and impingement against the acromion may play a role in the tenocyte apoptosis seen in the rotator cuff, as compression induces apoptotic cell death in other cell types [23, 74]. Age, inflammation, and oxidative stress could also play important roles in the development of tenocyte apoptosis at various stages of pathology [51, 87, 111]. Thus, apoptosis may result from distinct physiological processes occurring at various phases of injury.

Arnoczky et al. have proposed an interesting model in which isolated collagen fibril rupture results in the release of tension from associated fibroblast arrays, and that this sudden loss of tension could trigger apoptosis [11]. The current thesis suggests that tenocyte apoptosis occurs in the context of cellular proliferation and increased

microvessel density [60, 112]. Apoptosis in tendinosis may not be an element of pathology per se, but may be part of the normal repair response – for example, a reduction in cell number (e.g. myofibroblasts) at the end of the repair phase is associated with a resolution of the scar response [29].

OVERUSE TENDINOPATHY VS TENDINOPATHY IN SEDENTARY INDIVIDUALS

An important distinction is that between overuse tendinopathy, and age-related tendinopathy which occurs without any apparent aggravating mechanical load [64]. Chapters 2-4 were conducted with the same set of patient biopsies, all of which were obtained from highly competitive athletes in whom loading of the patellar tendon (via jumping activity) played a clear etiologic role. The average age of these patients was 30.4 years. In contrast, the average age of patients from whom Achilles tendon biopsies were obtained was 40.7 years. Unfortunately, the activity levels of the Achilles tendinopathy patients were not available, which limits the ability to directly attribute changes in VGluT2 expression levels to overuse as opposed to age-related tendinopathy. It was not the intention of this thesis to conduct a comparison of the two biopsy sets. However, it should be noted that the Achilles tendon biopsies examined in Chapter 5 may have differed from the tendons in Chapters 2-4 not only by age and anatomic location, but also by activity level and possibly symptom severity as well. Despite this limitation, the pathology observed under the microscope in the two sets of studies was, according to the features identified in the Bonar histopathological scale, highly analogous. Both sets of patient biopsies displayed the same morphological features which have been termed

"tendinosis" by many authors – namely, collagen abnormalities (disorganized or degenerated in appearance), increased proteoglycan, regional variations in cellularity (usually increased tenocyte numbers, with focal regions of decreased cell number), increased prominence of microvessels, fibrocartilage metaplasia, and a lack of inflammatory cells (neutrophils, macrohpages, lymphocytes). This similarity between the two sample sets suggests that, despite any possible variation in etiologic factors which may have been present in the two sets of biopsies, the end-result in the tissue was tendinosis.

LOAD-DRIVEN TENOCYTE RESPONSES – INITIAL STAGE OF TENDON OVERUSE PATHOLOGY?

The data from Chapter 6 showed that there was no evidence of cellular inflammation in rats which had been exposed to an increase in mechanical loading of the supraspinatus tendon by downhill running for 4-16 weeks. On the contrary, there were marked changes within the tenocytes and their surrounding extracellular matrix. The rounded tenocytes demonstrated increased IGF-I expression, both in terms of intensity and distribution, and this correlated with increased proliferation index. This study is the first to provide evidence that exercise is associated with the phosphorylation of ERK-1/2 within tendon. The antibody used against IRS1 recognizes an inhibitory phosphorylation site (serine 307), which suggests that with ongoing exercise, IGF-I signaling may be dampened by regulation of downstream events. IGF-I signaling is also modulated by the presence of IGF binding proteins which were not examined in the current study, but whose

expression levels have been shown to be regulated both peritendinously and systemically in response to exercise [78, 79].

