Tendon Cell Behavior and Matrix Remodeling in Degenerative Tendinopathy

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Tendon Cell Behavior and Matrix Remodeling in Degenerative Tendinopathy

Peescelgedrag en matrix remodellering in degeneratieve tendinopathie

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Voor mama

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List of Abbreviations

α-SMA	alpha smooth muscle actin
AA	Achilles tendon tissue adjacent to tendinotic lesion
AGC1	aggrecan
AGE	advanced glycation endproduct
AH	healthy Achilles tendon tissue
AT	tendinotic Achilles tendon tissue
BGLAP	bone gamma-carboxyglutamate = osteocalcin
BMSC	bone marrow-derived stromal cell
CBFA1	core-binding factor 1 = RUNX2
COL10A1	collagen type X, alpha 1 chain
COL2A1	collagen type II, alpha 1 chain
D7-FIB	11-fibrau, fibroblast antibody
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EGF	epidermal growth factor
FABP	adipocyte fatty acid binding protein
FACS	fluorescense activated cell sorter
FCS	fetal calf serum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
HGF	hepatocyte growth factor
HP	hydroxylysylpyridinoline
HPLC	high-performance liquid chromatography
Hyl	hydroxylysine
Hyp	hydroxyproline
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
LP	lysylpyridinoline
MEC	medical ethical committee
MMP	matrix-metalloproteinase
OA	osteoarthritis
PDGF	platelet-derived growth factor
PPARG	peroxisome proliferative activated receptor gamma
PPCR	platelet-poor clot releasate
PPP	platelet-poor plasma

PRCR	platelet-rich clot releasate
PRP	platelet-rich plasma
QPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
RFU	relative fluorescence unit
RT-PCR	reverse transcriptase polymerase chain reaction
RUNX2	RUNT-related transcription factor 2 = CBFA1
SOX9	SRY-box 9
SP7	Sp7 transcription factor = osterix
TDF	tendon-derived fibroblast
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
VEGF	vascular endothelial-derived growth factor A

Chapter 1

General Introduction

TENDINOPATHY

Tendon injuries are common in human athletes [1-4]. Furthermore, such injuries are also prevalent in the ageing sedentary population [5-7]. In recent decades, the incidence of tendon injuries has risen due to both an increase in an elderly population and a rise in participation in recreational and competitive sporting activities. In the general population the lifetime cumulative incidence of Achilles tendinopathy is 5.9 % among sedentary people and 50 % among elite endurance athletes [2]. Despite the high frequency, there are still many unsolved questions and differences of opinion concerning pathology, etiology, and even terminology.

Until several years ago the most often used word for tendon disease in the clinical practice of orthopaedic and sports medicine was 'tendonitis/ tendinitis', literally meaning tendon inflammation, reflecting the general idea that overuse tendinopathies were due to inflammation. However, this common wisdom was challenged by that time, as the histopathological feature usually described in tendinopathies was a degenerative process and inflammation was not typically seen [8-11]. Therefore Nicola Maffulli suggested to use the term 'tendinopathy' as a general descriptor of the clinical conditions in and around tendons arising from overuse [12, 13]. In addition the term 'tendinosis', literally meaning tendon degeneration, should be used after histopathological examination. This nomenclature is gradually being integrated now in research communication and clinicial practice.

The clinical presentation of tendinopathy is characterized by a combination of pain, swelling, and impaired performance. A variety of tendons in humans may be affected including the supraspinatus tendon in the shoulder, the forearm extensor and flexor muscle tendons in the elbow, and the Achilles tendon and the patellar tendon in the lower limb. The respons of tendinopathy to the currently available treatment options is often unsatisfactory requiring lengthy periods of rehabilitation or even surgical intervention [14, 15].

TENDONS

Function

Tendons are most often thought of as the bright white, parallel fibred connective tissues that join muscles to bones. While they exert no pulling force of their own, they enable locomotion by transferring muscle contractions to the bones. Energy storing tendons, like the Achilles tendon [16], not only transmit muscle-generated tensile forces to bone in order to move joints, but they additionally act like springs to store elastic energy, which enhances the efficiency of locomotion.

Structure

Tendons consist of highly specialized connective tissue, which is characterized by densely packed hierarchically arranged collagen filaments embedded in a hydrophilic matrix with a relatively small volume of cells. The main structural protein is tropocollagen which consists of three helically arranged polypeptide chains. Five of these tropocollagen molecules unite into a microfibril. These are organized together into larger longitudinal bundles. According to their size these are called subfibrils (primary fibre bundles), fibrils (secundairy fibre bundles), fascicles (tertiairy fibre bundles), and the tendon itself (Figure 1.1). The epitenon, a fine, loose connective-tissue sheath containing the vascular, lymphatic, and nerve supply to the tendon, covers the whole tendon and extends deep within it between the tertiary bundles as the endotenon. Superficially, the epitenon is surrounded by the paratenon, a loose connective tissue consisting of mainly collagen fibrils, elastin fibrils and an inner lining of synovial cells. Depending on its anatomical location and function a tendon can be covered by synovial sheaths and fibrous sheaths. The synovial sheath produces synovial fluid, the fibrous sheaths may form condensations, the pulleys, which act as fulcrums to aid tendon function. Vascularisation comes from the paratenon, the musculotendinous junction, and the osteotendinous junction. However tendons are sparsely vascularised, hence their white appearance.

Composition

The main extracellular matrix (ECM) component of tendons is collagen (approximately 65-80% of the dry weight). Collagen type I is the predominant type (around 95% of the total collagen) [18, 19], with small quantities of collagen type III (around 3% of the total collagen)[19] and minimal concentrations of collagen type II, IV, V, VI, IX, X, XI, XII, and XIV [20]. Collagens in the matrix

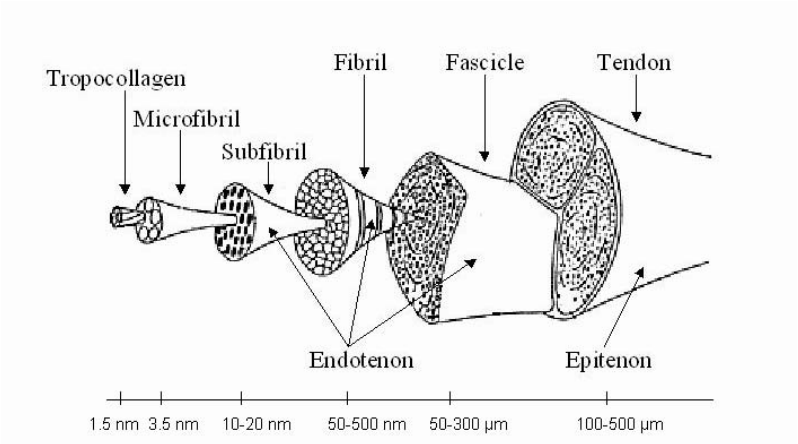

Figure 1.1 *Schematic picture of the hierarchical structure of tendon tissue (modified from Kastelic et al., 1978 [17]).*

are stabilized by cross-links. These cross-links influence the mechanical properties of the tendon tissue. Some cross-links are formed after enzymatic modifications. The best characterised of these are the hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links. Tendons have high HP content compared to other connective tissues. The amount of cross-links in a tendon is related to its mechanical function [21]. A greater cross-link concentration is generally found in compressed tendons associated with the fibrocartilaginous composition found at these sites [21, 22]. A second mechanism of intermolecular cross-linking of collagen is via non-enzymatic reactions with glucose. A well-identified non-enzymatic glycation endproduct is pentosidine. Pentosidine cross-links can be used to asses the remodeling rate and biological age of collagen networks because of the relatively slow collagen turnover rate and the linear time-related increase of spontaneously formed irreversible pentosidine cross-links [23, 24].

The major non-collagenous constituents of tendons are water (around 70% of the wet weight) and proteoglycans. The strongly hydrophilic glycosaminoglycan molecules attract water molecules and the collagenous network prevents the tissue from expanding beyond a certain limit, thus giving the tissue its strength. Differences in proteoglycan composition within and between tendons have been described during maturation and ageing, after immobilisation and exercise, in response to compressional mechanical forces, and after tendon injury [22, 25, 26].

Tenocytes are sparsely distributed within the ECM, constituting about 90-95% of the cell population. They can be considered highly specialized fibroblasts, but their phenotype has been poorly defined to date [27]. They are responsible for the production and maintainance of all the ground substances of the extracellular matrix. Adjacent cells are connected via large cytoplasmatic extensions with gap junctions, which are thought to be involved in effecting a coordinated cellular response to the environment. The cells can change their metabolic activity with respect to production of extracellular matrix components and matrix degrading enzymes [28]. For instance different types of loading (compression, tension) elicit different types of response [25, 29, 30].

The Achilles tendon

The thickest and strongest tendon in the body is the Achilles tendon (Figure 1.2, see also color inlay). It is an energy-storing tendon [16] that is subjected to tensional forces as large as 6-12 times the body weight [31, 32]. It originates from the calf muscles (gastrocnemius and soleus) where collagen fibers from within the muscles are continuous with those of the tendon. It is about 15 cm long and is inserted into the posterior surface of the calcaneus where its collagen fibers are mineralized and integrated into bone tissue. The tendon spreads out somewhat at its distal end so that the narrowest part, also called the mid-portion, is about 4-6 cm proximal to the insertion site. Along their run from proximal to distal the tertiairy bundles twist approximately 90 degrees around the tendons longitudinal axis, the largest part of this twist taking place in the mid-portion area [33]. The mid-portion area is also the most hypovascular region

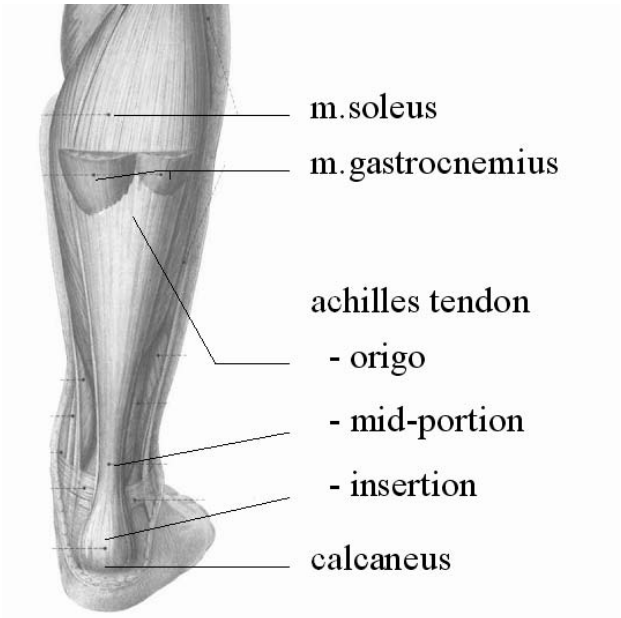

Figure 1.2 *Anatomy of the Achilles tendon (adapted from Wolf-Heidegger's Atlas of Human Anatomy, 4th edition). (See color inlay for a full color version of this figure.)*

of the Achilles tendon, hence also called the watershed region [34]. Besides insertional Achilles tendinopathies especially this mid-portion area of the Achilles tendon is frequently subject to degenerative pathology [1, 11, 14].

PATHOPHYSIOLOGY OF TENDINOPATHY

Histology

Histological alterations described in tendinotic lesions include an abnormal fiber structure and fiber arrangement, variations in cellular distribution with hypercellular and hypocellular areas, rounding of the tenocyte nuclei, decreased collagen stainability, increased GAG-stainability, and increased vascularity (Figure 1.3, see also color inlay)[8-11]. Occasionally signs of bleeding, fibrin deposits, calcifications, and lipid accumulations can be seen. Inflammatory cell infiltrates and acellular necrotic areas are exceptional and not regarded as normal elements of the degenerative process [9].

Biochemistry and molecular biology

There have been relatively few biochemical and molecular studies of tendinopathy, and most of them have been of material collected at the endstage after tendon rupture. Nonetheless, the

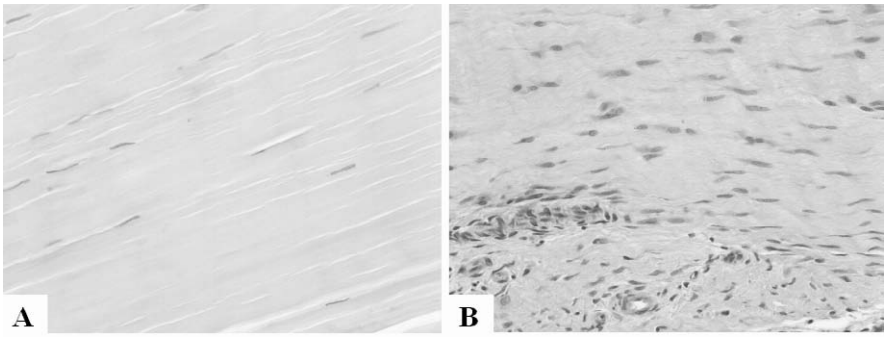

Figure 1.3 *Histological picture of (A) healthy and (B) tendinotic Achilles tendon tissue (Magnification 200x). (See color inlay for a full color version of this figure.)*

histological absence of inflammation in the chronic phase was confirmed using microdialysis techniques [35] and cDNA microarrays [36]. Due to lack of material from early tendinopathy possible inflammatory events in the early stages cannot be excluded yet.

The importance of matrix turnover in tendon pathology was demonstrated in many ways. In degenerative supraspinatus tendons a small but significant decrease in total collagen was found, with an increased proportion of type III collagen relative to type I, resembling fibrotic repair tissue [19]. A decreased accumulation of non-enzymatic pentosidine cross-links was found in supraspinatus tendinopathy suggesting a relatively high matrix remodeling rate linked to the onset of degenerative pathology [21]. Concurrent with the biochemical data, on gene expression level a large increase was found in collagen type I and III expression [36]. Also changes in gene expression level and activity of members of the matrix-metalloproteinase (MMP) family of matrix degrading enzymes were found [37-39].

It has been long recognized that in tendinopathy the amount of glycosaminoglycans is increased and the composition of these non-collagenous matrix components is changed towards a fibrocartilaginous composition [8-11, 22, 26]. These changes in tendinopathy have been considered a functional adaptation to compressional loading in these areas [25, 40-43] although it may have negative effects on the tendons tensional strength and ability to repair after injury [22].

Hypotheses

Extensive research during the past decades regarding the aetiological factors, the tissue alterations, and the cellular behavior associated with degenerative tendinopathy has led to the formulation of three main hypotheses regarding the pathophysiology, all three with proposedly synergistic interactions contributing to the disease:

1. Contribution of mechanical overuse (repetitive overload) [5, 44]

Classically, the aetiology of tendinopathy has been linked to the performance of repetitive activities, eliciting an overuse injury. A proposed algorithm for the onset of tendinopathy involves repetitive tensional loading with repeated strains below the injury threshold of the tendon, inducing microdamage. The microdamage and subsequent processes to repair the matrix composition and organisation cause a transient weakness of the tissue, which makes the tissue more susceptible to damage from continued loading. This damage then accumulates until the overt pathology of tendinopathy develops. Increased exercise levels increase the amount of microdamage. Ageing alters the tendons mechanical properties making the tissue more prone to microdamage. Poor vascularisation and low tenocyte metabolism contribute to the overload pathology by extending this vulnerable repair period. This theory explains how chronic repetitive damage to tendons could accumulate over time and perhaps why tendinopathy would be degenerative and not inflammatory in nature. However, it does not explain the pain associated with tendinopathy or the prevalence of tendinopathy in the sedentary population. Also it is somewhat counterintuitive that load well within the physiological range can actually harm the tendon.

2. Contribution of ageing [5, 45]

Tendon mechanical properties deteriorate with ageing. Tendons become stiffer and less elastic. There is substantial decrease in the solubility of the collagen, thought to be associated with the accumulation of advanced glycation endproduct (AGE) cross-links, such as pentosidine. Accumulated physical damage is seen in ageing tendons, with increased amounts of denatured collagen and increased proteolytic cleavage of matrix components. These changes are all associated with deterioration in the physical properties of the tendon. Also, tendon blood flow declines with age and after skeletal maturity the tendon cells appear to decrease matrix synthesis. More importantly, the cells decrease responsiveness to loading or exercise regarding their matrix synthesis. Though degeneration is not an inevitable consequence of ageing, the tissue changes in ageing tendons might at least partially explain the increased prevalence of degenerative changes associated with ageing. However this theory does not fully explain why certain ageing individuals do get tendinopathy and others do not. A synergistic effect of ageing and exercise in initiating tendon degeneration has been proposed.

3. Contribution of vascularisation [44, 46, 47]

During development, tendons are highly cellular and metabolically active and supplied with a rich capillary network. Mature tendons however are sparsely vascularised, hence their white appearance. The vascularity is even more compromised at junctional zones, and sites of torsion, friction, or compression. For example two hypovascular regions of the Achilles tendon are the insertional area and the mid-portion area. These avascular zones are commonly associated with degeneration and rupture. Furthermore tendon blood flow generally declines with increasing

age and mechanical loading. In many cases of chronic tendinopathy there is an angiofibroblastic response. The vasculoneural ingrowth may be a contributory factor to the pain in tendinopathy. This theory may explain why tendons have specific vulnerable sections and explain the relatively high prevalence of tendinopathy in hypovascular tendon regions like the mid-portion of the Achilles tendon. However, the role of neovascularisation in the pathology remains poorly understood.

TREATMENT OF TENDINOPATHY

Current treatments

There are numerous different treatment types in the management of tendinopathy. No good evidence so far is presented for physical therapies (cryotherapy, therapeutic ultrasound, and low-intensity laser treatment), popular manual therapies (deep transverse friction massage and soft tissue mobilisation stimulating blood supply), and biomechanical alterations (heelpads and other orthotics). Some pain relief may be achieved by oral and local non-steroidal anti-inflammatory drugs, possibly independent of the anti-inflammatory action. However, many consider the use of local intratendinous corticosteroid injections contraindicated given the high risk of spontaneous rupture of the tendon post-injection, particularly at the Achilles tendon. Injection of heparin, dextrose, aprotinin, or sclerosing agents also remains controversial. There is little evidence of benefit from extracorporeal shock wave therapy for tendon conditions other then calcifying tendinopathy of the shoulder and chronic heel pain [48, 49]. Topical application of glyceryl trinitrate showed improvement at 6 months, but these results are yet to be repeated [50]. Eccentric training for the treatment of mid-portion Achilles tendinopathy has gained popularity following recent successful randomized controlled trials [51, 52]. The success of this treatment for Achilles tendinopathy has led to efforts to see whether the results can be extended to other tendon disorders like the patella tendon and supraspinatus tendon. Early results look promising [53, 54], but why the treatment is successful remains uncertain.

The Dutch guidelines for treatment of mid-portion Achilles tendinopathy formulated in 2007 suggest maximal conservative treatment for a minimal duration of 6 months with at least 3 months of eccentric exercises [55]. After this period 20-25% of the patients will have not returned to their original exercise level and surgical treatment can be considered. Surgical intervention may include debridement of the lesional area, multiple longitudinal tenotomies, and cleaving of the peritendineum. Percutaneous tenotomy resulted in 75% of patients reporting good or excellent results at 18 months follow-up [56]. Open surgery for Achilles tendinopathy has shown that 67 % had returned to physical activity at 7 months after surgery [57].

In summary, tendinopathy responds poorly to the currently available treatment options of which very few are evidence-based. The nowadays abundant amount of treatment options for

tendinopathy inversily reflects the little amount of evidence regarding the working mechanisms and success rates of the practiced interventions.

Future treatments

As a result of the deficiencies of current treatments there is a great interest in investigating new therapies. Two treatments that hold promise for the future of tendon disease management are injection of autologous platelet-rich plasma (PRP) and a tissue engineering approach to regenerate healthy tendon tissue.

Clinical applications of autologous PRP in human medicine include periodontal and maxillofacial surgery, plastic surgery, treatment of bone fractures, and treatment of chronic skin and soft tissue ulcers. Numerous publications on PRP yielded excellent clinical outcomes [58, 59]. The only published cohort study in tendon research reported 93% reduction of pain for PRP-treated patients with chronic elbow tendinosis [60]. In natural healing processes in the body, platelets actively participate by rapidly releasing a variety of growth factors. PRP might provide an autologous source of these growth factors that play a key role in tendon repair mechanisms. Both controlled clinical studies and in-vitro studies are required to investigate in detail the possibly beneficial effects of PRP in tendon disorders.

Cell therapy with use of stem cells represents a potentially exciting alternative treatment in the future of tendon management. Whilst healing was seen when mesenchymal stem cells seeded in a collagen matrix were placed in a rabbit Achilles tendon defect, on histology the new cells exhibited morphology more similar to fibroblasts than tenocytes [61]. This illustrates the major challenge in tendon tissue engineering: mastering the knowledge on what drives a stem cell or fibroblast towards tenogenic differentiation and how to provide these biomechanical, biochemical, or biophysical cues in vivo or in vitro to facilitate healthy tendon tissue development or tendon repair. Early results in this research area are exciting and highlight the potential of cell therapy for tendinopathy in the future [62, 63].

AIMS AND OUTLINE

Tendinopathy often responds poorly to the currently available treatment options requiring lengthy periods of rehabilitation or even surgical intervention. The increasing scientific interest in tendon disorders and the concurrent application of biochemical and molecular techniques has led to rapid developments in the understanding of degenerative tendinopathies in the last decades. We believe that more insight in the behavior of the tendon cells and the biomechanical, biochemical, and biophysical signals that influence their conduct is a *conditio sine qua non* for the development of more effective *mechanism-based* therapeutical interventions for both prevention and repair of degenerative tendon lesions. The work in this thesis aims to improve fundamental knowledge of tendon cell behavior and matrix remodeling in tendinopathy, both

during the disease process as well as in reaction to treatment modalities. The focus in this research is on mid-portion Achilles tendinopathy.

We started out purely describing the changes observed in cell behavior, biochemical composition, and collagenous matrix turnover rate in vivo (**chapter 2**). We collected tendinosis biopsies and macroscopically healthy tendon biopsies adjacent to the lesion from patients undergoing surgery for mid-portion Achilles tendinopathy and healthy tendon biopsies from donors with asymptomatic Achilles tendons. The results were compared with findings in literature regarding supraspinatus tendinopathy.

From here hypotheses regarding pathogenic processes that might play a role in the development and persistance of tendinopathy were explored in vivo and in vitro. Firstly, we were intrigued by the analogy between tendon and cartilage tissue in both healthy and degenerative state. Therefore in **chapter 3** we hypothesized a role for Toll-like receptors (TLRs) in tendinopathy, a role that might be quite similar to their role in osteoarthritis and their even more distict role in rheumatoid and inflammatory arthritis. We explored this idea by examining both in vivo mid-portion Achilles tendinopathy samples as well as by performing in-vitro culture experiments stimulating healthy tendon explants with inflammatory mediators.