A caveat when discussing findings from the rat model in relation to human pathology is that the rat tendons never progressed to demonstrate the most common feature of the human biopsies – namely, an increased density of microvessels invading the tendon proper. The histological changes seemed to plateau between 12 and 16 weeks. One might question whether this rat model in fact represents tendon overuse pathology at all. IGF-I is the major physiological mitogen for tendons [16], so its expression in the tendon may be part of an adpative response rather than a feature of injury (although its expression has previously been reported in association with tendinopathies as well[43]). The original report of the rat model demonstrated a loss of mechanical properties in the supraspinatus tendon which was present by 4 weeks and remained (but did not progress) for the duration of the study [100]. This suggests that biomechanical testing may be a more sensitive indicator of change in the tendon extracellulatr matrix than the histology employed in the current study, as the collagen matrix appeared essentially normal until 12 weeks.

The collagen fragmentation and disarray observed in the rat study was distinct in appearance from the type of degeneration observed in human biopsies, in that it was localized to the immediate vicinity of the tenocytes, rather than affecting entire fields as in the human biopsies. Fibroblasts in ligament or tendon are capable of substantial intrinsic remodeling activity, therefore pericellular collagen remodeling may represent

intrinsic activity rather than injury per se [83, 84]. This is in contrast to an overt injury-repair scenario, where invading cells trigger a scar tissue phenotype and a loss of normal tendon architecture with increased vascularity [83]. The increased glycosaminoglycan surrounding the rounded, proliferating tenocytes may also represent a physiologic adapation to altered loading, i.e., it is possible that the tendon was adapting to repetitive compressive loading under the acromion during the rat gait cycle with downhill running, or that the fibrocartilage region adjacent to the enthesis was expanding as a means of adapting to the increased stress it was being placed under [17, 40, 65, 89, 99, 106]. Thus, in the absence of overt evidence of tendon injury including substantial breakdown of the matrix or proliferation of microvessels, the relevance of the rat model to human pathology remains open to question.

Further limitations of the rat model in understanding stages of tendinopathy

Additional, practical limitations were encountered with the rat model which may limit its applicability in researching new treatments. The rat supraspinatus tendon is too deep to be amenable to most non-invasive measurement techniques (e.g. laser doppler flowmetry or ultrasound), biomechanical testing using currently available technologies requires dissecting the specimen which introduces a large degree of uncertainty into the results, and it is too small to be readily amenable to drug, exercise or cellular implantation treatments. Also, there was no way of assessing whether the rats were experiencing any pain as a result of the exercise intervention. Finally, there is little control over the amount of stress and strain the supraspinatus tendon is placed under.

The rat shoulder demonstrates some marked differences from its human counterpart which may be relevant when discussing injury mechanisms, or the lack of substantial injury observed in the current series of rat experiments. Like other quadrupeds, the scapulae of the rat are aligned vertically, resting parallel on either side of the thorax, with the glenoid fossa pointing downwards [98]. Glenohumeral movement occurs almost entirely in the sagittal plane [98]. During typical gait cycle, the shoulder appears to reach just less than 90 degrees of elevation, although this measurement may not reflect glenohumeral motion alone [103]. Thus, despite the anatomic similarities highlighted by Soslowsky et al, including the fact that the supraspinatus tendon is prominent and passes repetitively underneath the acromion during daily activities, the differences are profound and include a reduced range of elevation and rotation compared to humans [52], both of which have been implicated in the development of rotator cuff injury [69].

In contast to the above limitations, the Backman model involves application of repetitive electrical stimulus to the rabbit calf musculature, and results in pathology analogous to human Achilles tendinosis within 6 weeks of training, 2 hrs/day, 3 times per week [13, 14]. In addition to solving the above practical problems in terms of its suitability as a model for testing treatments (discussed below) and greater control over the type and amount of loading, it offers interesting insights into pathophysiology which are relevant to the current discussion. By 6 weeks, tenocyte rounding and collagen matrix degeneration were observed involving areas surrounding multiple tenocytes, particularly in areas closely associated with paratendon inflammation. Macroscopically, the paratendon showed signs of scarring (i.e. fibrosis) and thickening, frequently with a

nodule in the midportion of the Achilles, as commonly seen in the human Achilles [13, 14]. Thus, the Backman model appears to more closely approximate the human condition of tendinosis in several respects. Future work with this model should help clarify the contributions of angiogenesis and inflammation-repair events to tendon overuse pathology which have been identified in the current set of studies.

CONCLUSIONS

1. Patellar tendinosis tissue demonstrated increased quantities of versican in the extracellular matrix compared to normal tendon. Versican was frequently associated with evidence of microvascular proliferation, which suggests that vascular proliferation is associated with extracellular matrix abnormality in tendinosis. This suggests that therapies which specifically target tendon vessels may be capable of influencing the tendon extracellular matrix, and that increased versican expression may represent a marker for tendinosis pathology and/or response to treatment.
2. Patellar tendinosis tissue demonstrated increased mast cell density compared with normal tendon. This evidence supports further research to test specific hypotheses regarding the potential roles of mast cells in regulating vascular function or vascular density in tendinosis, which could potentially lead to the use of mast cell inhibitors as a treatment for tendinopathy.
3. A subset of patellar tendinosis biopsies demonstrated VEGF expression, which was absent from normal adult patellar tendon. Patients with VEGF+ tendons demonstrated a shorter symptom duration than those with VEGF- tendons. This

suggests that VEGF expression may play a role in regulating angiogenesis following the development of tendon overuse injury, and that its expression may be temporally regulated. Whether blocking VEGF expression would be harmful or beneficial in the setting of early tendon overuse injury will be the focus of future experiments.

4. Human tenocytes express VGluT2, but there is no evidence of VGluT2 expression in nerve or vascular structures in human Achilles and patellar tendons. Thus, tenocytes, as opposed to nerves, may play a key role in regulating extracellular glutamate concentrations within tendon. Tenocyte-derived glutamate could impact on a variety of previously unexplored autocrine and paracrine functions within tendon including blood flow, nociception or sensitization, and tenocyte proliferation, apoptosis or mechanotransduction.
5. In rats, downhill running resulted in increased IGF-I expression and ERK-1/2 phosphorylation among proliferating tenocytes in the supraspinatus tendon. This finding highlights the importance of load-induced IGFI-I signaling in the response of tendon to increased mechanical demand. ERK-1/2 activation in loaded tendon may represent a means by which diverse signals, both mechanical and soluble, may influence key tenocyte behaviours such as proliferation, death and survival.
6. Achilles tenocytes underwent apoptosis and necrosis in response to prolonged hypoxia. Hypoxia, if it is present in injured tendons for prolonged periods of time or in concert with other environmental stresses, may contribute to the increased rates of cell death observed in tendinosis lesions.

7. IGF-I exerted a pro-survival effect including rapid phosphorylation of PKB in cultured tenocytes exposed to hypoxia. Thus, IGF-I expression in tendon may represent a physiological adaptation which supports tenocyte survival. In extensively injured tendons where apoptosis may play a role in reducing the repair potential, IGF-I administration may be beneficial.

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Certificate of Expedited Approval

Clinical Research Ethics Board Official Notification

PRINCIPAL INVESTIGATOR	DEPARTMENT	NUMBER
Miran-Khan, K.	Family Practice	**C05-0425**

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

UBC Campus, Vancouver Coastal Health Authority

CO-INVESTIGATORS:

Duronio, Vincent, Medicine; Guy, Pierre, Orthopaedics; Scott, Alexander, Medicine

SPONSORING AGENCIES

Canadian Institutes of Health Research

TITLE :

Pathogenesis of Tendinosis: Clinical and Laboratory Studies

APPROVAL DATE	TERM (YEARS)	DOCUMENTS INCLUDED IN THIS APPROVAL:
31 August 2005	1	Protocol version date 14 August 2005; Subject Consent Form version 2 dated 30 August 2005

CERTIFICATION:

In respect of clinical trials:

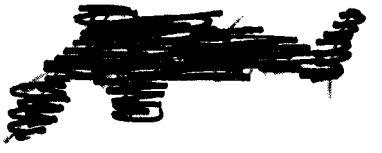
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.

2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.

3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the Chair of the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

The CREB approval for this study expires one year from the approval date.



Approval of the Clinical Research Ethics Board by one of:
Dr. Gail Bellward, Chair
Dr. James McCormack, Associate Chair