Mid-portion Achilles tendinotic lesions display an increased amount of glycosaminoglycans, resembling cartilage tissue in this aspect, and sometimes also contain calcifications and lipid accumulations. We hypothesized that alterations in tendon cellular differentiation might contribute to these tissue changes associated with tendinopathy. In **chapter 4** we studied whether a population of cells with intrinsic differentiation potential is present in tendon tissue. We performed in-vitro experiments with tendon-derived cells from non-degenerative human tendon tissue trying to differentiate these cells towards cells with chondrogenic, adipogenic, or osteogenic characteristics.

Considering the excessive or inappropriate fibrocartilaginous matrix production in tendons as a remarkable feature of the pathological process in mid-portion Achilles tendinopathy, we were especially interested in chondrogenic differentiation possibilities of the native tendon cells. In **chapter 5** this chondrogenic differentiation pattern was studied in mid-portion Achilles tendinopathy in vivo. Reasoning that opposing or removing the stimulus that causes the metaplasia in the diseased tendons might help the tenocytes to return to their normal tendon matrix production, we developed a tendon explant culture model to induce a chondrogenic differentiation quite similar to the in-vivo situation in tendinopathy. We explored the usefulness of this model to investigate early chondrogenic differentiation as a possible target for drug treatment of tendinopathic lesions. We also studied the effects of adding triamcinolone or platelet-rich plasma (PRP) to the chondrogenic differentiation model.

The following chapter (**chapter 6**) explored in further detail the working mechanisms of this platelet-rich plasma (PRP), being considered in the present literature as a clinically promising treatment intervention for tendinopathy although the exact effects on tendon cell behavior

are not known yet. We studied the effects of platelet-rich plasma (PRP) on the metabolism of explanted human tendon cells in-vitro.

In the final chapter (**chapter 7**) the main results described in this thesis are discussed in relation to eachother and in the context of current scientific knowledge. Also, certain limitations of the in-vitro work are summarized. Suggestions for follow-up experiments and future research directions are given throughout this general discussion section.

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Chapter 2

Achilles tendinosis: changes in biochemical composition and collagen turnover rate

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ABSTRACT

Background

Understanding of biochemical and structural changes of the extracellular matrix in Achilles tendinosis might be important for developing mechanism-based therapies.

Hypothesis

In Achilles tendinosis changes occur in biochemical composition and collagen turnover rate.

Methods

From ten patients undergoing surgery for Achilles tendinopathy, one tendinosis biopsy (AT) and one biopsy of macroscopically healthy tendon tissue adjacent to the lesion (AA) were collected. Furthermore, biopsies were collected from three donors with asymptomatic Achilles tendons (AH). Water content, collagen content, percentage of denatured collagen, amount of lysine hydroxylation, number of enzymatic and non-enzymatic cross links, matrix metalloproteinase (MMP) activity, and MMP and collagen gene-expression levels were analysed.

Results

In AT lesions the water content was highest, collagen content was subnormal with higher amounts of denatured/damaged collagen. Low pentosidine levels in AT tissue indicated the presence of relatively young collagenous matrix. More hydroxylated lysine residues were present in AT samples, but enzymatic crosslinks revealed no differences between AT, AA, and AH samples. In AT specimens MMP activity was higher, MMP gene-expression profile was altered, collagen type I and III gene expression were upregulated.

Conclusions

In Achilles tendinosis the collagen turnover rate is increased and the natural biochemical composition of the collagenous matrix is compromised.

Clinical Relevance

Although tendon tissue directly adjacent to an Achilles tendinosis lesion looks macroscopically healthy, histological and biochemical degenerative changes in adjacent tissue are evident, which may have implications for surgical interventions.

INTRODUCTION

Tendinopathy is a tendon disorder that occurs most frequently in athletes and middle-aged people [20]. Tendinopathy of the mid-portion of the Achilles tendon is one of the main causes of chronic Achilles tendon pain [5, 7]. Generally, treatments of Achilles tendinopathy are conservative and is mainly aimed at relief of symptoms, with frequently unsatisfying results due to a remarkable lack of knowledge concerning the underlying pathological mechanisms [24, 28]. On one hand, a prostaglandin-mediated inflammatory cascade does not seem to play a major role in the pathogenesis of tendinopathy [2]. Moreover, there is growing evidence that tendinopathy of the Achilles tendon mid-portion is often the clinical result of multiple degenerative processes in the tendon matrix called tendinosis [25]. In order to develop a mechanism-based therapy, it is crucial to obtain a better insight in the biochemical and structural changes of the extracellular matrix that are involved in the pathogenesis of Achilles tendinosis.

Maintenance and regeneration of the physiological biochemical composition of the collagenous and non-collagenous extracellular matrix is essential for optimal structure and function of the tendon [29]. There have been some studies on the compositional changes of the tendon in supraspinatus tendinopathy. However, there are no such studies available on the difference in biochemical collagen composition between healthy and degenerated mid-portion Achilles tendon tissue.

Assumptions on the biochemical collagen compositional changes in Achilles tendinosis can only be extrapolated from supraspinatus tendinopathy studies. This extrapolation might be justified by the observation that histological features in Achilles tendinosis and supraspinatus tendinopathy are similar [7, 16, 22, 26]. The alterations described in supraspinatus tendinopathy include: 1) a significantly increased water content [32]; 2) a small, but significant decrease in total collagen content [11, 32]; 3) an increased proportion of type III collagen compared to type I collagen [32]; 4) an altered mode of collagen crosslinking, namely an increase in the amount of hydroxylysine residues per collagen triple helix, and an increase of the enzymatic cross links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) [11], which is also reported in fibrotic tissue [36]; 5) a significant decrease in the amount of non-enzymatic pentosidine cross links per collagen molecule, which can be interpreted as a young biological age of the collagenous tissue [11]. All these findings taken into account, it seems likely that in supraspinatus tendinopathy the previously functional and carefully constructed native matrix is replaced by an aberrant collagen network.

The process of collagen network turnover is a dynamic equilibrium between synthesis and degradation [29]. Degradation of the extracellular matrix (ECM) is principally mediated by the enzymatic activity of matrix metalloproteinases (MMPs). MMP activity is the product of MMP synthesis, activation, inhibition, and degradation. MMP gene expression levels have been measured in healthy, tendinotic, and ruptured Achilles tendon [3, 19, 21, 27], but the actual MMP activity has not.

We hypothesized that changes in biochemical collagen composition and collagen turnover rate were present in Achilles tendinosis lesions. We report the results of a comparison between (1) Achilles tendinosis lesions, (2) less affected Achilles tendon tissue surrounding those lesions in the same individual, and (3) healthy Achilles tendon tissue, concerning histology, total water content, biochemical collagen composition (including total collagen content, percentage of degraded collagen, number of enzymatic and non-enzymatic cross links), MMP activity, and gene expression levels of MMPs and collagens.

MATERIALS AND METHODS

Patient characteristics and sample collection

Tissue specimens of 10 consecutive patients with chronic mid-portion Achilles tendinopathy were harvested during surgical debridement. Chronic Achilles tendinopathy was defined as Achilles tendon pain for at least 3 months in combination with clinically and / or radiologically (either MRI or ultrasonography) suspected mid-portion Achilles tendinopathy. Only patients without previous tendon injury, without radiologically evident partial tendon rupture, without use of chinolone antibiotics in the past three years, and with sufficient biopsy material to perform all analyses were included. At surgery, one biopsy specimen was taken from the macroscopically affected tendinotic lesion (AT) and a second biopsy specimen was taken from macroscopically healthy tendon tissue adjacent to the lesion (AA). Approval for this study was obtained from the Medical Ethical Committee of the Erasmus MC University Medical Center (MEC-2005-100). All patients signed informed consent.

Of the ten patients that underwent surgical debridement for mid-portion Achilles tendinopathy, five patients were male and five were female. The average age was 46 years (range 36-58). Seven of ten patients underwent surgery for their left Achilles tendon and three patients for their right Achilles tendon. Mean duration of symptoms was 22.5 months (range 9-48 months). Five of ten patients had contralateral tendon complaints as well. Two patients were competitive athletes before the beginning of symptoms (skating, football), seven were recreational athletes (swimming, running, squash, tennis, fitness), and one patient did not participate in any sports activity. Three of nine athletes had reduced their sports activities whereas six of nine had completely stopped. All patients underwent at least 3 months of eccentric training as described by Alfredson et al [4]. Although local corticosteroid treatment for conservative treatment of Achilles mid-portion tendinopathy is not recommended because of elevated risk of rupture 15, 34, two of ten patients (patient number 7 and number 9) reported to have received one corticosteroid injection for their Achilles tendon pain in the course of pre-operative treatment by a physician not related to this study.

Healthy Achilles tissue specimens of 3 donors (1 man, 2 women; average age 58, range 25-78) without clinically evident Achilles tendinopathy were collected as control (AH) (MEC-2006-069).

Surgical procedures for these patients included arthrodesis of the talocrural joint, upper leg amputation because of a septic revised total knee prosthesis, and extension of the Achilles tendon for spastic diplegia.

Tissue preparation

Specimens were divided into four samples for separate analyses, such as histology, biochemistry, and gene expression analyses. For histology, tissue was fixed overnight in 10% formalin and then embedded in paraffin. For the biochemical analyses, two wet tissue samples of each specimen were weighted, frozen, and then freeze dried until no further weight change was recorded. The water content of the tendon specimens was calculated as follows: (wet weight - dry weight) / wet weight x 100%. For gene expression analyses, tissue was snap frozen and stored at -80°C until further use.

Histology

Longitudinal sections (6 µm) of the paraffin embedded tendon samples were stained with haematoxylin and eosin or with thionin (for glycosaminoglycans). Two different researchers performed a blinded examination of the slides using a modified semiquantitative grading scale for tendinosis adapted from Åström et al. [7], including subscores for fiber structure / arrangement, regional differences in cell density, roundness of cell nuclei, collagen stainability, and GAG-stainability. Each item was scored 0 (normal), 1 (mildly deviant), 2 (moderately deviant), or 3 (severely affected). The scores of the two examiners were added up yielding a total sum of 0 for minimal histological severity and 30 for maximal severity.

Collagen composition

1. Collagen content, hydroxylysine, and cross links

After MMP extraction, tendon samples were hydrolyzed (108° C, 18–20 h) with 6 M HCl for high-performance liquid chromatography (HPLC) of amino acids (hydroxyproline (Hyp) and hydroxylysine (Hyl)), enzymatic collagen cross links (HP and LP) and non-enzymatic glycation cross links (pentosidine). The hydrolyzed samples were vacuum-dried and redissolved in an internal standard solution (2.4 mM homo-arginine, 10 µM pyridoxine (Fluka Buchs, Switserland) in water). Tissue collagen levels (Hyp) and Hyl were determined in 250 times diluted hydrolysates after FMOC labelling by reversed-phase HPLC as described before [9]. In the same tissue hydrolysates the HP, LP, and pentosidine levels were determined by reversed-phase HPLC after 5-fold dilution as described before [8]. The total collagen content, expressed as % of dry weight, was calculated assuming 300 Hyp residues per collagen molecule and molecular mass of 300 kDa. HP, LP, and pentosidine levels were expressed as the total amount of residues per collagen molecule.

2. Degraded collagen

The assay for degraded collagen is based on the selective proteolysis of denatured collagen by α-chymotrypsin (αCT) as described before [10]. Briefly, the tendon samples were extracted two times with 1 mL 4M guanidine-HCl to remove proteoglycans and make the tissue more accessible. After removal of the guanidine-HCl the denatured collagen was digested overnight at 37° C using 0.25 mg of αCT. After treatment with αCT, the digested collagen containing supernatant was separated from the remaining insoluble matrix containing the intact collagen. Collagen contents in both supernatant and pellet were determined after acid hydrolysis by measuring hydroxyproline levels using a colorimetric assay as described before [17]. The concentration of degraded collagen was calculated as follows: Hyp supernatant / (Hyp supernatant + Hyp pellet) x 100%.

MMP activity

Tendon tissue was extracted with 200 µL extraction buffer (overnight at 4° C under constant agitation). The supernatant obtained after centrifugation was used for measurement of MMP activity. General MMP activity and MMP3 activity were measured based on methods described previously [12, 13]. Briefly, MMP activity in 4 times diluted tendon extract was determined using fluorogenic MMP-specific substrates TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH2; 5 µM) and TNO003-F (Dabcyl-Gaba-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Cys[Fluorescein]-Gly-Lys-NH2; 5 µM) for general MMP assay and MMP3 assay respectively. EDTA-free Complete™ solution (Roche, Mannhein, Germany; 1 tablet per 10 mL) was added to all conditions to reduce non-MMP substrate conversion. All incubations and measurements were performed in sealed, black, clear-bottom 384-well plates. The increase in fluorescence, which results from cleavage of the substrates was measured for 6 hours at 30° C using Cytofluor 4000 (Applied Biosystems, Foster City, CA, USA) at 485/530 nm (excitation/emission). For both MMP activity assays the substrate conversion rate was determined with and without the selective MMP inhibitor BB-94 (10 µM), the difference between the two was called MMP activity. The amount of MMP activity was expressed as the relative increase in fluorescence per second (ΔRFU/s) per g tendon dry weight.

MMP and collagen gene expression

Specimens were snap frozen and quickly homogenized in a Mikro-Dismembrator® (BioTech International Inc, Needville, Texas) and suspended in 1.8mL/100mg RNA-BeeTM (TEL-TEST, Friendswood, TX, USA). RNA was isolated and purified using RNeasy® Micro Kit (Qiagen, Hilden, Germany), and 1 µg total RNA of each sample was reverse-transcribed into cDNA using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Primers were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet Taqman® or SYBR®Green requirements and were designed to bind to separate exons to avoid co-amplification of genomic DNA. BLASTn ensured gene specificity of all primers listed

Table 2.1 *Primer and probe nucleotide sequences of the tested genes*

Gene	Accession no.	Primer	Probe
18SrRNA	M10098.1	F: AGTCCCTGCCCTTTGTACACA R: GATCCGAGGGCCTCACTAAAC	CGCCCGTCGCTACTACCGATTGG
COL1	NM_000088.3	F: CAGCCGCTTCACCTACAGC R: TTTTGTATTCAATCACTGTCTTGCC	CCGGTGTGACTCGTGCAGCCATC
COL3	NM_000090.3.	F: TACTTCTCGCTCTGCTTCATCC R: GAACGGATCCTGAGTCACAGAC	*
MMP1	NM_002421	F: CTCAATTTCACTTCTGTTTTCTG R: CATCTCTGTCGGCAAATTCGT	CACAACTGCCAAATGGGCTTGAAGC
MMP2	NM_004530	F: TCAAGTTCCCCGGCGAT R: TGTTCAGGTATTGCACTGCCA	TCGCCCCCAAAACGGACAAAGA
MMP3	NM_002422	F: TTTTGGCCATCTCTTCCTTCA R: TGTGGATGCCTCTTGGGTATC	AACTTCATATGCGGCATCCACGCC
MMP9	NM_004994	F: TGAGAACCAATCTCACCGACAG R: TGCCACCCGAGTGTAACCAT	CAGCTGGCAGAGGAATACCTGTACCGC
MMP13	NM_002427	F: AAGGAGCATGGCGACTTCT R: TGGCCCAGGAGGAAAAGC	CCCTCTGGCCTGCTGGCTCA

*F: forward; R: reverse; * SYBR®Green assay.*

in Table 2.1. Amplification was performed in 20 ml reactions using either TaqMan® Universal PCR MasterMix (ABI, Branchburg, New Jersey, USA) or qPCRTM Mastermix Plus for SYBR®Green I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) according to the manufacturer's guidelines. Real-Time RT-PCR (QPCR) was done using an ABI PRISM® 7000 with SDS software version 1.7. Data were normalized to 18SrRNA which was shown to be stably expressed across samples. Relative expression was calculated according to the $2^{-\Delta CT}$ formula [23].

Statistical analysis

To determine sample size, a power analysis was performed using PS Power and Sample Size software version 2.1.31. Calculation was based on the collagen contents measured by Bank et al. in supraspinatus tendons [11]. A difference ($p<0.05$) between the paired AA and AT samples concerning total collagen content could be demonstrated with a power of 0.90 if we used 10 samples per groups.

Further statistical analyses were performed using SPSS 11.0 software (SPSS Inc., Chicago, USA). A Wilcoxon Signed Ranks Test was used to assess the differences between tendinotic (AT) and adjacent (AA) tissue. A Mann-Whitney U Test was used to assess the differences between healthy (AH) and tendinotic (AT), and between healthy (AH) and adjacent (AA) tissue. Significance level was set at $p<0.05$.

RESULTS

Histology

None of the tendon samples showed macroscopically or microscopically evidence of tendon rupture or tendon inflammation. Histological severity of the AA and AT samples ranged from 4 to 24 out of 30, thus representing a range from mild to moderate stages of tendinosis (Table 2.2). Although the tissue specimens adjacent to the lesions were macroscopically healthy, in all patients not only the lesion biopsy but also the adjacent tissue specimen showed histological signs of degeneration. The median histological severity score of the 10 tendinotic (AT) specimens was 18 (range 9-24) and of their adjacent (AA) specimens 14.5 (range 4-19). This difference was statistically significant (p=0.01).

Histological severity of the three healthy (AH) samples ranged from 1 to 4 out of 30, which was significantly different from AT (p=0.011) as well as from AA (p=0.014) samples. Microscopically normal tendon tissue morphology was found in all AH samples: a minimal tendinosis grade, no

Table 2.2 *Histological severity score of tendon specimens of patients (n=10) and controls (n=3: A,B,C)*

Patient	Specimen	Score per examiner		Total score
		I	II	
1	AA	9	10	19
	AT	8	10	18
2	AA	7	8	15
	AT	7	9	16
3	AA	3	5	8
	AT	5	4	9
4	AA	6	8	14
	AT	9	11	20
5	AA	4	3	7
	AT	9	4	13
6	AA	8	8	16
	AT	9	9	18
7*	AA	6	9	15
	AT	9	11	20
8	AA	6	5	11
	AT	6	6	12
9*	AA	3	1	4
	AT	9	9	18
10	AA	10	7	17
	AT	14	10	24
A	AH	1	2	3
B	AH	0	1	1
C	AH	1	3	4

*AH: healthy specimen. AA: biopsy specimen adjacent to tendinosis lesion. AT: tendinosis lesion. *patient received corticosteroid injection before surgery. Total score can range from 0 (normal) to 30 (maximal severity).*

inflammatory cell infiltration, no granulation, no small ruptures, no chondroid metaplasia, and no calcifications were seen.

Total water content

Mean water content, expressed as the percentage of wet weight, was 76.3% in the tendinotic tissue (AT) which was significantly higher than 73.3% in the adjacent less affected samples (AA) (p=0.028). In the three healthy samples (AH) the mean water content was lowest, namely 66.2% (p=0.028 for the difference between AH and AT)(Table 2.3).

Collagen composition

Although the total collagen content per tendon dry weight was not significantly different between AT and AA specimens (p=0.114), a trend was seen towards a subnormal collagen content in the AT specimens (Table 2.3). This trend was largely due to the relatively low collagen content in the tendinotic specimens of patients 2, 7, and 9, compared to their adjacent tissue biopsies (Figure 2.1). The percentage of denatured collagen was significantly higher in AT specimens (Table 2.3). The number of pentosidine cross links per collagen triple helix was significantly lower in tendinotic lesions compared to the adjacent tissue, reflecting an increased

Table 2.3 *Biochemical analyses of healthy Achilles tendons (AH), Achilles tendon tissue adjacent to tendinosis lesion (AA), and Achilles tendinosis lesions (AT).*

Biochemical parameter	Healthy tendon tissue (AH) *n=3* *mean +/- stdev*	Adjacent tendon tissue (AA) *n=10* *mean +/- stdev*		Tendinosis lesion (AT) *n=10* *mean +/- stdev*	
Watercontent *% of wet weight*	66.2 +/- 5.5	73.3 +/- 4.7		76.3 +/- 6.3	† §
Total collagen *% of dry weight*	64.5 +/- 4.6	61.4 +/- 8.1		56.6 +/- 9.7	
Denatured collagen *% of total collagen*	6.00 +/- 7.97	7.51 +/- 2.65		10.40 +/- 2.85	†
Pentosidine *mol/mol collagen triple helix*	0.0172 +/- 0.0127	0.0073 +/- 0.0027		0.0047 +/- 0.0032	†
Hydroxylysine *mol/mol collagen triple helix*	29.2 +/- 3.2	35.9 +/- 2.6	§	37.7 +/- 3.4	† §
HP crosslinks *mol/mol collagen triple helix*	1.10 +/- 0.19	1.30 +/- 0.19		1.26 +/- 0.29	
LP crosslinks *mol/mol collagen triple helix*	0.075 +/- 0.012	0.077+/- 0.026		0.079 +/- 0.032	
MMP activity TNO211-F *ΔRFU/sec/ g of dry weight*	51.7 +/- 21.9	88.2 +/- 107.9		308.6 +/- 392.6	†
MMP activity TNO003-F *ΔRFU/sec/ g of dry weight*	20.1 +/- 22.0	65.8 +/- 43.8		103.9 +/- 64.8	§

ΔRFU: Relative fluorescence unit; p-value<0.05 is considered statistically significant. § p<0.05 compared to AH in an unpaired non-parametric test (mann-whitney u test). † p< 0.05 compared to AA in a paired non-parametric test (wilcoxon signed ranks test)

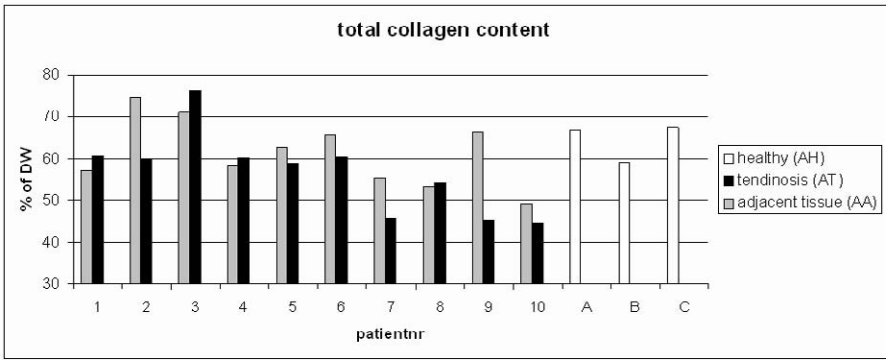

Figure 2.1 *Total collagen content in biopsies of healthy Achilles tendons (AH, n=3), in biopsies adjacent to a tendinosis lesion (AA, n=10), and in biopsies of Achilles tendinosis lesions (AT, n=10). Total collagen content is expressed as % of dry weight. Patient number 7 and 9 reported having received one corticosteroid injection before surgery. Interestingly, compared to the other patients, patient numbers 2, 7, and 9 had a relatively large difference in collagen content between the two biopsy sites.*

remodeling rate of the collagen network with mature collagen being degraded and replaced with newly synthesized matrix (Table 2.3).

The number of hydroxylysine residues per collagen triple helix was also significantly higher in the tendinotic specimens compared to the adjacent specimens. However, the number of HP and LP cross links per collagen triple helix in the tendinotic lesions was not significantly different from the adjacent tendon tissue (all Table 2.3). The HP/LP ratio did not differ significantly between the three conditions (data not shown).

MMP activity

General MMP activity (TNO211-F, which is mainly selective for MMP2, MMP9, MMP13) as well as MMP3 activity (TNO003-F) per tendon dry weight was significantly higher in tendinotic lesions compared to the less affected adjacent tendon tissue or the healthy control samples (Table 2.3).

MMP and collagen gene expression

MMP3 gene expression was significantly downregulated in the tendinotic lesions compared to their adjacent tendon tissue (p=0.007) as well as compared to the three healthy samples (p=0.028). MMP2, MMP9, and MMP13 all showed a near-significant trend towards upregulation in tendinotic lesions compared to adjacent biopsies and the three healthy samples. MMP1 was expressed at very low levels among all samples and no differences between groups were found (all Figure 2.2).

Collagen type I and collagen type III gene expression were upregulated in AT samples compared to the control AH samples (Figure 2.3). No statistically significant differences were

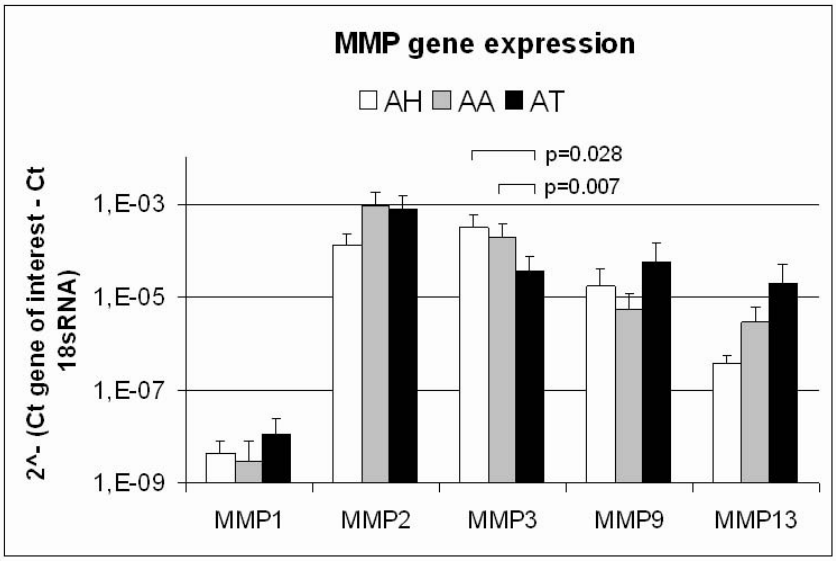

Figure 2.2 *MMP gene expression levels in biopsies of healthy Achilles tendons (AH, n=3), in biopsies adjacent to a tendinosis lesion (AA, n=10), and in biopsies of Achilles tendinosis lesions (AT, n=10).*

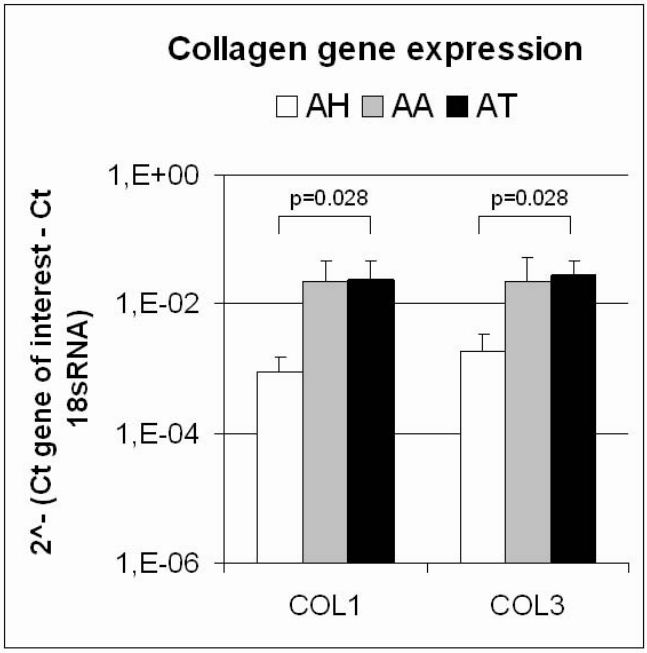

Figure 2.3 *Collagen gene expression levels in biopsies of healthy Achilles tendons (AH, n=10), in biopsies adjacent to a tendinosis lesion (AA, n=10), and in biopsies of Achilles tendinosis lesions (AT, n=10).*

found between AT and AA samples. The ratio between collagen type I and collagen type III gene expression did not differ between the three conditions (results not shown).

DISCUSSION

This comparison of several tissue characteristics between healthy Achilles tendon tissue, tendinotic Achilles tendon tissue, and its adjacent less affected Achilles tendon tissue revealed various differences concerning biochemical composition, MMP activity, and MMP gene expression levels. These differences indicate an aberrant collagenous matrix composition and a relatively high collagenous matrix turnover rate in degenerated Achilles tendon tissue. On one hand, the significant increases of both MMP activity and degraded collagen are signs of increased matrix degradation. On the other hand, higher collagen gene expression levels and a low pentosidine level, reflecting a relatively young collagenous matrix, are indicative for increased matrix synthesis.

Our results are in line with the findings in supraspinatus tendinopathy [11, 32], indicating that both Achilles and supraspinatus tendinopathies result at least partly from similar underlying pathological processes. First, we found an increased water content in tendinotic Achilles tendon specimens, which is consistent with previous biochemical findings in supraspinatus tendinopathy [32]. Probably, the combination of matrix disintegration and an increased water content due to increased amounts of glycosaminoglycans [31], leads to the tissue swelling and signal intensity changes as visualized in MRI images of Achilles tendinopathies [6]. Second, we found lower levels of non-enzymatic pentosidine cross-links in tendinotic Achilles tendon specimens, representing a young collagenous matrix. This was also observed in previous studies of supraspinatus tendinopathy [11]. Other results require more elaboration and/or were different from the supraspinatus tendinopathy studies, and are therefore addressed in more detail in the following paragraphs.

The absence of a significant change in total collagen content in Achilles tendinotic tissue, is consistent with findings in equine superficial digital flexor tendon degeneration [14]. In supraspinatus tendinopathy, however, total collagen content has been reported to be lower than in the healthy specimens [11, 32].

One explanation for this discrepancy might be the different preoperative treatments given to Achilles and supraspinatus tendinopathy patients. In the supraspinatus studies, all patients received at least one local corticosteroid injection before surgery, whereas the control patients (cadaver material from subjects with no known history of tendinopathy or shoulder pathology) did not. In our study, the AT and AA samples were harvested from the same Achilles tendon from the same patient, and had therefore also undergone exactly the same treatment. Of the three patients in our study who had a relatively low collagen content in their tendinotic sample (patients 2, 7, and 9), two patients (patient number 7 and 9) reported having received a local

corticosteroid injection pre-operatively. In-vitro culture in the presence of corticosteroids at pharmacological concentrations decreases collagen accumulation (and proliferation) by tenocytes within two weeks [33]. In this way, a higher tissue turnover rate in AT samples compared to AA samples, as was seen in this study, together with impaired collagen synthesis due to corticosteroid treatment, might lead to lower total collagen content in tendinosis samples subjected to corticosteroids. We are not aware of any literature reporting on the in-vivo effects of local corticosteroids application on collagen content.

An alternative explanation for this discrepancy might be that a change in collagen content does take place during early stages of tendinosis development. In our study, the tissue adjacent to the lesion (AA), despite of being macroscopically normal, was already mildly affected (median histological severity score 14.5, range 4-19). It could thus be argued that in the adjacent samples (AA) the total collagen content had already reached a lower level (see Table 2.3 for mean collagen contents in AH, AA, and AT samples), making it impossible to ascertain a difference between affected (AT) and less affected (AA) tissue. The number of healthy control tissues (AH, n=3) from our extra control group is too small to reach statistically significant differences between healthy and tendinotic tissue concerning this parameter. This number of AH samples was severy limited by our choice of material for healthy tendon tissue. However, we preferred to obtain the fresh tendon tissue instead of post-mortem material for this extra control group because we do not know how the biochemical parameters change post-mortem. Furthermore, gene expression analysis cannot be performed on post-mortem material because RNA is lost.

At gene expression level we saw an upregulation of collagen type I and collagen type III in Achilles tendinosis (AT) specimens without a change in ratio between the two collagen types. Ireland et al. also found an increased expression of both collagen types in Achilles tendinosis but did not report on the ratio between them [19]. At protein level others have found an increase in collagen type III protein relative to collagen type I protein in degenerated equine tendon tissue [14], human supraspinatus tendinopathy [32], and ruptured human Achilles tendon [18]. With our method of measuring total collagen content, we cannot discriminate between collagen type I and collagen type III at protein level in our Achilles tendinosis specimens.

MMP activity levels have been reported to differ significantly between supraspinatus and biceps tendon, representing a higher level of protein turnover in supraspinatus tendons in response to the higher mechanical demands and/or repeated injury exerted upon the supraspinatus tendon [30]. The increase in total MMP activity in our study potentially mediates the higher collagen turnover rate that we ascertained in the affected Achilles tendon specimens. Affirming the collective activity of MMPs, we also found a higher amount of denatured/damaged collagen.

Consistent with the increase in general MMP activity (TNO211-F, which is mainly converted by MMP2, MMP9, and MMP13) is the trend towards an increase in gene expression of MMP2, MMP9, and MMP13 in the tendinotic tissue. Although upregulation of MMP9 and MMP13 gene expression has been described in ruptured Achilles tendon [19, 21], the studies in question

reported no upregulation in painful Achilles tendons. In our tendinotic samples, this might suggest that even though the tendon matrix did not yet show evidence of a macroscopic rupture, microruptures may already have taken place. The decrease of MMP3 gene-expression level confirms earlier results on MMP3 gene expression in painful as well as ruptured Achilles tendons [3, 19, 21]. Although MMP3 gene expression was downregulated, we found an increase in MMP3 activity in Achilles tendinotic specimens. MMP3 activity therefore appears to be mainly regulated on posttranscriptional level (increased activation or less inhibition). MMP3 is active against a broad range of substrates and is also capable of activating other MMPs [37], thus playing an important role in the MMP cascade. The elevated MMP3 activity may also explain the relatively large increase in general MMP activity that we found in the tendinotic specimens as compared to the gene expression levels for MMP2, MMP9, and MMP13, which merely showed a non-significant trend towards upregulation. Generally, a fibrotic repair process is accompanied by increased formation of hydroxylysine residues and of enzymatic HP cross links [36]. Both changes were seen in supraspinatus tendinopathy [11], but only an increase in the amount of hydroxylysine residues was found in our Achilles tendinosis samples. Corticosteroid injection in the rotator cuff can cause fragmentation of collagen bundles, inflammatory cell infiltration and necrosis [1, 35]. In response to damage or inferior tissue repair, fibrosis is a common pathophysiological process. This fibrotic repair process might be much more advanced in the supraspinatus tendinopathy samples than in our Achilles tendinosis samples. In this case, the trend towards upregulation of MMP2, MMP9, and MMP13 gene expression in our tendinotic Achilles specimens, might be interpreted as a sign of microruptures taking place (see previous paragraph) and the increase in hydroxylysine residues as an early sign of the start of a concomitant fibrotic process.

To develop methods for intervention and thereby improve the clinical management of tendon degeneration, it is crucial to obtain a detailed understanding of the biochemical and structural changes involved in the development and worsening of tendinotic lesions. Similar pathological processes appear to underlie both Achilles and supraspinatus tendinopathy in middle-aged people. It remains unclear whether these results can be applied to a younger athletic populations as well. For studying the general disease process there may be three advantages of using the Achilles tendon: 1) corticosteroid injections that presumably influence the pathological process are less often used in conservative treatment of Achilles tendinosis than of supraspinatus tendinopathy; 2) the Achilles tendon has a more accessible anatomical localisation and a larger diameter; 3) it is easier to clinically monitor the effect of intervention studies in the Achilles tendon. With respect to the third point mentioned, an ultrasonographic imaging technique, facilitating the discrimination of various stages of integrity of tendon tissue by means of computerized ultrasonographic tissue characterisation, is currently being evaluated as a tool for diagnosis and subsequent monitoring of tendon pathology, in order to improve clinical management of Achilles tendinopathy [38-42].

We conclude that in Achilles tendinosis an increase in tissue turnover rate as part of an exaggerated repair process, possibly resulting from a failure to regulate specific MMP activities, leads to the deposition of a compromised, non-physiological tendon matrix. Also, the tendon tissue directly surrounding a tendinotic lesion, despite of looking macroscopically healthy, reveals both histologically and biochemically degenerative changes. Changes in extracellular matrix composition seen in Achilles tendinosis and supraspinatus tendinopathy are largely alike and may be the result of similar underlying pathological processes. The MMP gene expression pattern in our early and mild stages of Achilles tendinosis suggests the occurrence of microruptures, and our crosslink analyses may at least be suggestive for an early fibrotic repair process.

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Chapter 3

Tendon degeneration is not mediated by regulation of Toll-like receptors 2 and 4 in human tenocytes

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ABSTRACT

Objective

We hypothesized that expression of Toll-like receptors (TLRs) 2 and 4 by tenocytes is involved in the catabolic processes of tendon degeneration. We investigated TLR2 and TLR4 expression by tenocytes in healthy and tendinotic tendons. We also investigated whether TLR2 and TLR4 could be upregulated in tendon explants using proinflammatory cytokines IL-1β and TNFα.

Methods

Peroperatively harvested healthy (n=5) and tendinotic (n=13) Achilles tendon samples were examined by real-time RT-PCR and immunohistochemical staining for TLR2 and TLR4. In addition the catabolic process in tendinopathy was analysed by real-time RT-PCR for matrix metalloproteinases MMP1, MMP3, MMP9, and MMP13. Furthermore, healthy tendon explants were cultured in the presence of 20 ng/ml IL-1β (n=10) or 10 ng/ml TNFα (n=8) for 4, 24, 48, and 72 hours before analysis of TLR and MMP expression levels.

Results

Although mRNA levels for both TLR2 and TLR4 were detected in healthy and tendinotic Achilles tendons, we could not confirm expression of these receptors by immunohistochemical staining in neither healthy or tendinotic tendon samples. Both receptors didn't show significant transcriptional regulation in tendinopathy, although MMP3 was downregulated and MMP9 was upregulated in tendinopathy. In tendon explant cultures TLR2 mRNA was upregulated by TNFα ($p<0.05$) and IL-1β (not significant). TLR4 gene expression was not altered by addition of IL-1β or TNFα.

Conclusion

Tendon tissue can be stimulated to increase TLR2 gene expression by addition of 'catabolic factors' TNFα or IL-1β. However, the catabolic processes in tendinopathy can not be attributed to regulation of TLR2 and TLR4 by tenocytes.

INTRODUCTION

Toll-like receptors (TLRs) are phylogenetically conserved transmembrane receptors belonging to the family of pattern-recognition receptors (PRR). In humans, eleven TLR homologs have been identified thus far, expressed by numerous cell types like monocytes and dendritic cells [1], and for example by chondrocytes [2]. TLRs detect microbial components, but TLR2 and TLR4 also recognize endogenous ligands like heat-shock proteins, necrotic cells, hyaluronan, and fibronectin [1]. Ligand recognition elicits an immune response with upregulation and activation of proinflammatory cytokines and matrix-metalloproteinases (MMPs)[2]. Hence, via endogenous ligand recognition the TLR-mediated catabolic signalling pathways can directly contribute to degradative tissue reactions even in the absence of microbial components. TLRs are implicated in inflammatory and autoimmune diseases, but also in disorders like atherosclerosis and cancer [3, 4]. Furthermore they play a role in non-infected rheumatoid arthritis (RA) and in the primarily degenerative joint disease osteoarthritis (OA)[1-3, 5, 6].

In degenerative tendinopathies the balance between matrix synthesis and degradation is disturbed and increased MMP activity as well as an increased percentage of degraded collagen are found [7-9]. An increase in NO synthase expression was found in a supraspinatus tendon overuse model [10] and an increase in COX2, a key regulator of prostaglandin synthesis was found in patellar tendinopathy [11]. These catabolic activities can be influenced by TLR signalling, as shown in experiments with chondrocytes [2, 6]. Furthermore in Achilles tendinopathy increased expression of subunit B of the endogenous TLR ligand fibronectin has been found in Achilles tendinopathy [12]. The presence of other endogenous TLR ligands known so far (heat-shock proteins, necrotic cells, hyaluronan) in tendinopathy is very likely considering the nature of the pathology.

We hypothesized that TLR2 and 4 expression on tenocytes is involved in the abovementioned catabolic processes of tendon degeneration. Therefore, we investigated whether TLR2 and TLR4 were constitutively expressed by tenocytes in healthy tendon and were higher expressed by tenocytes in tendinotic lesions. Also, we investigated whether TLR2 and TLR4 could be upregulated in healthy tendon explant cultures by adding proinflammatory cytokines IL-1β and TNFα.

MATERIALS AND METHODS

Tendon biopsies

Tissue specimens of 13 patients with chronic mid-portion Achilles tendinopathy (6 men, 8 women; average age 46, range 36-58) were harvested from the macroscopically affected tendinotic lesion during surgical debridement. Approval was obtained from the Medical Ethical Committee of the Erasmus MC University Medical Center (MEC-2005-100). All patients signed

informed consent. Healthy Achilles tissue specimens of 5 donors (3 men, 2 women; average age 44, range 20-78) without clinically evident Achilles tendinopathy were collected as control (MEC-2006-069). Of every patient one specimen was frozen in liquid nitrogen for RNA extraction, one was embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, the Netherlands) for cryosectioning and immunohistochemical staining of TLR2 and TLR4, and one was fixed overnight in 10% formalin and embedded in paraffin for histological evaluation of tendinosis severity.

Culture experiments

We obtained non-degenerative hamstring tendon tissue from healthy adolescents (all under 18 yrs of age) undergoing hamstring-tendon release for treatment of knee-contractures (MEC-2006-069). After peritendineum and fat tissue were carefully removed, tendons were cut into 3 mm^3 sections and cultured at 37˚C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). After 24 hours, medium was replaced with DMEM containing 2% FCS with or without either 20 ng/ml IL-1β (Peprotech, Rocky Hill, NJ) or 10 ng/ml TNFα (Biosource, Camarillo, CA). All media contained 50 µg/ml gentamycin, and 1.5 µg/ml fungizone (both Invitrogen). Explants were frozen in liquid nitrogen for RNA extraction after 4, 24, and 48 hours for gene expression analysis and after 72 hours for immunohistochemical staining of TLR2 and TLR4.

Histology

Longitudinal frozen sections (5 µm) were blocked with normal swine serum followed by 60 minutes of incubation with polyclonal antibodies against human TLR2 (H-175)(10µg/ml) or TLR4 (H-80)(12µg/ml)(both Santa Cruz Biotechnologies, Santa Cruz, California). The secondary antibody, biotinylated swine anti-rabbit Ig (Dako Diagnostics, Glostrup, Denmark), was incubated for 30 minutes. Slides were stained with streptavidin peroxidase, developed with Diaminobenzidine (DAB)(Sigma, St. Louis, MO), and counterstained with hematoxylin for 30 seconds. Control sections were stained with irrelevant primary isotype-specific IgG antibodies.

Longitudinal paraffin sections (6 µm) were stained with haematoxylin and eosin or with thionin for glycosaminoglycans. Two different researchers performed a blinded examination of the slides using a modified semiquantitative grading scale for tendinosis [7], yielding a total sum of 0 for minimal histological tendinosis severity and 30 for maximal severity.

RNA isolation and real-time RT-PCR

Specimens were snap frozen and quickly homogenized in a Mikro-Dismembrator® (BioTech International Inc, Needville, Texas) and suspended in 1.8mL/100mg RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was isolated and purified using RNeasy® Micro Kit (Qiagen, Hilden, Germany), and 1 µg total RNA of each sample was reverse-transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Primers were

designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet SYBR®Green (TLRs only) or Taqman® requirements. BLASTn ensured gene specificity of the following primers: TLR2 (F: 5'-CTACTGGGTGGAGAACCTTATGGT-3'; R: 5'-CCGCTTATGAAGACA-CAACTTGA-3'), TLR4 (F: 5'-GGCATGCCTGTG

CTGAGTT-3'; R: 5'-CTGCTACAACAGATACTACAAGCACACT-3'), MMP1, MMP3, MMP9, MMP13 [7], and GAPDH [13] as calibrator. Amplification was performed using an ABI PRISM® 7000 with SDS software version 1.7 in 20 µl reactions using either qPCR™ Mastermix Plus for SYBR®Green I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) or TaqMan® Universal PCR MasterMix (ABI, Branchburg, New Jersey, USA) according to the manufacturer's guidelines. Data were normalized to GAPDH. Relative expression was calculated according to the $2^{-\Delta CT}$ formula.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc 1994-2007, www.graphpad.com). A Mann-Whitney U Test was used to assess the differences between healthy and tendinotic tendon tissue. A Kruskall-Wallis H test and post-hoc Dunn's multiple comparison test was used to asses the differences between growth factor stimulated cultures and control cultures. Significance level was set at $p<0.05$.

RESULTS

Achilles tendinopathy

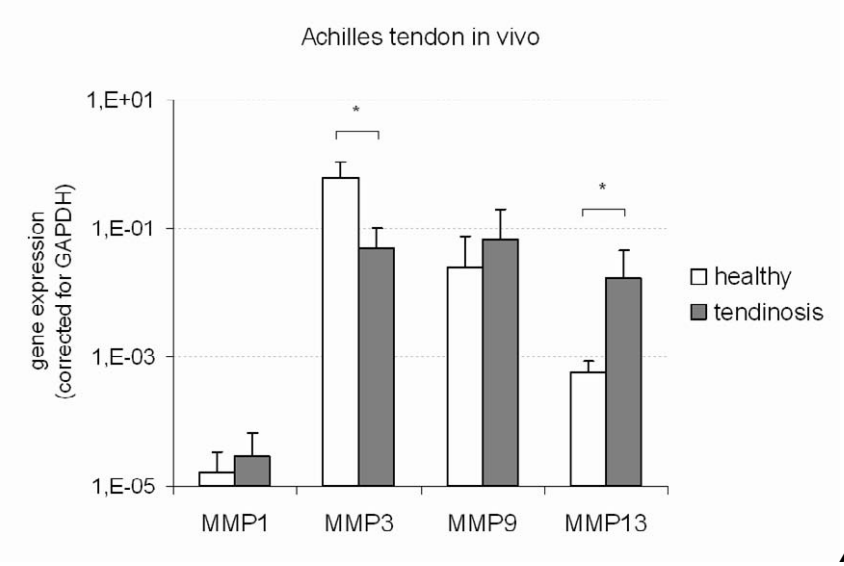
Tendinosis severity ranged from 1 to 10 (median 3) in healthy Achilles tendon samples, and from 9 to 24 (median 18) in tendinotic Achilles tendon samples (p=0.02). MMP activity was significantly higher in tendinotic Achilles samples than in healthy controls [7]. On gene expression level MMP3 was significantly downregulated, MMP13 significantly upregulated (both $p<$ 0.05), and MMP1 and MMP9 were not altered (Figure 3.1A).

Transcripts of both TLR2 and TLR4 were detected in healthy Achilles tendon tissue at basal levels, but no significant differences in TLR2 or TLR4 expression were found between healthy and tendinotic Achilles tendon tissue (Figure 3.1B).

Immunohistochemical staining for TLR2 and TLR4 were performed on frozen sections of healthy and tendinotic Achilles tendon biopsies. Human RA synovium was used as positive control. None of the tenocytes in either healthy or tendinotic Achilles tendon samples stained positive for TLR2 or TLR4 (Figure 3.2, see also color inlay).

Explant cultures

Healthy tendon explants were harvested after 4, 24, and 48 hours of culture in the presence of IL-1β or TNFα. MMP1, MMP3, and MMP13 gene expression levels were significantly upregulated by IL-1β treatment after 24 hours (all $p<$ 0.05). MMP1 and MMP9 gene expression were



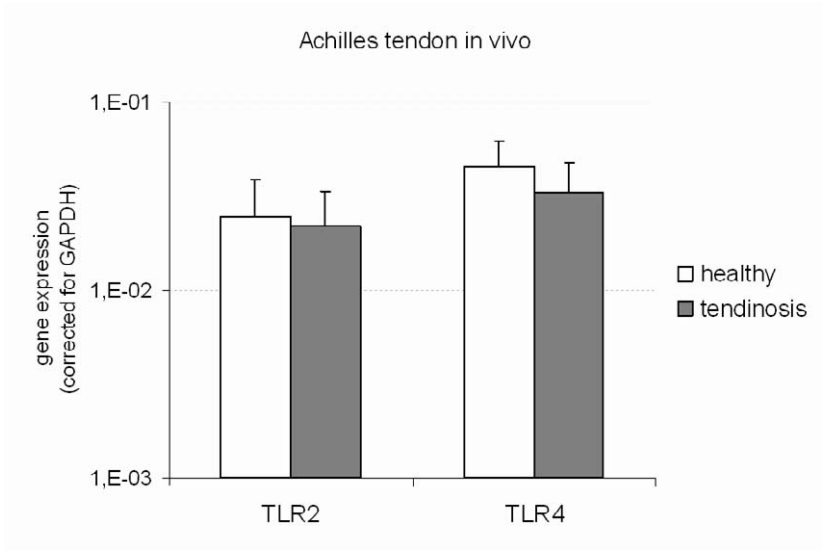


Figure 3.1 *Gene expression of* ***A.*** *MMP1, MMP3, MMP9, MMP13, and* ***B.*** *TLR2 and TLR4 in healthy (n=5) and tendinotic (n=13) Achilles tendon samples. Ct values were corrected for GAPDH, which was stably expressed across samples. Mean +/- standard deviation are displayed. * Indicates a p-value<0.05.*

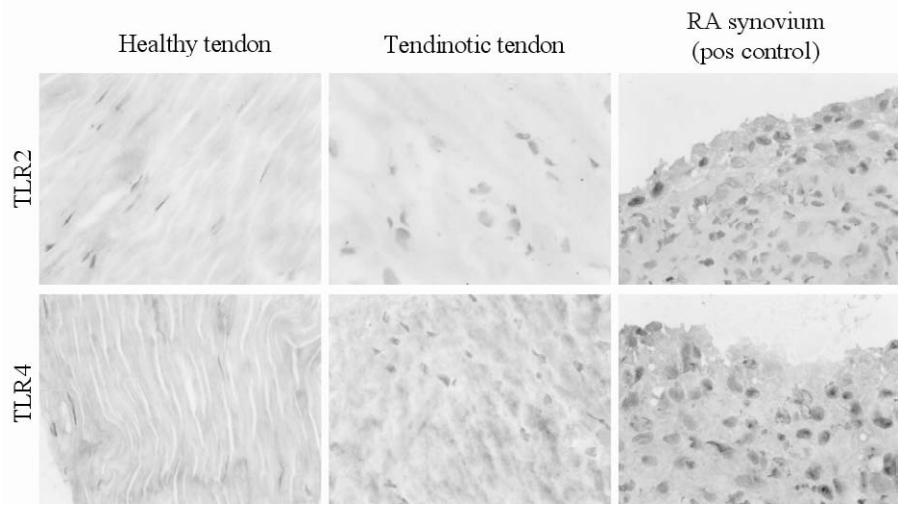


Figure 3.2 *Immunohistochemical staining for TLR2 and TLR4. Representative photomicrographs are shown from frozen longitudinal sections of healthy Achilles tendon samples (n=5) and tendinotic Achilles tendon samples (n=13). Human rheumatoid arthritis (RA) synovium was used as positive control. Magnification 400x. (See color inlay for a full color version of this figure.)*

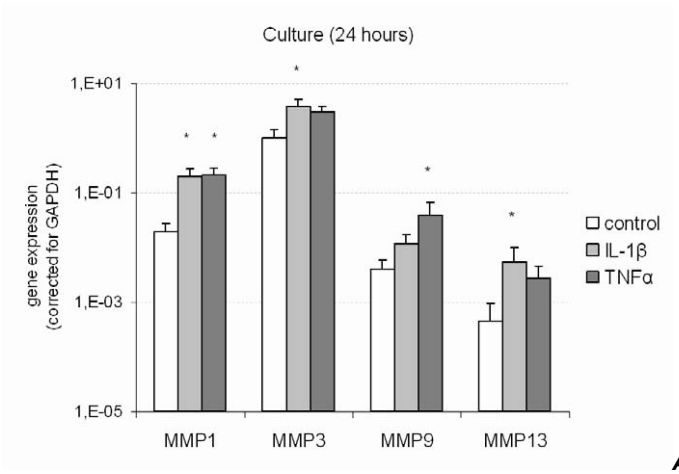
significantly upregulated by TNFα after 24 hours (both $p< 0.05$). MMP3 and MMP13 showed a clear but non-significant trend towards upregulation by TNFα treatment. These data indicate that IL-1β and TNFα treatments in culture were effective (Figure 3.3A).

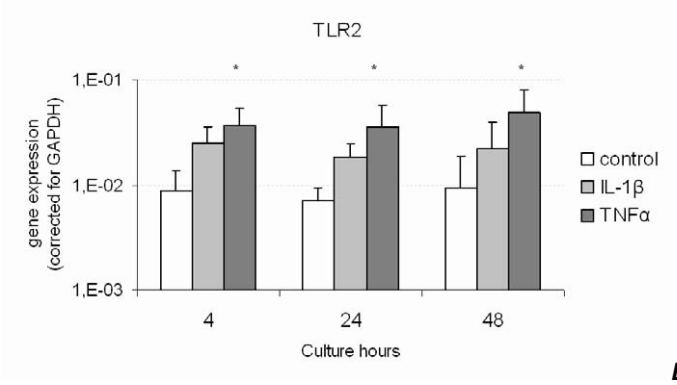
Gene expression of TLR2 appeared upregulated by IL-1β and TNFα at all time points, but this general trend reached significance ($p<0.05$) only for TNFα stimulation (at all time points; Figure 3.3B). Gene expression of TLR4 was not significantly affected by either IL-1β treatment or TNFα treatment (Figure 3.3C).

Immunohistochemical staining on frozen sections of explants cultured for 3 days in the presence or absence of IL-1β or TNFα showed no staining for TLR 2 or TLR4 in any of the tenocytes.

DISCUSSION

We hypothesized that TLR2 and TLR4 presentation by tenocytes is involved in tendon degeneration. However, in tendon samples in vivo no TLR2 or TLR4 expression by tenocytes was seen in any of the healthy or tendinotic tendon samples on immunohistochemical staining. Basal mRNA levels of TLR2 and TLR4 were detected in healthy Achilles tendon tissue, but were not upregulated in tendinotic Achilles tendon lesions. The catabolic factors TNFα and IL-1β were able to stimulate TLR2 mRNA expression in tendon explant cultures (significantly for TNFα). Therefore we conclude that tendons can be stimulated to increase TLR2 expression by addi-





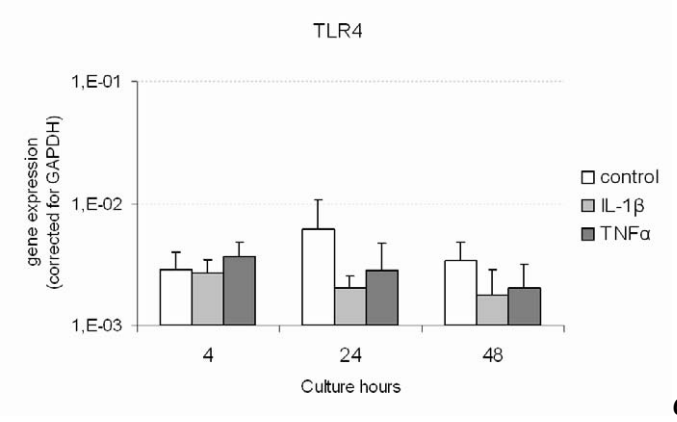


Figure 3.3 *Gene expression of A. MMP1, MMP3, MMP9, MMP13, B. TLR2 and C. TLR4 in healthy tendon explants cultured in the presence of either 20 ng/ml IL-1β (n=10) or 10 ng/ml TNFα (n=8). Ct values were corrected for GAPDH, which was stably expressed across samples. Mean +/- standard deviation are displayed. * Indicates a p-value<0.05 compared to control cultures.*

tion of the 'catabolic' cytokines TNFα or IL-1β, but the catabolic processes demonstrated in tendinopathy can not be attributed to regulation of TLR2 and TLR4 by the tenocytes.

The exact molecular processes involved in tendinopathy are largely unknown. Our hypothesis was mainly based on the catabolic processes known so far to occur in tendinopathy. Tendon tissue has many aspects in common with cartilage tissue. Both are embryologically derived from the mesenchyme with the basic components of the extracellular matrix (ECM) consisting of large collagen bundles and a glycosaminoglycan(GAG)-rich viscous substance interspersed between the collagenous matrix. The main cell type in tendons are tenocytes, the only cell type in cartilage are chondrocytes. These specialized cells are responsible for maintenance of the ECM. However, in both tissues they are distributed over the ECM at very low density and they function under hypoxic conditions because tendon and cartilage respectively have limited to no blood supply. Consequently, both tissues have very limited regeneration capacity, rendering them susceptible to degenerative diseases. Cartilage degeneration in osteoarthritis (OA) and tendon degeneration in tendinopathy appear to have many aspects in common as well. Both OA and tendinopathy are considered overuse injuries characterized by catabolic tissue reactions associated with increased expression of mediators of inflammation [14, 15]. The central event in both diseases seems to be degradation of the ECM, although the changes in the complex balance between activities of matrix degrading enzymes (e.g. MMPs), cytokines, growth factors, and other signalling molecules have been studied in far more detail in cartilage pathology than in tendon pathology.

The interplay between TLRs and catabolic processes has been studied in joint pathology (cartilage, synovium). Human articular chondrocytes were shown to express a variety of TLRs in vivo [6]. In cartilage degeneration TLR2 and TLR4 reappear on the chondrocyte membrane [2, 6], both underlining the involvement of an inflammatory component and concurrently reminiscing TLR expression during embryological developmental stages. In primary OA chondrocytes in culture this upregulation of TLR2 (and TLR4, though not significantly) was induced by the proinflammatory cytokines IL-1β and TNFα [2]. Our study is the first to investigate TLR expression in tendon tissue. We found basal mRNA levels for both TLR2 and TLR4 in Achilles tendon tissue, but no upregulation was seen in tendinotic Achilles tendon samples. Moreover immunohistochemical staining for TLR2 and TLR4 remained negative for all tenocytes in the healthy and degenerated Achilles tendon samples.

Although we conclude that TLR expression on tenocytes does not contribute to the tendon degeneration process, we do not exclude a role for TLRs in tendinopathy. The presence and regulation of TLRs on cells in the peritendineum and endotenon might be able to contribute to the degradative tissue reactions seen in the tendinotic lesions. Analogous to the role of TLR-presenting cells in synoviitis accompanying OA and RA [5], monocytes and other inflammatory cells can invade the peritendineum and the endotenon in peritendinitis, which is a frequently seen co-morbidity with tendinopathy. TLR presentation by these cells and the subsequently induced catabolic pathways might influence the degenerative process in tendinopathy.

In conclusion, based on the degenerative processes present in tendinopathy we hypothesized that TLR2 and TLR4 are involved in the pathology of tendon degeneration, analogous to their involvement in OA. However, our results add to the current knowledge indicating that the catabolic processes in tendinopathy can not be attributed to regulation of TLR2 and TLR4 by tenocytes. Nonetheless tendon tissue can be stimulated to increase mRNA expression of TLR2 by addition of "catabolic factors" TNFα or IL-1β. Therefore, further investigation is warranted into the role of TLR-presenting cells that invade the endotendon/peritenon, hereby inducing general catabolic responses and thus contributing to the degenerative process in tendinopathy.

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Chapter 4

Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study

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ABSTRACT

Background

Tendinosis lesions show an increase of glycosaminoglycan amount, calcifications, and lipid accumulation. Therefore, altered cellular differentiation might play a role in the etiology of tendinosis. This study investigates whether adolescent human tendon tissue contains a population of cells with intrinsic differentiation potential.

Methods

Cells derived from adolescent non-degenerative hamstring tendons were characterized by immunohistochemistry and FACS-analysis. Cells were cultured for 21 days in osteogenic, adipogenic, and chondrogenic medium and phenotypical evaluation was carried out by immunohistochemical and qPCR analysis. The results were compared with the results of similar experiments on adult bone marrow-derived stromal cells (BMSCs).

Results

Tendon-derived cells stained D7-FIB (fibroblast-marker) positive, but α-SMA (marker for smooth muscle cells and pericytes) negative. Tendon-derived cells were 99% negative for CD34 (endothelial cell marker), and 73% positive for CD105 (mesenchymal progenitor-cell marker). In adipogenic medium, intracellular lipid vacuoles were visible and tendon-derived fibroblasts showed upregulation of adipogenic markers FABP4 (fatty-acid binding protein 4) and PPARG (peroxisome proliferative activated receptor γ). In chondrogenic medium, some cells stained positive for collagen type II and tendon-derived fibroblasts showed upregulation of collagen type II and collagen type X. In osteogenic medium Von Kossa staining showed calcium deposition although osteogenic markers remained unaltered. Tendon-derived cells and BMSCs behaved largely comparable, although some distinct differences were present between the two cell populations.

Conclusions

This study suggests that our population of explanted human tendon cells has an intrinsic differentiation potential. These results support the hypothesis that there might be a role for altered tendon-cell differentiation in the pathophysiology of tendinosis.

BACKGROUND

Tendinosis is a chronic degenerative tendon disorder occurring particularly among athletes and middle-aged people [1]. As its pathophysiology is still largely unknown, only symptomatic treatment options are available, with limited success rates [1, 2]. A better understanding of the cellular processes involved in the development of tendinosis lesions may ultimately improve treatment and prevention.

Histopathological findings in tendinosis have been described in detail [3, 4]. In brief, hypercellularity and rounding of the cell nuclei indicate a relatively high metabolic activity. Likewise, altered extracellular matrix composition reflects changes in cellular behavior. For instance, in tendinosis lesions there is a higher amount of glycosaminoglycans [3]. Lipid accumulation and calcium deposition have also been described [5]. Thus, the histopathological findings may indicate the presence of cells with diverse phenotypes, different from that of tenocytes under healthy conditions.

Cells with multilineage differentiation potential likely play an important role in the body's capacity to naturally remodel, repair, and regenerate various tissue types where necessary [6]. However, the multilineage differentiation potential of cells might also be involved in pathological processes. Although the pathophysiology of tendinosis is largely unclear, histological findings suggest that multipotent cells might be implicated in its development. The origin of these multipotent cells is unknown. They may be recruited from the bone marrow in response to tendon tissue injury, and migrate through the circulation to the site of tissue damage [7]. They might also be present in the tendon tissue itself.

Local progenitor cells with multilineage potential have previously been found in many locations within the musculoskeletal system, e.g. in bone marrow, skin, periosteum, bone, muscle and adipose tissue [8-15]. On the other hand, progenitor cells are not the only cells with multilineage potential: some highly differentiated cells are capable of transdifferentiation, i.e. switching their phenotype to another lineage. This transdifferentiation has been demonstrated for highly differentiated chondrocytes [16, 17].

Multipotent cells have been found in virtually all tissues of the musculoskeletal system, but it is not known if tendon tissue has a cell population with multilineage potential. In this study, we investigated whether the population of cells derived from non-degenerative tendon tissue has differentiation potential similar to bone marrow-derived stromal cells (BMSCs). Specifically, we characterized human tendon-derived fibroblasts by immunohistochemical staining and FACS-analysis. Then, after a culture period of 21 days in adipogenic, chondrogenic, and osteogenic medium, we evaluated changes in their phenotype using immunohistochemical and histochemical stainings as well as gene expression analysis.

METHODS

Study design

Cells were explanted from human adolescent non-degenerative hamstring tendon tissue (n=5). After the phenotype of the cells was analyzed by immunohistochemical staining and FACS-analysis, cells were cultured for 21 days on osteogenic, adipogenic, or chondrogenic medium. The differentiation potential of the tendon-derived cell population was evaluated by immunohistochemical and histochemical staining and real-time RT-PCR, and was compared with the differentiation potential of human femoral-shaft-derived BMSCs (n=5).

Isolation of tendon-derived cells and BMSCs

Human tendon-derived cells were cultured from explants from hamstring tendon tissue of five adolescents (age 12-17 years) undergoing hamstring-tendon release for treatment of knee-contractures (MEC-2006-069). In this clinical condition the tendon is primarily not affected, but is exposed to continuously high tensile strains. After the peritendineum had been carefully removed, the tendon was cut into 3 mm^3 sections, transferred into six-well plates (Corning, NY, USA) and cultured in expansion medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (all Invitrogen, Scotland, UK)). Tissue cultures were maintained at 37˚C in a humidified atmosphere of 5% CO2 for ten days, with three medium changes. During this time, fibroblasts migrated out of the tissue and adhered to the bottom of the culture dish. Cells were subcultured and trypsinized at subconfluency and cells from the third to the fifth passage were used for the differentiation experiments.

Human bone marrow stromal cells (BMSCs) were isolated from femoral shaft biopsies of six patients (age 42-72 years) undergoing total hip replacement for treatment of osteoarthritis (MEC-2004-142). BMSCs were isolated from aspirated marrow acccording to procedures described earlier [18]. Briefly, heparinized femoral-shaft marrow aspirate was plated out and after 24 hours, non-adherent cells were removed with 2% FCS in 1xPBS. Adherent cells were subcultured in medium with 10% FCS and trypsinized at subconfluency. Cells from the second to the fourth passage were used for the differentiation experiments. The used serum lot was selected specifically for the maintenance of multipotential cells.

Phenotypic characterisation of tendon-derived cells

Explants harvested on day 6 of the explantation period were fresh frozen in liquid nitrogen and 6 µm frozen sections were fixated in acetone. Cells in monolayer cultures, passage 1 and 4, were fixated in ice-cold 70% ethanol.

Ki-67, D7-FIB, and α-SMA staining

Cells and histological sections were incubated with either mouse monoclonal antibody against 11-fibrau (Clone D7-FIB; diluted 1:400; Imgen, Netherlands), a marker for fibroblasts [19], or

monoclonal antibody against α-SMA (Clone 1A4; diluted 1:1000; Sigma, St.Louis, Missouri, USA), a marker for smooth muscle cells and pericytes [20], for two hours. Cells were rinsed in 1xPBS and IHC detection was performed using Link-Label (Biotin-based) Multilink® IHC Detection Kit (Biogenex, San Ramon, CA). Finally, a new fuchsin substrate was added to obtain a pink signal in positive cultures. Cells were counterstained with Gill's haematoxilin (Sigma). For Ki-67 staining histological sections were pre-incubated in 1% H2O2 (Sigma) in methanol (Sigma) and then incubated with mouse monoclonal antibody Ki-67 (M7187; diluted 1:25; Dako, Glostrup, Denmark). IHC detection was performed using StrAviGen Multilink® Kit (Biogenex, San Ramon, CA), substrate development was performed using the SK-4800 Vector® NovaREDTM Substrate kit (Vector Laboratories, Burlingame, CA), and no counterstaining was performed.

FACS-analysis

Trypsinized first to fifth passage cells were incubated at 4ºC for 30 minutes with saturating amounts of human antibodies CD105-PE (dilution 1:20; BD Biosciences, San Jose, USA), a marker for mesenchymal progenitor cells [21], and CD34-PE (dilution 1:20; Ancell, Bayport, USA), a marker that remains negative in non-hematogenic progenitor cells [20] and is positive for hematogenic progenitor cells, endothelial cells, and pericytes [22-24]. Cells were washed and resuspended in 300 µl HBN buffer (Hank's Balanced Salt Solution (HBSS; GIBCO, Breda, The Netherlands) + 0.5% (wt/vol) Bovine Serum Albumin + 0.05% (wt/vol) sodium azide) and analyzed by flow cytometric analysis using a FACSCalibur flow cytometer and Cellquest software (BD Biosciences, San Jose, USA) with a minimum of 10,000 events acquired.

Differentiation experiment

After trypsinisation, cells were seeded in six-well plates and cultured in a modified version of three differentiation media described earlier [18]. Briefly, cells were seeded at 3,000 cells/cm^2 to induce osteogenic differentiation and then cultured in an osteogenic induction medium containing DMEM plus 10% FCS and freshly added β-glycerophosphate 10 mM (Sigma, St. Louis, USA), dexamethasone 0.1 µM (Sigma) and L-ascorbic acid 2 phosphate 0.5 mM (Sigma). Cells were seeded at 20,000 cells/cm2 to induce adipogenic differentiation, and cultured in adipogenic induction medium containing DMEM with 10% FCS, supplemented with dexamethasone 1 µM, indo-methacin 0.2 mM, insulin 0.01 mg/ml, and 3-isobutyl-l-methyl-xanthine 0.5 mM (all from Sigma). To induce chondrogenic differentiation, cells were cultured in 1.2% low viscosity alginate beads at a density of 4×10^6 cells/ml in serum-free chondrogenic induction medium containing DMEM supplemented with TGF-β2 10 ng/ml (R&D Systems, UK), L-ascorbic acid 2 phosphate 25 µg/ml (Sigma), sodium pyruvate 100 µg/ml (Invitrogen), proline 40 µg/ml (Sigma) and ITS+ (diluted 1:100; BD Biosciences, Bedford, MA). All media contained 50 µg/ml gentamicin and 1.5 µg/ml fungizone. Cells were cultured in differentiation media for 21 days, with media changes twice a week. On day 21 of culture, two wells were harvested for RNA

extraction and one well was used for histochemical evaluation. One well was cultured for 21 days on expansion medium as control condition for the histochemical stainings.

Gene expression analysis

At harvesting, monolayer cell cultures were suspended in RNA-BeeTM (TEL-TEST, Friendswood, TX, USA). Alginate beads were dissolved in 150 µl of 55 mM sodium citrate in 150 mM sodium chloride per bead (both Fluka, Steinheim, Switzerland) and cell pellets were subsequently suspended in RNA-BeeTM. RNA was precipitated with 2-propanol, purified with lithium chloride, and 1 µg total RNA of each sample was reverse-transcribed into cDNA using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Primers were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet Taqman® or SYBR®Green requirements and were designed to bind to separate exons to avoid co-amplification of genomic DNA. BLASTN ensured gene specificity of all primers listed in Table 4.1. As osteogenic markers osterix (SP7), RUNT-related transcription factor 2 (RUNX2), and osteocalcin (BGLAP) were studied, while SOX9, aggrecan (AGC1), collagen type II (COL2A1), and collagen type X (COL10A1) were used as chondrogenic markers. Adipogenic markers studied were fatty acid binding protein 4 (FABP4) and peroxisome proliferative activated receptor γ (PPARG). Amplifications were performed as 25 µl reactions using either TaqMan® Universal PCR MasterMix (ABI, Branchburg, New Jersey, USA) or qPCRTM Mastermix Plus for SYBR®Green

Table 4.1 *Primer and probe nucleotide sequences of the tested genes*

Gene	Accession no.	Primer	Probe
GAPDH	NM_002046.2	F: ATGGGGAAGGTGAAGGTCG R: TAAAAGCAGCCCTGGTGACC	CGCCCAATACGACCAAATCCGTTGAC
RUNX2	NM_001024630.1 NM_00101505.1 NM_0043468.3	F: GCCTTCAAGGTGGTAGCCC R: CGTTACCCGCCATGACAGTA	CCACAGTCCCATCTGGTACCTCTCCG
BGLAP	NM_199173.2	F: GAAGCCCAGCGGTGCA R: CACTACCTCGCTGCCCTCC	TGGACACAAAGGCTGCACCTTTGCT
PPARG*	NM_138712.2 NM_015869.3 NM_005037.4 NM_138711.2	F: AGGGCGATCTTGACAGGAAA R: TCTCCCATCATTAAGGAATTCATG	
SOX9	NM_000346.2	F: CAACGCCGAGCTCAGCA R: TCCACGAAGGGCCGC	TGGGCAAGCTCTGGAGACTTCTGAACG
AGC1	NM_001135.1 NM_013227.1	F : TCGAGGACAGCGAGGCC R : TCGAGGGTGTAGCGTGTAGAGA	ATGGAACACGATGCCTTTCACCACGA
COL2A1	NM_033150	F: GGCAATAGCAGGTTCACGTACA R: CGATAACAGTCTTGCCCCACTT	CCGGTATGTTTCGTGCAGCCATCCT
COL10A1	NM_000493.2	F: CAAGGCACCATCTCCAGGAA R: AAAGGGTATTTGTGGCAGCATATT	TCCAGCACGCAGAATCCATCTGA

*F: forward; R: reverse; * SYBR®Green assay. Commercially available, so-called assays-on-demand (Applied Biosystems, Foster City, CA, U.S.A.) were used to detect osterix (SP7; Hs_00541729_m1) and fatty acid binding protein 4 (FABP4; Hs_00609791)-specific mRNA.*

I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) according to the manufacturer's guidelines. Real-Time RT-PCR (QPCR) was done using an ABI PRISM® 7000 with SDS software version 1.7. Data were normalized to GAPDH which was stably expressed across sample conditions (not shown). Relative expression was calculated according to the $2^{-\Delta CT}$ formula [25] using averages of duplicate samples.

Histochemical and immunohistochemical stainings

Von Kossa staining

Cells were fixed in formalin, hydrated in milliQ water, immersed in 5% silver nitrate solution (Sigma) for 10 minutes, rinsed and exposed to light for 10 minutes. Excess silver nitrate was removed with 5% sodium-thiosulphate (Sigma) and cells were rinsed in distilled water, followed by a counterstaining with azophloxine (Sigma).

Oil Red O staining

Cells were fixed in 10% formalin, treated with 0.3% Oil red O solution (Sigma) for 15 min, and then repeatedly washed with tap water.

Collagen type II staining

Alginate beads were dissolved in sodium citrate, cytospins were prepared and stored at -80˚C. Cytospins were fixed in acetone and treated with 1% hyaluronidase (Sigma) for 20 min. Cell monolayers were fixed with 70% ethanol, treated with 50 mM NH4Cl (Sigma), and permeabilised in a 0.1% Triton X-100 (Sigma) solution. Cells were incubated with mouse monoclonal antibody against collagen type II (II-II6B3, diluted 1:100; Developmental Studies Hybridoma Bank) for 2 hours. Anti-mouse Fab fragments conjugated with alkaline phosphatase (GAMAP, diluted 1:100; Immunotech, Marseille, France) were added. Finally, alkaline phosphatase conjugated anti-mouse antibodies in combination with a new fuchsin substrate were added to obtain a pink signal in positive cultures. Counterstaining with Gill's haematoxilin (Sigma) was performed.

RESULTS

Characterisation of tendon-derived fibroblasts

Histological examination of the adolescent hamstring tendon explants confirmed normal tissue morphology. Specifically, no degenerative lesions, inflammatory cell infiltration, (partial) ruptures, chondroid metaplasia, or calcifications were seen.

During the explant culture period, proliferating cells (Ki-67 positive) were located between the highly organized collagen fibres of the tendon tissue and also in the connective tissue of

the endotenon. These cells stained positive for fibroblast-marker D7-FIB. On the other hand, proliferating cells were also seen in the vascular walls, staining negative for D7-FIB but positive for α-SMA, a marker for pericytes and smooth muscle cells (Figure 4.1, see also color inlay).

Cells explanted from the tendon tissue had a characteristic spindle-shaped fibroblastic morphology. Through the first four passages in monolayer culture all tendon-derived cells stained positive for D7-FIB but stained negative for α-SMA (Figure 4.1, see also color inlay). On further characterisation by FACS-analysis 99.1 +/- 1.1 % of the tendon-derived fibroblasts were CD34 negative and 72.6 +/- 22.9 % were CD105 positive (average of passage 2 to 5 tendon-derived cells, n=4). The BMSCs had 99.7 +/- 0.4 % CD34 negative cells and 93.8 +/- 4.6 % CD105 positive cells (average of passage 1 to 5 BMSCs, n=8).

Adipogenic markers

Light microscopy revealed the presence of vacuoles within approximately one third of the cells in all adipogenic cultures of tendon-derived fibroblasts. Oil Red O staining confirmed that these were lipid vacuoles (Figure 4.2A, see also color inlay). Only cells aggregated into clusters stained positively for lipid vacuoles. Tendon-derived fibroblasts cultured on control medium (Figure 4.2B, see also color inlay), on osteogenic, or on chondrogenic medium (not shown) did not

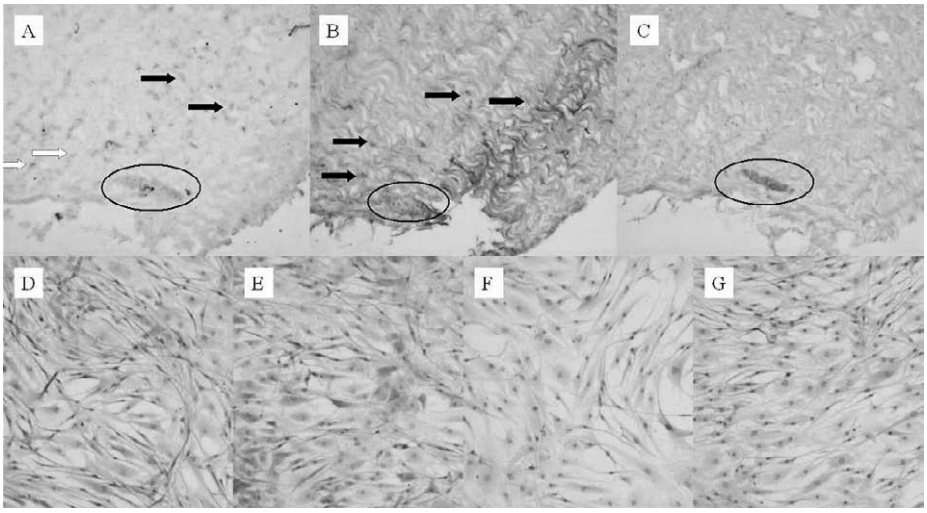

Figure 4.1 *Ki-67, D7-FIB, and α-SMA staining on tendon explants (day 6 of explantation period) and on tendon-derived fibroblasts (TDF) in monolayer culture. Ki-67 positive (proliferating) cells in the explants were located in the tendinous tissue (A, black arrow), in the endotenon (A, white arrow), and in the vascular walls (A, circle). Cells in the tendon tissue and in the endotenon stained positive for fibroblastmarker D7-FIB (B). Cells in the vascular walls remained negative for D7-FIB (B) and instead stained positive for α-SMA, a marker for pericytes and smooth muscle cells (C). All TDFs in monolayer culture stained positive for D7-FIB from passage one (D) to passage four (E) and remained negative for α-SMA from passage one (F) to passage four (G). (See color inlay for a full color version of this figure.)*

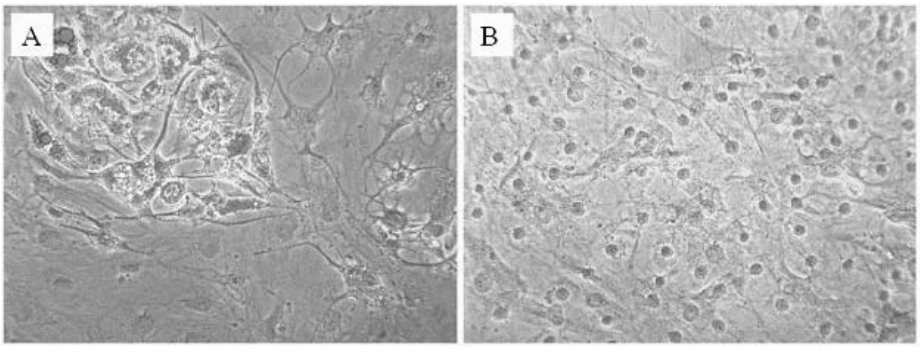

Figure 4.2 *Oil Red O staining on tendon-derived fibroblasts cultured for 21 days in adipogenic medium (A) (note that not all cells but merely clusters of cells formed Oil Red O positive lipid vacuoles inside the cell's main body) or in control medium (B). Like cells in control medium, cells cultured in osteogenic or chondrogenic medium were negative (figures not shown). (See color inlay for a full color version of this figure.)*

develop any lipid vacuoles. Cellular distribution of Oil Red O positive BMSCs cultured in adipogenic medium was more homogenous with approximately 75% of cells staining positively (results not shown).

In addition to this, culture of tendon-derived fibroblasts in adipogenic medium significantly upregulated expression of FABP4 and PPARG compared to those cultured in osteogenic (both p=0.009) and chondrogenic medium (both p=0.025). Similar findings were seen in the BMSC cultures although the difference in PPARG expression between the osteogenic and adipogenic medium condition did not reach statistical significance in the BMSC cultures (Figure 4.3). In

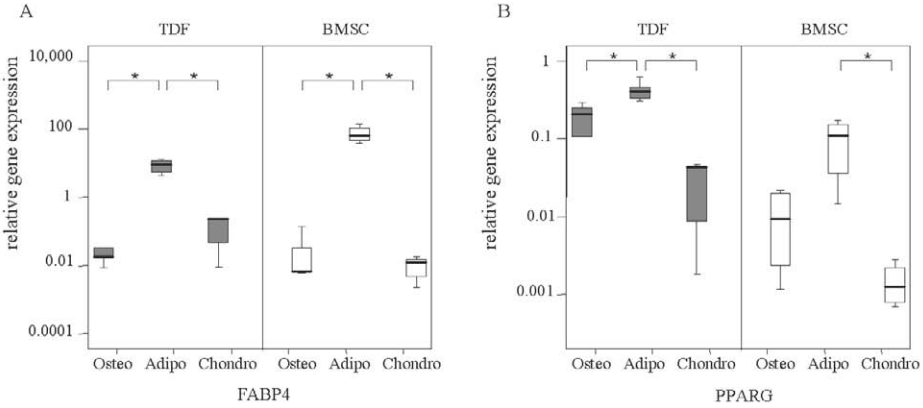

Figure 4.3 *Expression levels of adipogenic markers in tendon-derived fibroblasts (TDF) and bone marrow-derived stromal cells (BMSC). Cells were cultured for 21 days on osteogenic (N=5 for TDF, N=4 for BMSC), adipogenic (N=5 for TDF, N=5 for BMSC), or chondrogenic (N=3 for TDF, N=5 for BMSC) induction medium. The relative, GAPDH-normalized, expression levels of fatty acid binding protein 4 (FABP4)(A) and peroxisome proliferator activated receptor γ (PPARG)(B) is displayed on the vertical axis. * Indicates a P-value<0.05.*

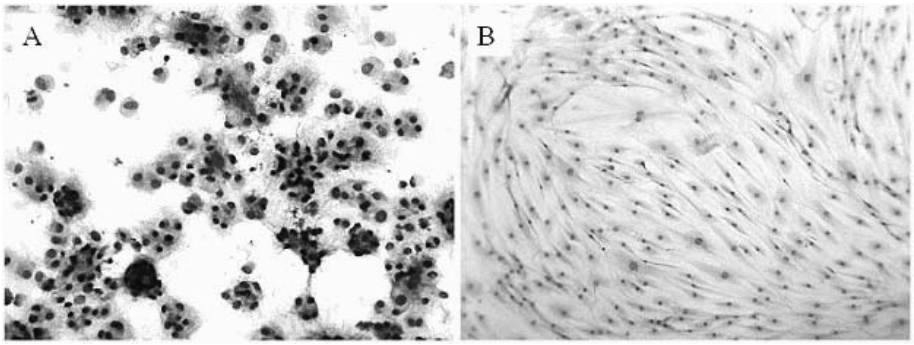

Figure 4.4 *Immunohistochemical staining for collagen type II on tendon-derived fibroblasts. 5% of the cells cultured for 21 days in alginate beads in chondrogenic medium stained positive (A). Cells cultured in monolayer in control medium remained negative (B) as did cells in adipogenic or osteogenic media (figures not shown). (See color inlay for a full color version of this figure.)*

the BMSC cultures PPARG expression was significantly higher in adipogenic medium compared to chondrogenic medium (p=0.021); FABP4 expression was upregulated in the adipogenic medium compared to osteogenic medium (p=0.021) and chondrogenic medium (p=0.021).

Chondrogenic markers

Immunohistochemical staining for collagen type II was performed on tendon-derived fibroblasts cultured in chondrogenic, adipogenic, osteogenic, and control medium for 21 days. In all chondrogenic medium conditions approximately 5% of the cells stained positive for collagen type II (Figure 4.4A, see also color inlay). Tendon-derived fibroblasts cultured in control medium (Figure 4.4B, see also color inlay), as well as adipogenic and osteogenic medium were immunonegative for collagen type II (not shown). BMSC cultures showed a similar amount of collagen type II staining in chondrogenic medium (not shown).

Culture of tendon-derived fibroblasts in chondrogenic medium significantly increased expression of chondrogenic markers COL2A1 and COL10A1 (the latter is considered to be a marker for hypertrophic cartilage formation) compared to the osteogenic condition (p=0.025 for both genes) and adipogenic condition (p=0.025 for both genes). Expression of SOX9 and AGC1 in chondrogenic medium compared to osteogenic and adipogenic medium was not significantly different (Figure 4.5).

BMSC cultures also showed a significantly higher expression of COL2A1 and COL10A1 in chondrogenic medium compared to osteogenic medium (p=0.014 for both genes) and adipogenic medium (p=0.028 for COL2A1 and p=0.009 for COL10A1). SOX9 expression in BMSCs showed the same trend as in the tendon-derived fibroblast cultures, but the differences only reached significance in the BMSC cultures (osteogenic versus adipogenic medium p=0.014 and osteogenic versus chondrogenic medium p=0.014). Expression of AGC1 in the BMSCs did not differ significantly between the three medium conditions. Interestingly, BMSCs cultured in

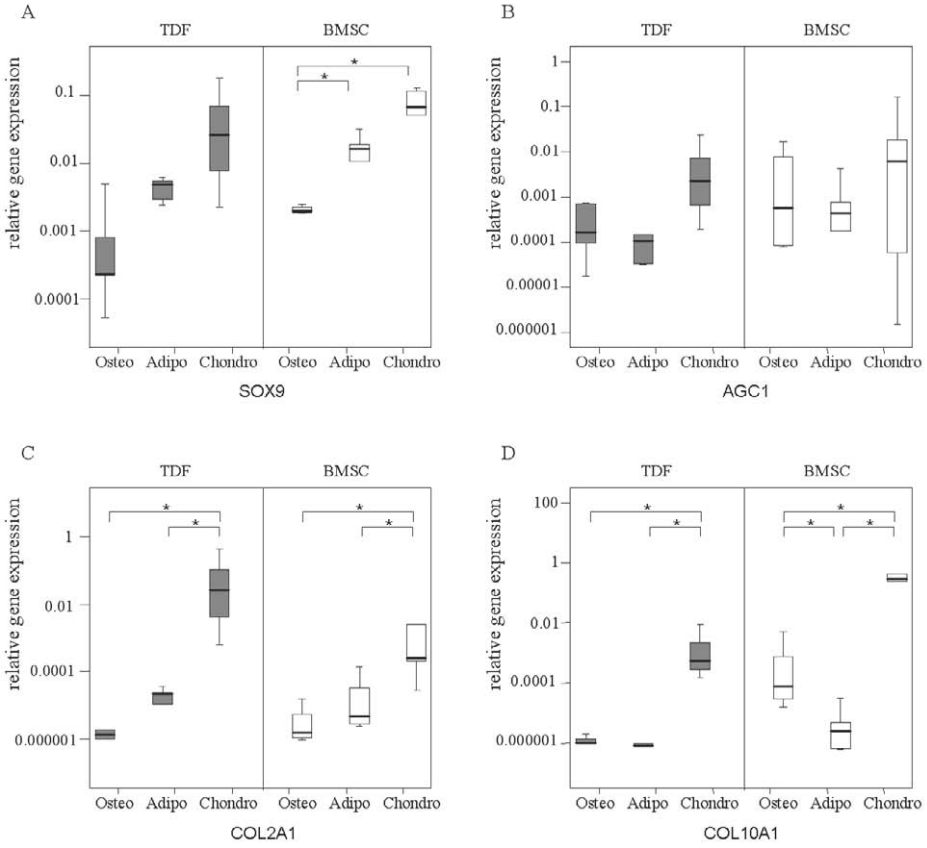

Figure 4.5 *Expression levels of chondrogenic markers in tendon-derived fibroblasts (TDF) and bone marrow-derived stromal cells (BMSC). SOX9 (A), aggrecan (AGC1)(B), collagen type II (COL2A1)(C) and collagen type X (COL10A1)(D). See figure 3 for reminder of key.*

osteogenic medium had significantly upregulated COL10A1 compared to the adipogenic condition (p=0.027). This phenomenon was not seen in the tendon-derived fibroblasts (Figure 4.5).

Osteogenic markers

Von Kossa staining of tendon-derived fibroblasts in the osteogenic condition showed clustered areas of calcium deposition, whereas the tendon-derived fibroblast cultures in control medium had no calcium deposition (Figure 4.6, see also color inlay). Also, tendon-derived fibroblast cultures in adipogenic and chondrogenic medium remained negative for calcium (results not shown). Similarly, in BMSC cultures, calcium deposition was found only in the osteogenic condition (not shown).

Although we did find expression of osteogenic markers RUNX2, osterix, and osteocalcin, culture of tendon-derived fibroblasts in osteogenic medium did not induce statistically significant upregulation of any of these genes. Similar results were found by QPCR of these markers

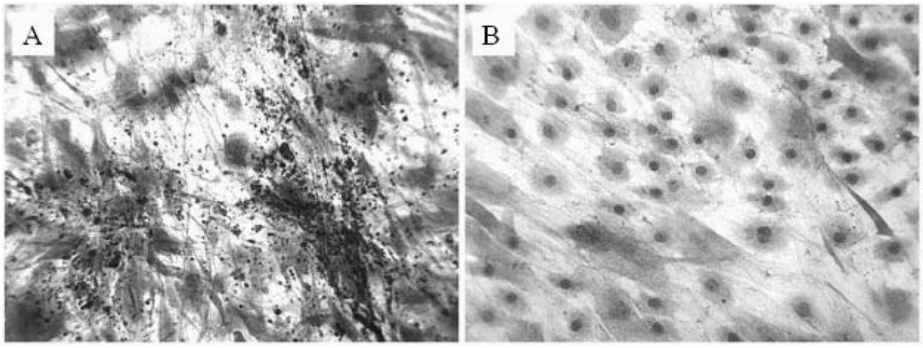

Figure 4.6 *Von Kossa staining on tendon-derived fibroblasts cultured for 21 days in osteogenic (A) or control medium (B). Calcium deposition was seen in osteogenic medium (A), not in control medium (B) or in adipogenic or chondrogenic media (figures not shown). (See color inlay for a full color version of this figure.)*

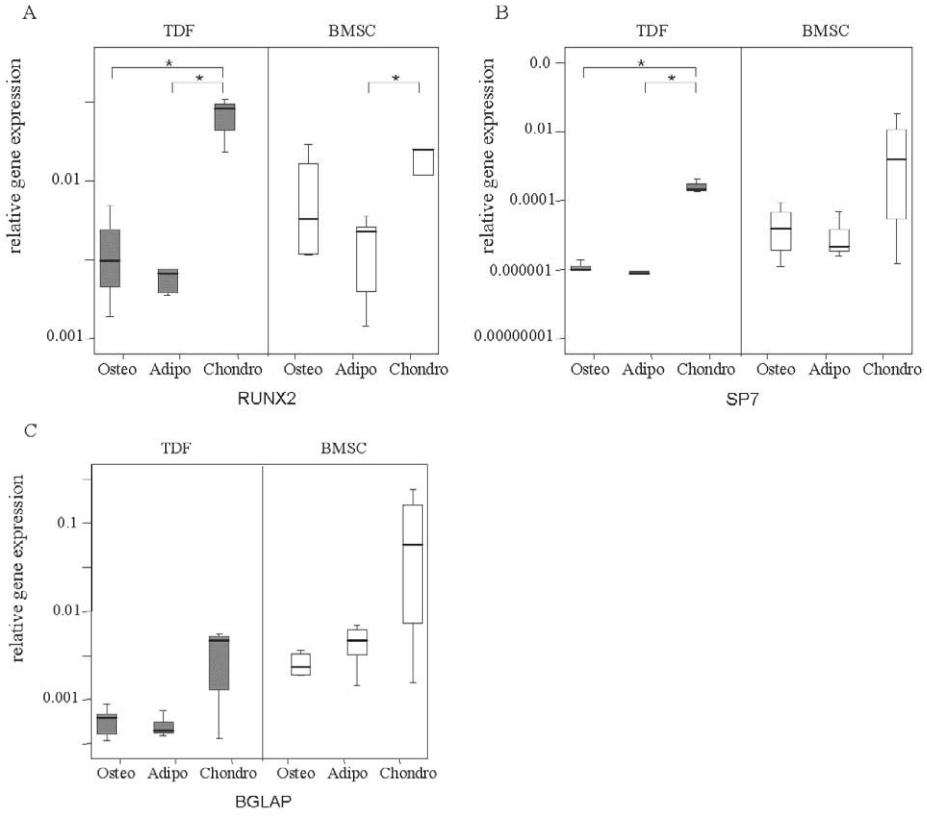

Figure 4.7 *Expression levels of osteogenic markers in tendon-derived fibroblasts (TDF) and bone marrow-derived stromal cells (BMSC). RUNT-related transcription factor 2 (RUNX2) (A), SP7 (B), and BGLAP (C). See figure 3 for reminder of key.*

in BMSCs cultured in osteogenic medium (Figure 4.7). In the tendon-derived fibroblast cultures SP7 and RUNX2 (both also known to play an important role in chondrogenic differentiation and hypertrophic cartilage formation [26]) were significantly upregulated in the chondrogenic medium compared to the osteogenic (p=0.025 for both genes) and adipogenic medium (p=0.025 for both genes)(Figure 4.7). BMSCs also showed an upregulation of SP7 and RUNX2 in the chondrogenic medium. RUNX2 upregulation was significant (p=0.016 for the difference in gene expression of RUNX2 between adipogenic and chondrogenic medium in BMCSs), but SP7 upregulation in chondrogenic medium did not reach significance (Figure 4.7). In summary, chondrogenic medium not only stimulated expression of chondrogenic marker COL2A1, but also of COL10A1, RUNX2, and SP7.

DISCUSSION

This in-vitro differentiation study suggests that a proportion of the cell population explanted from adolescent human tendon tissue may have adipogenic and chondrogenic differentiation potential. In adipogenic medium lipid vacuoles were visible and tendon-derived fibroblasts showed upregulation of FABP4 and PPARG. In chondrogenic medium, positive collagen type II staining was visible around some of the tendon-derived fibroblasts and the tendon-derived fibroblasts showed upregulation of COL2A1 and COL10A1. In osteogenic medium Von Kossa staining showed calcium deposition, although osteogenic markers remained unaltered, as assessed by qPCR. Compared to the BMSCs, the diffentiation capacity of our tendon-derived fibroblasts was similar, although some differences were visible, mainly concerning the number of Oil Red O positive cells.

To our knowledge, this is the first study evaluating the intrinsic differentiation potential of human tendon cells in vitro. Previously, Salingcarnboriboon et al [27] established three murine tendon cell lines by clonal expansion and showed that these single cell clones could differentiate towards multiple mesenchymal lineages upon culture in appropriate differentiation media. Therefore, they suggested that cells with mesenchymal stem-cell-like characteristics might exist in murine tendon tissue. Our experiments cannot distinguish between individual cells with multilineage potential and a cell population containing more or less strongly committed cells. We did find that not all of the tendon-derived fibroblasts appeared to be capable of differentiating towards other lineages, e.g. not all fibroblasts but merely clusters of fibroblasts created lipid vacuoles in adipogenic medium and only a small proportion of approximately 5% of the cells stained positive for collagen type II. In addition to this observation, only a subpopulation of 72.6 +/- 22.9 % of these tendon-derived fibroblasts stained positive for CD105 and this subpopulation might be responsible for the observed differentiation potential.

Due to their spindle-shaped morphology in monolayer culture and because all explanted cells stained D7-FIB positive in passage one through passage four, we identified these cells

as tendon-derived fibroblasts. Based on the results of the Ki-67 staining, it could be surmised that this mixed population may be partly derived from the tendon tissue and partly from the endotenon. It is possible that these cells were already preselected for during the explantation procedure, based on cellular motility, chemotactic responses or plastic adherence characteristics. Within this culture population, mature tendon-derived fibroblasts with transdifferentiation capacity or a specific subpopulation of tendon-derived progenitor cells might exist. Several authors have found that pericytes isolated from different tissues can be induced to differentiate into various connective tissue phenotypes [8]. It seems unlikely that the presence of vascular pericytes in tendon tissue, which might be another multipotent cell source in tendon tissue [28], can account for our findings. Not only is tendon a poorly vascularized tissue, but also the tendon-derived fibroblasts remained negative for pericyte marker α-SMA through the first four passages. Furthermore, our explanted cell population was 98.5 +/- 0.7 % negative for CD34 on FACS-analysis. It seems unlikely that the small portion of 1.5% CD34-positive tendon-derived fibroblasts accounts for the results of the immunohistochemical staining and the changes in gene expression pattern.

A cell population with multilineage potential that might be present in tendon tissue, is likely involved in tendon repair. Such a population might also contribute to the development of tendinosis, as this tendon disorder is associated with fatty degeneration, glycosaminoglycan accumulation, and calcifications. In addition to these internal multipotent cells other cells with multilineage potential may arrive at the site of overuse or tendon damage through the vascular system and contribute to the development or repair of tendinosis: upregulation of VEGF was found in human Achilles tendinosis lesions [29] and VEGF can act as a chemotactic stimulus for mesenchymal cells [30]. In-vivo control of differentiation of cells with multilineage potential might prove useful in the future for prevention of tendinosis lesions or induction of in-situ repair of these lesions.

The exact changes in the tendon microenvironment outside the cells that play a role in cellular differentiation are still the subject of many investigations. First, the capability of specific growth factors, cytokines, and other inflammatory mediators to influence the cellular differentiation process has been demonstrated. Changes in the concentration of various growth factors have also been found in tendinosis lesions: for instance, a higher number of cells expressing TGF-β2 and TGF-βRII (a TGF-β receptor) in chronic Achilles tendinosis lesions [31] and increased expression of TGF-β1 in patellar tendinosis [32] have been reported. TGF-β molecules are also used in vitro to induce chondrogenic differentiation of mesenchymal progenitor cells [21]. Second, changes in the degree of vascularisation of the tissue, as reported in Achilles tendinosis lesions [33], might influence the tendon cell differentiation state in vivo. For instance, oxygen tension influences the redifferentiation potential of dedifferentiated chondrocytes in vitro [34] and hypoxia not only promotes the differentiation of bone mesenchymal stem cells along a chondrocyte pathway [35], but can also promote the formation of an adipocyte-like phenotype with cytoplasmic lipid inclusions in human MSCs [36]. Third, following repetitive tendon

overload and its resulting microruptures in tendinosis lesions [37], tendon cells may experience an altered mechanical microenvironment, which in turn might influence chondrogenic, osteogenic, or tenogenic differentiation [38].

Our findings demonstrate that an intrinsic differentiation capacity is present in tendon tissue of adolescent individuals. However, age plays an important role in the response of musculoskeletal tissues in response to environmental changes. It has been demonstrated that adult but not juvenile cartilage has lost its ability to regenerate (cited by Hunter [39]) and BMSCs gradually loose their differentiation potential as subjects grow older [40]. Therefore, the adolescent tendon samples used in this study might not be representative of tendon tissue in adult tendinosis lesions. Since tendon cell populations derived from adult and from late fetal equine tendons have demonstrated similar levels of a weak progenitor cell ability [41], it might be justified to speculate that tendon-derived fibroblasts from older subjects may still have some differentiation capacity. However, this certainly needs further investigation.

A tendon-cell population with intrinsic differentiation capacity might be used in vivo for repair of lesions and might play a role in tendinosis. However, extrapolating results from in-vitro cultures to the in-vivo situation must be done with tremendous caution, particularly as the expansion-culture period prior to experimentation may have led to the loss of the original tendon fibroblast phenotype (due to dedifferentiation): the latter being well known in chondrocyte-cultures [42]. Whether cells in vivo can be stimulated to display this differentiation potential remains to be elucidated.

CONCLUSIONS

Obtaining insight in the cellular behavior and pathogenesis in tendinosis is crucial in order to develop mechanism-based therapies. Our study suggests that adolescent tendon tissue has an intrinsic differentiation potential. This study conducted on human tenocytes corroborates the findings that cells with mesenchymal stem-cell-like characteristics might exist in murine tendon tissue. Our results support the hypothesis that altered tendon-cell differentiation might play a role in the pathophysiology of tendinosis.

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Chapter 5

In-vitro model to study chondrogenic differentiation in tendinopathy

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ABSTRACT

Background

Fibrocartilaginous metaplasia can be a sign of tendinopathy. We investigated whether chondrogenic differentiation is present in mid-portion Achilles tendinopathy and subsequently developed an in-vitro model for chondrogenic differentiation of tendon cells to investigate possible targets for future treatment.

Methods

Peroperatively harvested tissue from human mid-portion Achilles tendinotic lesions (n=12) and healthy Achilles tendons (n=5) were analysed by microscopy and real-time reverse transcription polymerase chain reaction (QPCR). Secondly, chondrogenic differentiation of non-degenerative human tendon explants was induced using transforming growth factor beta (TGF-β) to develop an in-vitro model for chondrogenic differentiation of tendon tissue. To evaluate the usefulness of the model, we investigated the possibility to modulate the chondrogenic differentiation in vitro by removing the chondrogenic stimulus or adding triamcinolone or platelet-rich plasma (PRP).

Results

Mid-portion Achilles tendinotic lesions had significantly increased glycosaminoglycan staining and more rounded cell nuclei than healthy tendons. Chondrogenic markers (sex-determining region Y)-box9 (SOX9), aggrecan (AGC1), collagen type II (COL2A1), RUNT-related transcription factor 2 (RUNX2), but not collagen type X (COL10A1) were upregulated. Non-degenerative tendon explants cultured on chondrogenic medium for 7, 14, and 21 days had significantly higher expression of AGC1, COL2A1, and COL10A1, but not SOX9 and RUNX2. Removing the chondrogenic stimulus at day 14 resulted in a reduced expression of AGC1, COL2A1, and COL10A1 at day 21. Adding triamcinolone (10^{-4} M) from day 14 resulted in less COL2A1 expression, whereas other genes were not affected. PRP (20% vol/vol) also appeared to likely influence chondrogenic gene expression pattern.

Conclusions

In conclusion, mid-portion Achilles tendinopathy showed chondrogenic differentiation and the in-vitro model that was developed in order to investigate chondrogenic differentiation appeared to be useful for the evaluation of innovative therapies.

INTRODUCTION

Chronic tendinopathies are a common problem in orthopaedic and rheumatologic practice, occurring in athletes as well as in the general population. Chronic Achilles tendinopathy involves either mid-portion tendinopathy, insertional tendinopathy, or peritendinitis, or any combination of these. Chronic mid-portion Achilles tendinopathies are often difficult to treat, and surgical interventions may be necessary [1, 2]. Although several hypotheses have been formulated during the last decade [3-7], the pathophysiology of tendinopathies in general, and of mid-portion Achilles tendinopathy in particular, is still largely unknown.

Considerable alterations in the extracellular matrix (ECM) composition have been described in chronic mid-portion Achilles tendinopathy [8]. Firstly, an increased amount of glycosaminoglycans is present in tendinotic tissue compared to healthy tendon tissue [9, 10]. Secondly, the tendon cells in chronic mid-portion Achilles tendinopathy display an altered gene expression pattern of the major proteoglycans towards that seen in fibrocartilaginous tissue [11, 12]. These findings led us to consider whether in Achilles mid-portion tendinopathy some of the tendon cells may deviate from tendon matrix production towards fibrocartilaginous matrix production. Fibrocartilaginous differentiation along the length of the mid-portion area of the Achilles tendon, which is a 'direct' tendon area [12] (an area that runs a straight course and experiences mainly tensile forces), most probably results in impaired elasticity and tensile stress resistance of the tendon. This can compromise the tendon's function as elastic energy-saving spring and force transmitter, and thus can make the tissue more prone to degeneration and rupture.

Fibrocartilaginous metaplasia is a normal feature of insertional tendon areas (entheses) and of compressed areas of 'wrap-around' tendons that bend around a bony pulley [12]. This process is considered to be a functional adaptation to compressive loads and/or stress shielding (lack of tensile loads) [3, 13]. However, it can also be a sign of tendon pathology. Maffulli *et al.* found chondral metaplasia in calcific insertional Achilles tendinopathy [14]. Archambault *et al.* found cartilage-specific markers to be upregulated in an overuse model of the rat supraspinatus tendon [15]. Excessive or inappropriate fibrocartilaginous matrix production in tendons may therefore be considered part of the pathological processes involved in developing tendinopathies. Consequently, removing or ceasing the stimulus that causes the fibrocartilaginous metaplasia might help tenocytes to return to their normal tendon matrix production. From this perspective, understanding the mechanical and/or molecular signals that can influence chondrogenic differentiation may be key factors to eventually improve prevention and treatment opportunities of developing tendinopathies.

Because tendinopathies are exposed to triamcinolone and PRP in clinical as well as experimental settings, both compounds are of interest to study in relation to chondrogenic differentiation of tendon cells. Triamcinolone is a synthetic analogue of cortisol which has a potent anti-inflammatory effect [16]. Although inflammation does not appear to play a major role in chronic tendinopathies [15, 17], triamcinolone is used as a symptomatic treatment for various tendinopathies and for peritendinitis. As peritendinitis is a frequently diagnosed co-morbidity

of tendinopathy, tendinotic tendon tissue may be exposed to rather high local steroid concentrations at some point during their disease course. Platelet-rich plasma (PRP) has yielded promising outcomes in numerous clinical applications. A cohort study reported 93% reduction of pain for PRP-treated patients with chronic elbow tendinosis [18], although the exact working mechanism still remains to be elucidated.

We hypothesized that chondrogenic differentiation, demonstrated in insertional and compressed tendon areas, also takes place in mid-portion Achilles tendinopathy. Therefore, we investigated whether a chondrogenic differentiation pattern was seen in mid-portion Achilles tendinopathy in patients. Secondly, we hypothesized that this chondrogenic differentiation might be used as a target for drug treatment of developing tendinotic lesions. Therefore, the second part of this research aimed at developing an in-vitro culture model of chondrogenic tendon differentiation, for future use in evaluating treatment opportunities for tendinopathy.

MATERIALS AND METHODS

Study design

To investigate whether mid-portion Achilles tendinotic lesions display a chondrogenic differentiation pattern, peroperatively harvested biopsies of human mid-portion Achilles tendinotic lesions and of macroscopically healthy Achilles tendons were analysed by microscopy and real-time RT-PCR. Secondly, to develop a culture model for chondrogenic differentiation of tendons, healthy adolescent hamstring tendon explants and healthy adult Achilles tendon explants were stimulated in-vitro with TGF-β2. The subsequently induced chondrogenic differentiation was modulated by switching to non-chondrogenic medium, or by adding either triamcinolone or platelet-rich plasma (PRP) to the chondrogenic culture medium.

Tendon biopsies

For the in-vivo study, macroscopically affected tissue specimens of 12 consecutive patients with chronic mid-portion Achilles tendinopathy (6 male and 6 female; average age 46 years, range 36-58; mean duration of symptoms 25 months, range 9-54 months) were harvested during surgical debridement of the lesion. All lesions were located between 3-5 cm proximal from the insertion. Chronic Achilles tendinopathy was defined as Achilles tendon pain for at least 3 months in combination with clinically and/or radiologically (either MRI or ultrasonography) suspected mid-portion Achilles tendinopathy. Patients with previous tendon injury, radiologically evident partial tendon rupture, or use of chinolone antibiotics in the past three years were excluded. Approval for this study was obtained from the Medical Ethical Committee of the Erasmus MC University Medical Center (MEC-2005-100). All patients signed informed consent. Healthy Achilles tissue specimens (all between 3-5 cm proximal from the insertion) of 5 donors (2 male, 3 female; average age 55, range 24-78) without clinically evident Achilles tendinopathy

were collected as control. Surgical procedures for these patients included arthrodesis of the talocrural joint, upper leg amputation because of a septic revised total knee prosthesis, lower leg amputation after trauma, and extension of the Achilles tendon for spastic diplegia (MEC-2006-069).

For the differentiation culture studies we harvested a) non-degenerative hamstring tendon tissue from 9 adolescents (all under 18 yrs of age) undergoing hamstring-tendon release for treatment of knee-contractures (MEC-2006-069) and b) non-degenerative Achilles tendon tissue from 2 adults (ages 24 and 61 years) undergoing leg amputation following acute trauma (MEC-2006-069).

Chondrogenic differentiation experiment

After the peritendineum and the fat tissue had been carefully removed, the tendons were cut into 3 mm^3 sections, transferred into six-well plates (Corning, NY, USA), and cultured at 37˚C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS or in chondrogenic induction medium containing DMEM supplemented with TGF-β2 10 ng/ml (R&D Systems, UK), L-ascorbic acid-2-phosphate 25 µg/ml (Sigma), sodium pyruvate 100 µg/ml (Invitrogen), proline 40 µg/ml (Sigma) and ITS+ (diluted 1:100; BD Biosciences, Bedford, MA). All media contained 50 µg/ml gentamycin and 1.5 µg/ml fungizone (both Invitrogen). Medium was refreshed twice a week. Explants were frozen in liquid nitrogen for RNA extraction at day 7 (adolescent hamstring tendons n=5 donors) and day 14 (adolescent hamstring tendons n=5 donors, adult Achilles tendons n=2 donors).

Modulation of chondrogenic differentiation

Explants were cultured during 21 days (adolescent hamstring tendons from 2 donors, adult Achilles tendons from 2 donors). Explants were first maintained for 14 days on chondrogenic medium. At day 14, either the chondrogenic medium was switched to control medium, or triamcinolone acetonide (10^{-4} M; Sigma, T6501) was added to the chondrogenic medium, or PRP (20% vol/vol) was added to the chondrogenic medium. In addition, explants were cultured for 21 days on chondrogenic medium or for 21 days on control medium. Explants were harvested in duplicate at day 21 for RNA extraction.

PRP preparation

After informed consent, whole blood (500 mL) from a healthy, male donor was collected in 70 ml of anticoagulants (citrate-phosphate-dextrose [CPD], Sanquin Blood Supply Foundation) and processed within 24 hours. Briefly, whole blood was centrifuged at 300x*g* for 10 min. The buffy coat was centrifuged again at 300x*g* for 10 min to separate PRP (supernatant) from erythrocytes and leucocytes (bottom layer). The PRP was then centrifuged at 480x*g* for 20 min to precipitate the platelets. After removal of the supernatant, the platelets were resuspended in a smaller volume to increase platelet concentration. Clotting upon addition of 22.8 mM $CaCl_2$

at 37°C for 1 hr activated platelets to release their growth factors. The soluble releasate from the clotted preparation, containing growth factors, was aspirated, stored at 4°C, and used within two weeks. Platelet concentration in PRP, measured on an ABC Animal Blood Counter (Scil, Viernheim, Germany), was 2.8 times higher than baseline concentration in whole blood.

RNA isolation and Real-Time RT-PCR

Explants and biopsies were quickly homogenized in a Mikro-Dismembrator® (BioTech International Inc, Needville, Texas) and suspended in >1.8ml/100mg RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was further purified using RNeasy® Micro Kit (Qiagen, Hilden, Germany), and 1 µg of total RNA of each sample was reverse-transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Primers were designed using PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA, USA) to meet Taqman® requirements and were designed to bind to separate exons to avoid co-amplification of genomic DNA. BLASTN ensured gene specificity of the following primers and probes: (sex-determining region Y)-box9 (SOX9) [19], aggrecan (AGC1) [19], and collagen type II (COL2A1) [19] were used as chondrogenic markers; RUNT-related transcription factor 2 (RUNX2) [19] and collagen type X (COL10A1) [19] were used as markers for early and late hypertrophic cartilage formation respectively; gene expression of transforming growth factor beta (TGF-β)

(F: 5'-GTGACAGCAGGGATAACACACTG-3'; R: 5'-CATGAATGGTGGCCAG GTC-3'; Probe: 5'-ACATCAAGGGGTTCACTACCGGC-3') was assessed in the patient samples. Amplifications were performed as 20 µl reactions using TaqMan® Universal PCR MasterMix (ABI, Branchburg, New Jersey, USA) according to the manufacturer's guidelines. Real-Time RT-PCR (QPCR) was performed using an ABI PRISM® 7000 with SDS software version 1.7. Two housekeeping genes, GAPDH and 18SrRNA, were tested on both the in-vivo and the in-vitro datasets. In-vivo data were normalized to 18SrRNA [20] which was shown to be most stably expressed across those samples. In-vitro data were normalized to GAPDH [19] which was shown to be most stably expressed across these samples. Relative expression was calculated according to the $2^{-\Delta CT}$ formula [21].

Histology

Longitudinal sections (6 µm) of paraffin embedded tendon specimens were stained with hematoxylin and eosin or with thionin (for glycosaminoglycans). Two different researchers each performed a blinded examination of the sections twice. Both GAG-stainability of the tendon matrix and morphology of the cell nuclei were scored 0 (normal), 1 (mildly deviant), 2 (moderately deviant), or 3 (severely affected). For each section the mean scores of the two examiners were added up yielding a total minimum score of 0 and total maximum score of 6.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). A Mann-Whitney U Test was used to assess the differences between healthy and tendinotic Achilles tendon samples as well as to assess the differences between explants cultured on chondrogenic and control medium. The modulation experiments were analysed using a Kruskall-Wallis H test and post-hoc Dunn's multiple comparison test. For all tests $p<0.05$ was considered statistically significant.

RESULTS

In vivo chondrogenic differentiation pattern

To investigate whether mid-portion Achilles tendinotic lesions display a chondrogenic differentiation pattern, peroperatively harvested biopsies of human mid-portion Achilles tendinotic lesions and of macroscopically healthy Achilles tendons were analysed.

Microscopically, the healthy Achilles tendons were confirmed to have normal tendon morphology: uni-axially arranged tendon bundles interspersed with tenocytes with slim nuclei, and no signs of degeneration, inflammatory cell infiltration, granulation, small ruptures, fibrocartilaginous metaplasia, or calcifications. Tendinotic specimens appeared to have a remarkably less organized matrix and cells had a significantly rounder and prominent nucleus (Figure 5.1, see also color inlay). Thionin staining revealed that tendinotic tendon samples had significantly higher GAG scores compared to the healthy tendon samples (Figure 5.2, see also color inlay).

Gene expression analysis revealed that the tendinotic samples expressed significantly higher levels of chondrogenic markers SOX9 (p=0.005), AGC1 (p=0.031), and COL2A1 (p=0.007), and of early marker for hypertrophic cartilage formation RUNX2 (p=0.043). However, COL10A1 expression was not significantly upregulated in the tendinosis samples (p=0.429) (Figure 5.3).

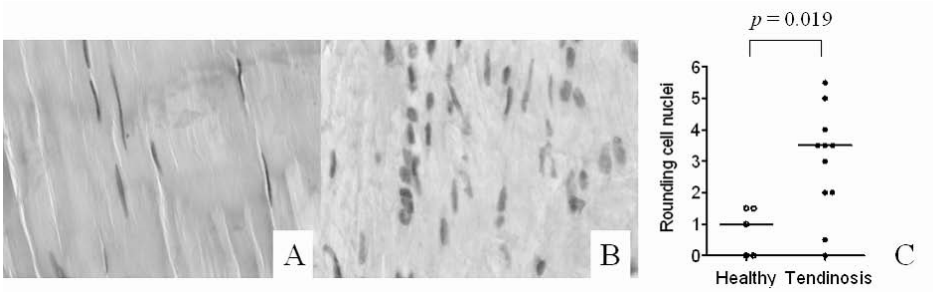

Figure 5.1 *Changes in cell morphology in mid-portion Achilles tendinosis. Hematoxilin and eosin staining of tendon samples. Representative photomicrographs (400x magnification) of healthy Achilles tendon samples (n=5, A) and of Achilles tendinotic samples (n=12, B) are shown; rounding of the cell nuclei was scored for each individual sample; total score can range from 0 (normal) to 6 (maximum deviant), line represents median score (C). (See color inlay for a full color version of this figure.)*

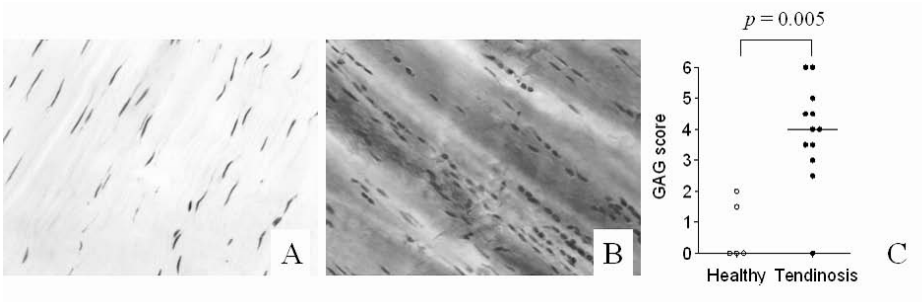

Figure 5.2 *Fibrocartilaginous metaplasia in mid-portion Achilles tendinosis demonstrated by thionin staining for glycosaminoglycans (GAGs). Representative photomicrographs (200x magnification) of healthy Achilles tendon samples (n=5, A), and of Achilles tendinotic samples (n=12, B); GAG stainability was scored for each individual sample, total score can range from 0 (normal) to 6 (maximum GAG staining), line represents median GAG score (C). (See color inlay for a full color version of this figure.)*

Gene expression of TGF-β was significantly upregulated in the tendinotic samples compared to the healthy samples (p=0.005) (Figure 5.3).

In-vitro culture model for chondrogenic tendon differentiation

To simulate chondrogenic differentiation of tendon tissue in an in-vitro model, we cultured the hamstring tendon explants on chondrogenic and control medium for 7 days (Figure 5.4). The expression level of early chondrogenic marker SOX9 was not significantly upregulated

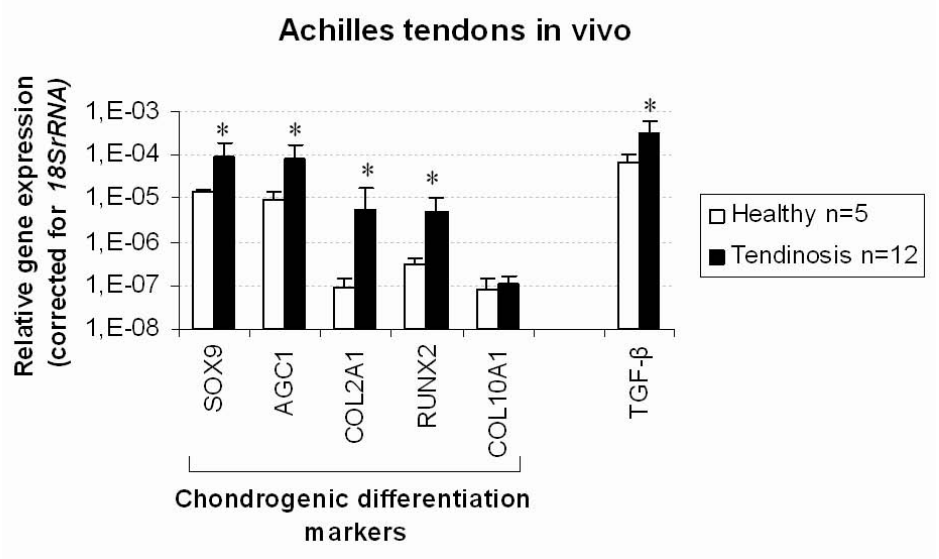

Figure 5.3 *Gene expression of chondrogenic markers and of TGF-β in mid-portion Achilles tendinosis. Healthy (n=5) and tendinotic (n=12) Achilles tendons were harvested peroperatively. Ct values were corrected for 18SrRNA, which was stably expressed across samples (data not shown). * Indicates a p-value<0.05 compared to healthy Achilles tendons.*

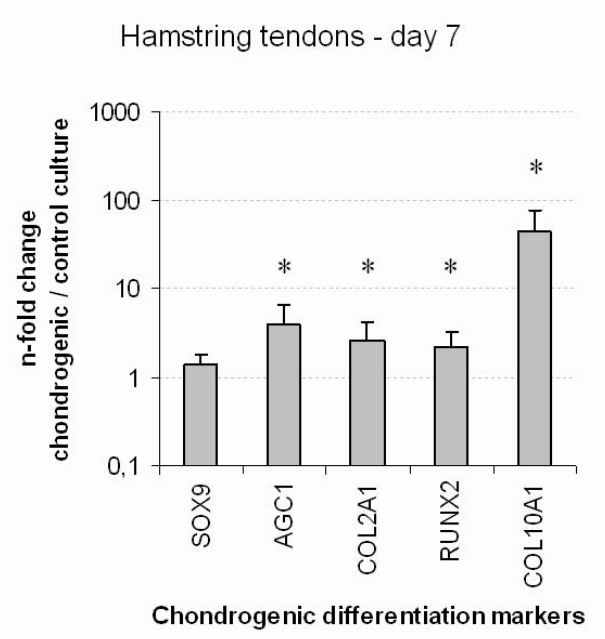

Figure 5.4 *Chondrogenic differentiation in vitro. Gene expression of chondrogenic markers in non-degenerative adolescent hamstring tendon tissue explants (n=5) cultured for 7 days on chondrogenic medium. Relative gene expression levels (corrected for GAPDH) are expressed as n-fold changes compared to control cultures. * Indicates a statistically significant difference between the chondrogenic and control medium (p-value<0.05).*

in chondrogenic medium compared to control medium (p=0.110). However, all other chondrogenic differentiation markers were significantly upregulated: AGC1 (p=0.009), COL2A1 (p=0.009), RUNX2 (p=0.006), and COL10A1 (p=0.002).

To examine whether this chondrogenic differentiation in culture could be extended and was not limited to adolescent hamstring tendons (easily available for research), we repeated the experiment with different tendon types. Therefore we cultured non-degenerative adolescent hamstring tendons (n=5) and non-degenerative adult mid-portion Achilles tendon explants (scarcely available, n=2) for 14 days on chondrogenic medium (Figure 5.5). Expression of AGC1 (p=0.021), COL2A1 (p=0.009), and COL10A1 (p=0.009) was significantly upregulated in chondrogenic medium compared to control medium at day 14. SOX9 and RUNX2 upregulation were not significant (p=0.117 and p=0.347 respectively, Figure 5.5). Gene expression of Achilles tendon explants showed a chondrogenic differentiation pattern very similar to that of hamstring tendon explants.

Modulation of the chondrogenic differentiation model

To test whether the chondrogenic differentiation in this model could be modulated, we used two different approaches. Firstly, after 14 days of culture in chondrogenic medium, we removed

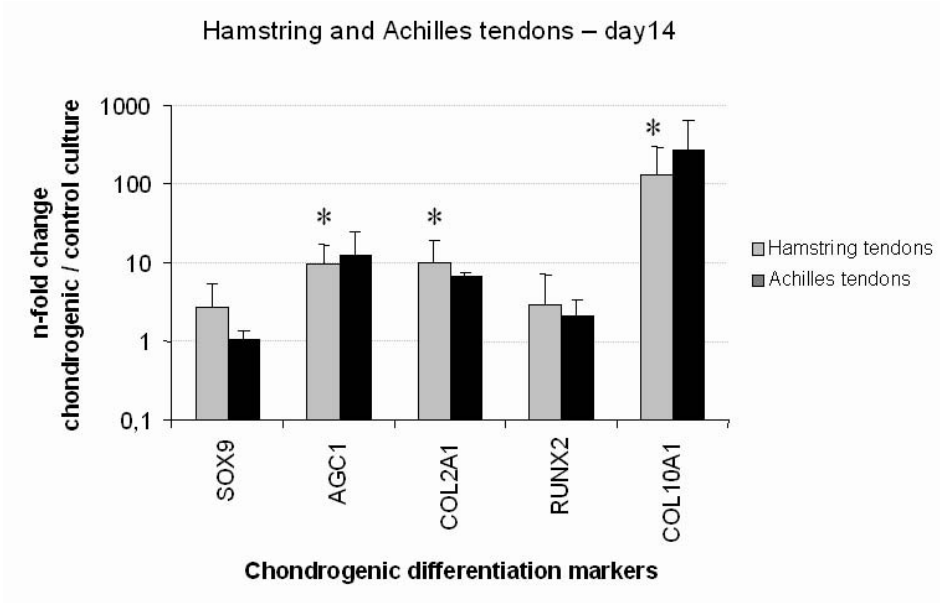

Figure 5.5 *Comparison of chondrogenic differentiation in hamstring and Achilles tendons in vitro. Explants of non-degenerative adolescent hamstring tendon tissue explants (n=5) and non-degenerative adult Achilles tendons (n=2) were cultured for 14 days on chondrogenic and control medium. Relative gene expression levels (corrected for GAPDH) are expressed as n-fold changes compared to control cultures. * Indicates a statistically significant difference between the chondrogenic and control medium (p-value<0.05). Statistics were not performed on Achilles tendon explants due to small sample size.*

the chondrogenic environment by switching back to control medium for another 7 days. Secondly, after 14 days of culture in chondrogenic medium, we added either triamcinolone acetonide (10^{-4} M) or PRP (20% vol/vol) to the medium for another 7 days. Switching back to control medium at day 14 resulted in a significant decrease in expression of these genes at day 21 (p= 0.018 for AGC1, p=0.028 for both COL2A1 and COL10A1). Adding triamcinolone in a concentration of 10^{-4} M at day 14 resulted in a significant decrease of COL2A1 expression (p=0.028), whereas expression of the other genes was not affected (Figure 5.6A). Adding PRP (20% vol/vol) to the chondrogenic medium at day 14 resulted in a significant decrease of chondrogenic markers SOX9 and COL2A1 but not COL10A1 (Figure 5.6B).

DISCUSSION

This study shows chondrogenic differentiation on transcriptional level in mid-portion Achilles tendinopathy. Early and late chondrogenic markers were upregulated in affected tissue, but hypertrophic cartilage formation (as indicated by collagen type X upregulation) was not seen in vivo. However, note that RUNX2, indicated as an early marker for hypertrophic cartilage

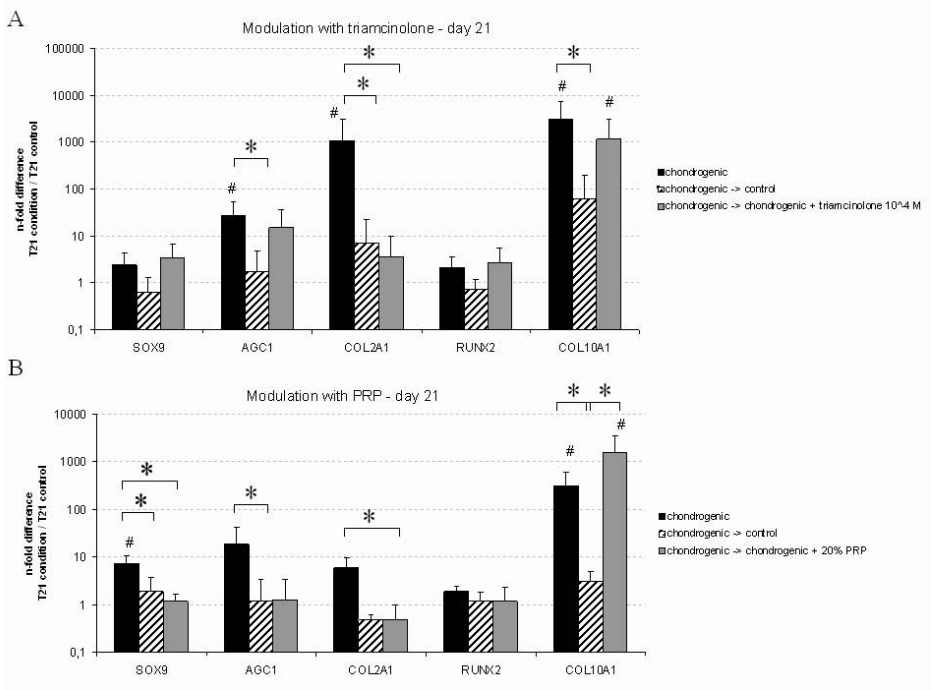

Figure 5.6 *Modulation of chondrogenic differentiation in vitro. Tendon explants were maintained for 14 days on chondrogenic medium.* ***(A)*** *At day 14, the chondrogenic medium was switched to control medium, or triamcinolone acetonide (10–4 M) was added to the chondrogenic medium (n=1 adolescent hamstring tendon in duplo and n=2 adult Achilles tendons in duplo were pooled for statistical analysis).* ***(B)*** *At day 14, the chondrogenic medium was switched to control medium, or PRP (20% vol/vol) was added to the chondrogenic medium (n=3 adolescent hamstring tendons in duplo were pooled for statistical analysis). Relative gene expression levels (corrected for GAPDH) are expressed as n-fold changes compared to control cultures. * Indicates a statistically significant difference between two conditions (p-value<0.05). # Indicates a statistically significant difference between the condition and 21 days on control medium (p-value<0.05).*

formation, was upregulated in the affected tissue. In an in-vitro model simulating this chondrogenic tendon differentiation, the early chondrogenic marker SOX9 was not upregulated anymore, whereas besides collagen type II also the hypertrophic marker collagen type X was upregulated in both tendon types. This suggests that the model in its current state may slightly overstimulate the cells towards a hypertrophic chondrogenic lineage compared to the in-vivo situation. However, all upregulated chondrogenic markers (including collagen type X) could still be significantly downregulated by removing the chondrogenic stimulus. Importantly, we also showed that chondrogenic differentiation of tendon cells could be modulated by addition of a biochemical signal in the presence of the chondrogenic environment. The model can therefore be used to test substances for their potentially therapeutic role in controlling chondrogenic differentiation in tendinopathy.

We used both healthy adolescent hamstring tendons and healthy adult Achilles tendons and found no striking difference between these two tissue sources regarding their behavior in the model. Without stating that chondrogenic differentiation capacity is independent of age or tissue source, we think our results justify the use of easily available non-degenerative adolescent hamstring tendons as a standard tissue source for the model. This is especially interesting because it may often be difficult to obtain large amounts of non-degenerative rotator cuff, elbow, and Achilles tendon tissue In this context, it might even be interesting to test the benefits of an animal tissue source for the experimental model.

Three main theories exist about the origin of cells that play a role in fibrocartilaginous metaplasia in vivo. Firstly, differentiated cells may change their pattern of differentiation in a process termed transdifferentiation. Secondly, local progenitor cells may become reprogrammed by changing molecular or biomechanical signals. Thirdly, circulating stem cells may be recruited from the blood into the tissue and develop into a chondrocyte-like phenotype. Recently, evidence for the presence of cells with multilineage potential in tendon tissue has been collected by several groups. Salingcarnboriboon *et al.* [22] showed that single cell clones from murine tendon cell lines could differentiate towards multiple mesenchymal lineages upon culture in appropriate differentiation media. In-vitro differentiation experiments from our group have shown that third to fifth passage tendon-derived fibroblasts explanted from adolescent hamstring tendons, have a differentiation capacity similar to that of adult bone marrow-derived stromal cells [19]. Our chondrogenic differentiation model suggests that at least part of the resident cells, either local progenitor cells or local differentiated cells, are involved. It cannot be excluded, however, that in the in-vivo situation, also circulating progenitor cells are recruited from the periphery.

Our findings indicate that in mid-portion Achilles tendinopathy at least part of the tendon cells deviate in their metabolism towards fibrocartilaginous matrix production. However, what can cause this chondrogenic differentiation? Mid-portion Achilles tendinopathy has historically been attributed to tensional overload, but the unique anatomical structure of the triceps surae muscle-tendon unit, being a fusion of several muscles bodies into one tendon, might also implicate a role for compressional overload in two ways. Firstly, the tertiairy bundles in the Achilles tendon twist approximately 90 degrees around the tendon's longitudinal axis, the largest part of this twist taking place in the mid-portion area [23]. During tensional loading, the mid-portion may experience torsional compressive and shearing forces (similar to wringing out a towel) [23, 24]. Secondly, individual force contributions of the triceps surae components cause non-uniform stress in the Achilles tendon, which may result in frictional and compressive forces between tendon fibres [25]. Anatomical variations and exercise level, both influencing the magnitude and duration of these intratendinous forces, may predispose to develop a lesion. Apart from mechanical factors, also biochemical changes in the environment of the cells can exert an effect on chondrogenic differentation. Growth factors, like TGF-ß in this study, can influence chondrogenic differentiation. In mid-portion Achilles tendinopathy, such biochemical

signals could be due to ageing, inflammatory processes, wound healing processes, or mechanical alterations. In summary, the pathways involved in tensional mechanical overuse leading to microdamage of the fibrils rendering them inactive and causing tensional stress deprivation of tenocytes [26] might act simultaneously or consecutively with the pathways involved in compressional mechanical overload, possibly even enhancing eachother, ultimately leading to cumulative tendon damage and degeneration.

In an in-vitro culture system to simulate chondrogenic differentiation in tendon explants, we applied TGF-β as chondrogenic stimulus in a stress-deprived culture condition. TGF-β is a well accepted chemical substance to induce chondrogenic differentiation in stem cell research, even without loading [27]. Addition of TGF-ß can be done easily in a (high-throughput) model system. Furthermore, an increase in TGF-β on protein level has been demonstrated in Achilles and patellar tendinopathy [28, 29], which is confirmed on transcriptional level in the present study. Also mechanical loading effects on chondrogenic behavior have been demonstrated to be dependent on TGF-β mediated pathways [30-32]. New insights on the role of stress deprivation in the development of tendinopathy provide good reason for the use of a stress deprived culture condition in a model for tendinopathy [33]. However, caution is required to extrapolate data derived from the model directly to the in-vivo situation.

The outcome parameters in our model were restricted to analyses on transcriptional regulation level, which makes the model useful to detect early cellular responses in relatively short term experiments and creates the possibility for high throughput screening. Follow-up with longer culture periods would be necessary to detect changes in matrix composition using histology and biochemistry. Furthermore, the model is intended for testing of early interventions. Advanced degenerative tissue or cartilage tissue formation can very likely not be treated with drugs. In this model we intended to simulate chondrogenic celldifferentiation that is still reversible with drug treatment.

Triamcinolone was studied as a first example to see whether the chondrogenic tendency could be influenced by adding a biochemical substance. Triamcinolone, and glucocorticoids in general, have been studied in the light of chondrogenic differentiation before. Glucocorticoids appear to promote chondrogenic differentiation in general [34-36]; on the other hand, triamcinolone acetonide led to a decrease in the proliferative rate of chondroprogenitor cells along with an inhibition of the chondrogenic differentiation pathway in an in-vitro study with neonatal mouse cartilage explants [37]. Therefore, triamcinolone was expected to exert a certain effect on the chondrogenic differentiation state of tendon cells (either stimulatory or inhibitory). In our model, triamcinolone acetonide successfully downregulated COL2A1 expression, but had no effect on any of the other chondrogenic markers. As a second example we tested the effect of platelet-rich plasma, a promising new substance in the treatment of tendinopathy [18, 38], in our model. In autologous PRP applications, the effects thus far have been attributed to the presence of high concentrations of multiple growth factors and cytokines that promote wound healing by stimulating cell migration, proliferation, collagen production, and differentiation

[38-41]. In this experiment, heterologous platelet-rich plasma appeared to decrease the chondrogenic differentiation state of tendon explants. However, the exact working mechanism remains to be elucidated.

In conclusion, we have shown that chondrogenic differentiation is present in mid-portion Achilles tendinopathy. Our study also demonstrates the use of an in-vitro model to investigate early chondrogenic differentiation as a possible target for drug treatment of tendinopathic lesions.

ACKNOWLEDGEMENTS

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Chapter 6

Can platelet-rich plasma enhance tendon repair: a cell culture study

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ABSTRACT

Background

Autologous platelet-rich plasma (PRP) application appears to improve tendon healing in traumatic tendon injuries, but basic knowledge on how PRP promotes tendon repair is needed.

Hypothesis

PRP has a positive effect on cell proliferation and collagen production, and induces the production of matrix-degrading enzymes and endogenous growth factors by human tenocytes.

Methods

Human tenocytes were cultured 14 days in 2% fetal calf serum medium complemented with 0%, 10%, or 20% vol/vol platelet-rich clot releasate (PRCR, the active releasate of PRP) or platelet-poor clot releasate (PPCR). At day 4, 7, and 14, cell amount, total collagen, and gene expression of collagen Iα1 (COL1) and IIIα1 (COL3), matrix metalloproteinases (MMP1, MMP3, MMP13), vascular endothelial-derived growth factor A (VEGF-A) and transforming growth factor β1 (TGFβ1) were analysed.

Results

Platelet numbers in PRP increased to 2.55 times baseline. Growth-factor concentrations of VEGF and PDGF-BB were higher in PRCR than PPCR. Both PRCR and PPCR increased cell number and total collagen, whereas they decreased gene expression of COL1 and COL3 without affecting COL3/COL1 ratio. PRCR, but not PPCR, showed upregulation of MMP1 and MMP3 expression. MMP13 expression was not altered by either treatment. PRCR increased VEGF-A expression at all timepoints and TGFβ1 expression at day 4.

Conclusions

In human tenocyte cultures PRCR, but also PPCR, stimulates cell proliferation, total collagen production. PRCR, but not PPCR, slightly increases the expression of matrix degrading enzymes and endogenous growth factors.

Clinical Relevance

In-vivo use of PRP, but also of PPP to a certain extent, in tendon injuries might accelerate the catabolic demarcation of traumatically injured tendon matrix and promote angiogenesis and formation of a fibro-vascular callus. Whether this will also be beneficial for degenerative tendinopathies remains to be elucidated.

INTRODUCTION

Traumatic tendon injuries and tendinopathies are a growing problem in sports medicine and orthopedic practice [4, 17]. Most tendons have the ability to heal after injury, but the repair tissue is functionally inferior to normal tendon tissue and is accompanied by increased risk of further injury [25]. The poor vascularisation seems to be a major reason for this limited healing capacity [14, 28]. Treatment of tendon lesions, either primary traumatic or degenerative tendinopathies, is often hampered by contradictory descriptions of the underlying pathology, with a limited repertoire of successful and evidence-based treatments [31]. New treatment strategies, such as the use of platelet-rich plasma (PRP), might improve healing.

Clinical applications of autologous PRP in human medicine include periodontal and maxillofacial surgery, plastic surgery, treatment of bone fractures, and treatment of chronic skin and soft tissue ulcers [3, 10, 15, 20, 23, 27]. Numerous publications on PRP yielded excellent clinical outcomes [3, 10, 15, 20, 23, 27, 32]. The only published cohort study in tendon research reported 93% reduction of pain for PRP-treated patients with chronic elbow tendinosis [24]. However, this was a pilot study with a small number of patients and without randomized control group.

Platelets actively participate in healing processes in the body [4]. Platelets contain different growth factors, such as PDGF [4], TGFβ [4, 27], IGF [27], EGF [27], VEGF [27], and FGF [27], which are released from their α-granules upon platelet activation and delivered to the injured site to facilitate healing [4]. Platelet-rich plasma is, by clinical definition, a volume fraction of the plasma, having a platelet concentration above baseline (whole blood) [27]. In activated PRP, compared to activated whole blood, significant increases of growth factors can be observed, e.g. VEGF (6.2-fold), PDGF-BB (5.1-fold), EGF (3.9-fold), and TGFβ1 (3.6-fold) [12]. Specific roles of growth factors in tendon and ligament healing have been studied before. PDGF, peaking shortly after tendon damage, plays a central role in the healing process [12, 25] by chemotaxis, proliferation of fibroblasts, collagen synthesis, and the stimulation of TGFβ1 and VEGF. TGFβ1 increases collagen production and cell viability [25]. VEGF is a powerful stimulator of angiogenesis [25].

To summarize, platelets rapidly release a variety of growth factors and PRP might provide an autologous source of these growth factors that play a key role in tendon repair mechanisms [25]. Not only controlled clinical studies but even more *in-vitro* studies are required to investigate in detail the effects of PRP on human tendon cell metabolism.

In *in-vitro* tendon research, the effects of culturing *equine* flexor digitorum superficialis tendon explants with *100%* PRP (vol/vol) and other blood products were examined [30]. Enhanced anabolic gene expression patterns (COL1A1, COL3A1 and COMP), with no concomitant increase in catabolic genes (MMP3 and MMP13), after 3 days of 100% PRP (vol/vol) treatment were reported. The only study with *human* tendon cells (tenocytes) cultured in *20%* PRP (vol/vol) reported an increase in cell proliferation and in VEGF and HGF production [4]. Effects of PRP on collagen production and degradation of human tenocyte cultures remain to be elucidated.

The purpose of this study was to investigate the effects of releasates from 10% (vol/vol) and 20% (vol/vol) platelet-rich and platelet-poor plasma on human tenocytes in culture. We examined whether platelet-rich plasma releasate affects cell proliferation, collagen production, and production of matrix-degrading enzymes and endogenous growth factors by human tendon cells.

MATERIALS AND METHODS

Isolation of tendon cells

Human tendon-derived cells were explanted from hamstring tendon tissue of three children (age 13-15 years) undergoing hamstring-tendon release for treatment of knee-contractures. Approval was obtained from the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam. After removal of peritendineum, the tendon was cut into 3 mm3 sections, transferred into six-well plates (Corning, NY, USA) and cultured in expansion medium (Dulbecco's modified Eagle's medium, 10% FCS, 50 µg/ml gentamycin and 1.5 µg/ml fungizone (all Invitrogen, Scotland, UK)). Cultures were maintained at 37˚C in humidified atmosphere of 5% CO_2 for ten days. During this time, fibroblasts migrated out of the tissue and adhered to the bottom of the culture dish. Cells were subcultured and trypsinized at subconfluency. Cells from fifth and sixth passage were used.

Plasma preparations

After informed consent, whole blood (500 ml) from nine healthy, male donors (median age 47 years, range 31-69 years) was collected in 70 ml of anticoagulants (citrate-phosphate-dextrose [CPD], Sanquin Blood Supply Foundation) and processed within 24 hours as a non-autologous source of platelets. None of the donors used medication that is known to influence platelet function. Processing protocols for platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were adopted from literature [4-6] and tested in different combinations. The best combination of these protocols (with highest platelet counts and lowest blood cell counts) was used in this study.

Briefly, whole blood was centrifuged at 300x*g* for 10 minutes. The supernatant was centrifuged at 4500x*g* for 12 minutes, to obtain a superficial layer of PPP. The buffy coat of the first centrifugation was centrifuged at 300x*g* for 10 minutes again to separate it into PRP (supernatant) and erythrocytes and leucocytes (bottom layer). In order to increase the platelet concentration, PRP was then centrifuged at 480x*g* for 20 min to precipitate the platelets. Half the superficial plasma layer was removed and the platelet pellet was suspended in the remaining half of the plasma volume. Clotting upon addition of 22.8 mM $CaCl_2$ at 37°C for 1 hr activated platelets to release their growth factors. The soluble releasate from the clotted preparations (PRCR and PPCR), containing growth factors, was aspirated, stored at 4°C and used within two weeks.

Concentrations of platelets and red and white blood cells were measured on ABC Animal Blood Counter (Scil, Viernheim, Germany) in samples of whole blood, PRP and PPP (before clotting), and PRCR and PPCR (after clotting). For growth factor measurements, 1 ml of freshly prepared PPCR and PRCR was collected separately and immediately centrifuged at 1000x*g* for 10 minutes at 4˚C, and stored at –80˚C until further use. Growth factor concentrations in PRCR and PPCR were measured in triplicate using commercially available sandwich ELISA kits for VEGF and PDGF-BB (both R&D systems, Abingdon, UK) according to manufacturers protocol.

Cell culture experiment

Trypsinized tenocytes were plated at a density of 4,000 cells/cm^2 and maintained in 10% FCS for 24 hours prior to replacement by medium with 2% FCS and 0.1 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, Missouri, USA), with or without (a) PPCR 10% (vol/vol), (b) PRCR 10% (vol/vol) (c) PPCR 20% (vol/vol), or (d) PRCR 20% (vol/vol). Cells were cultured at 37˚C in humidified atmosphere of 5% CO_2 for 14 days. Medium was refreshed at day 4, 7 and 11. At day 4, 7 and 14, amount of cells (DNA assay), total collagen (hydroxyproline assay), and gene expression of collagen types Iα1 (COL1) and IIIα1 (COL3), matrix metalloproteinases (MMP1, MMP3, and MMP13), vascular endothelial-derived growth factor A (VEGF-A) and transforming growth factor β1 (TGFβ1) were analysed.

DNA assay

Cells were suspended in 0.1% phosphate buffered saline (PBS) / Triton X-100 (Sigma-Aldrich, St. Louis, USA). Samples were sonificated for 10 seconds, incubated with 200 µl of 8.3 IU/ml heparin solution (LEO Pharma BV, Breda, Netherlands) and 100 µl of 0.05 mg/ml ribonuclease A for 30 min at 37˚ C. This was followed by adding 100 µl ethidium bromide solution (25 µg/ml) (Sigma-Aldrich). Quantification of incorporated dye was performed in triplicate on the Wallac 1420 victor2 (PerkinElmer, Wellesley, MA) using an extinction filter of 340 nm and an emission filter of 590 nm [11]. For standards, calf thymus DNA (Sigma) was used.

Hydroxyproline assay

Cells were suspended in milli-Q, hydrolyzed at 108°C for18-20 hours in 6 M HCl, dried and redissolved in 100 µL water. Hydroxyproline contents were measured by colorimetric method [8] (extinction 570 nm), with chloramine-T and dimethylaminobenzaldehyde (DMBA) as reagentia and hydroxyproline as standard (all Merck, Damstadt, Germany).

Gene expression analysis

Cells were suspended in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). Downstream processing and real-time PCR is described elsewhere [9]. Briefly, RNA was purified using RNeasy® Micro Kit (Qiagen, Hilden, Germany), and 1 µg of total RNA of each sample was reverse-transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany).

Primers were designed to meet Taqman® or SYBR®Green requirements and ensure gene specificity. COL1 [9] and COL3 [9] were studied as markers for collagen production. MMP1 [9], MMP3 [9] and MMP13 [9] were used as indicators of collagen degradation. Also, gene expression of growth factor VEGF-A (F:5'-CTTGCCTTGCTGCTCTACC-3'; R:5'-CACACAGGATGGCTTGAAG-3') and TGFβ1 (F:5'-GTGACAGCAGGGATAACACACTG -3'; R:5'-CATGAA TGGTGGCCAGGTC-3'; PROBE:5'-ACATCAACGGGTTCACTACCGGC-3') were assessed. Amplifications were performed as 20 µL reactions using TaqMan® Universal PCR MasterMix (ABI, Branchburg, NJ, USA) or qPCR™ Mastermix Plus for SYBR® Green I (Eurogentec, Maastricht, Netherlands) according to the manufacturer's guidelines on an ABI PRISM® 7000 with SDS software version 1.7. Data were normalized to 18SrRNA (F:5'-AGTCCCTGCCCTTTGTACACA-3'; R:5'-GATCCGAGGGCCTCACTAAAC-3'; PROBE:5'-CGCCCGTCGCTACTACCGATTGG-3'), which was stably expressed across samples (not shown). Relative expression was calculated according to $2^{-\Delta CT}$ method [21].

Statistical analysis

The experiment was performed in duplicate for all three donor explants (n=6). 2% FCS condition without PRCR or PPCR was used as control and was set to 1 at each timepoint. All conditions were expressed as n-fold difference from the control at the corresponding timepoint. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). A Kruskall-Wallis H test and post-hoc Dunn's multiple comparison test was used. $p<0.05$ was considered to indicate statistically significant differences.

RESULTS

Plasma preparations

Whole blood baseline platelet concentrations were all within physiological range (119 x10^6 to 195 x10^6 platelets/ml) (Table 6.1). The platelet concentration procedure increased platelet numbers in the PRP group on average to 2.55 times baseline concentration in whole blood. The PPP group showed an average decrease in platelet number to 0.02 times baseline. After clotting PRP and PPP, platelet numbers decreased respectively to 0.08 and 0.05 times baseline. In addition, the centrifugation and clotting procedure decreased white blood cell counts to 0.02 and red blood cell counts to 0.01 in PRCR, and in PPCR to respectively 0.02 and 0.00 times baseline.

VEGF concentration in PPCR was below the detection limit of 20 pg/ml in all preparations. On the other hand, VEGF concentration in PRCR was 107 ± 83 pg/ml (mean ± SD). PDGF-BB concentration in PPCR was 123 ± 151 pg/ml (mean ± SD) and in PRCR 3114 ± 2709 pg/ml (mean ± SD).

Table 6.1 *Platelet (PLT), white blood cell (WBC), and red blood cell (RBC) numbers in five different preparations from three donors.*

Plasma preparation	Donor	Concentration (x10^6 / ml)			n-fold change in PLT concentration (preparation / whole blood) (mean ± SD)
		PLT	WBC	RBC	
Whole blood	1	186	5.00	4430	1.00 ± 0.25
	2	195	4.17	4050	
	3	119	3.63	4313	
Platelet-Poor Plasma (PPP)	1	4.33	0.10	0.00	0.02 ± 0.00
	2	5.00	0.10	0.00	
	3	3.00	0.10	10.00	
Platelet-Rich Plasma (PRP)	1	451	14.47	2570	2.55 ± 0.16
	2	488	4.27	1750	
	3	324	8.90	2437	
Platelet-Poor Clot Releasate (PPCR)	1	6.00	0.10	10.00	0.05 ± 0.02
	2	11.00	0.10	10.00	
	3	8.00	0.10	10.00	
Platelet-Rich Clot Releasate (PRCR)	1	7.00	0.10	10.00	0.08 ± 0.07
	2	7.00	0.10	10.00	
	3	19.00	0.10	90.00	

Cell morphology

All cells at day one showed a spindle-shaped, fibroblast-like morphology. Control cells maintained their fibroblast-like morphology during the experiment, but in all PRCR and PPCR conditions the cells altered their morphology towards a more stretched, oblong shape during the 14 days of culture (Figure 6.1, see also color inlay).

DNA assay (amount of cells)

DNA content under control conditions increased in time. Both 20% PRCR (vol/vol) and 20% PPCR (vol/vol) significantly increased DNA content compared to control cultures ($p<0.05$) (Figure 6.2). The effects of PRCR and PPCR were dose-related. No significant changes between PRCR and PPCR were found.

Hydroxyproline assay (amount of collagen)

Total amount of collagen in the control condition increased in time. From day 7 on PRCR and PPCR both increased total amount of collagen, up to at least 3.3 times the control at day 14. However, only the 20% (vol/vol) conditions reached significance at day 7 (Figure 6.3). No differences were found between PRCR and PPCR, although at day 14 the 20% PPCR did not reach significance but the 20% PRCR did.

Gene expression

Gene expression of COL1 significantly decreased with PRCR but not PPCR treatment at day 7 and 14 (Figure 6.4A). Differences between PRCR and PPCR were not significant. COL3 gene expression was not significantly decreased upon addition of PPCR untill day 14, but PRCR

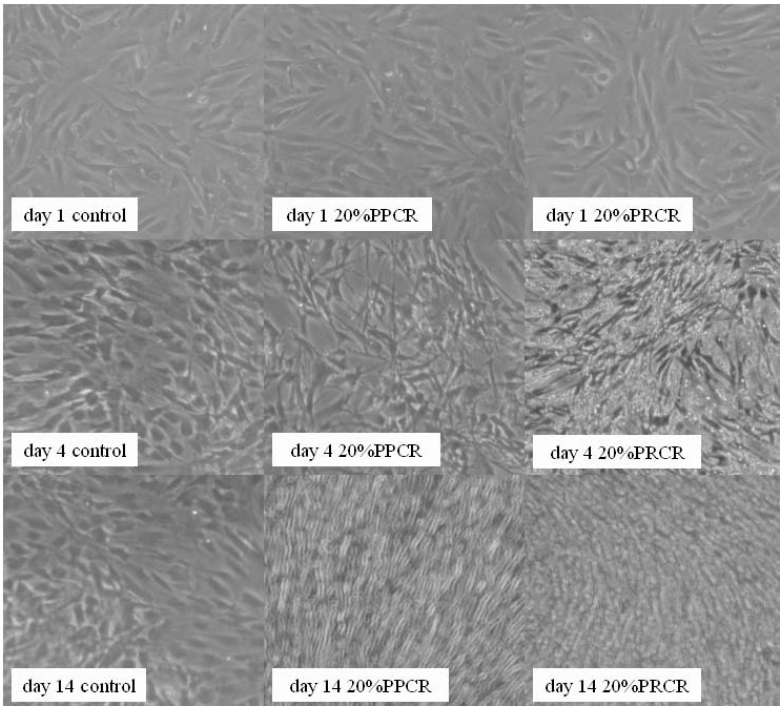


Fig. 6.1 *Photomicropgraphs of tenocyte cultures with or without PRCR or PPCR. Representative photomicrographs (200x magnification) are shown of control, 20% PPCR, and 20% PRCR conditions on day 1, 4, and 14 of the experimental culture period. (See color inlay for a full color version of this figure.)*

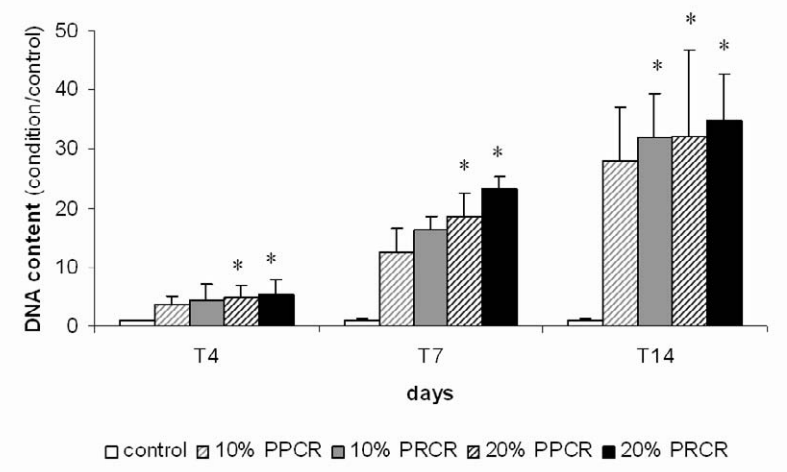


Fig. 6.2 *DNA content of tenocyte cultures with or without PRCR or PPCR. Cells were harvested at day 4, 7, and 14. Control cultures (2% FCS) are set to 1 at each timepoint. DNA content for each condition is expressed as n-fold difference from control cultures at the corresponding timepoint. Results represent mean ± SD (n=6). * p<0.05 as compared to control; # p<0.05 comparison between two specified groups.*

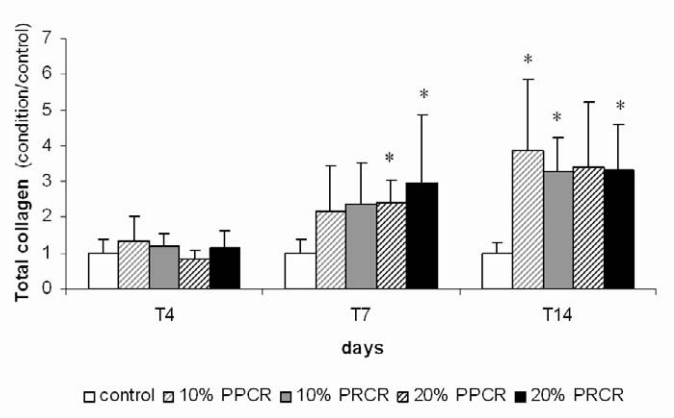
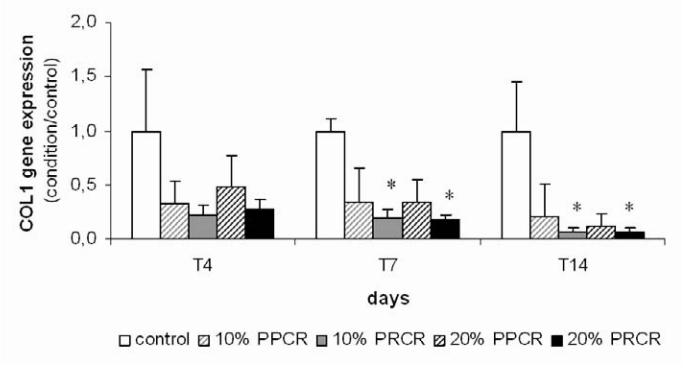


Fig. 6.3 *Total amount of collagen synthesized by tenocytes cultured with or without PPCR or PRCR. Collagen was measured at day 4, 7, and 14. Total amount of collagen for each condition is expressed as n-fold difference from control cultures at the corresponding timepoint. Control is set at 1 and results represent mean ± SD (n=6). * p<0.05 as compared to control.*



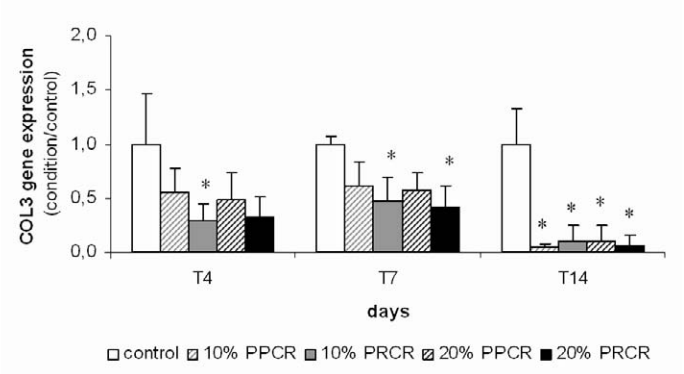
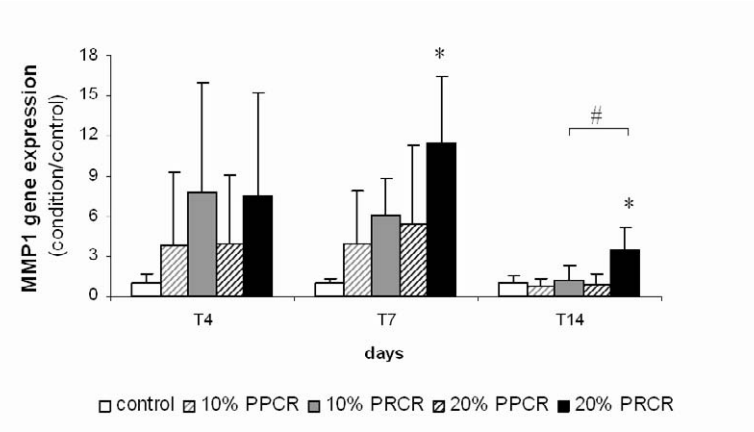
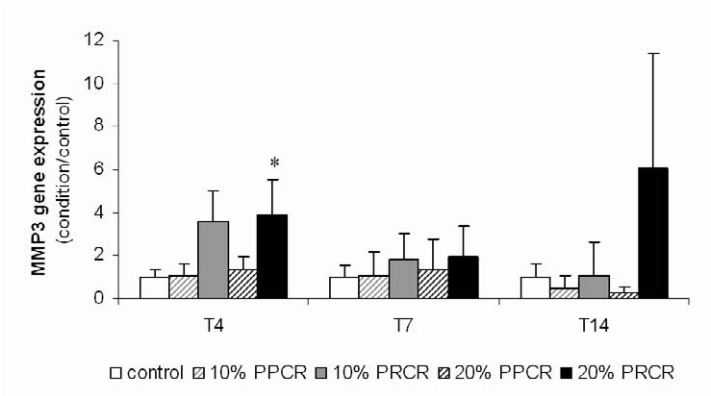


Fig. 6.4 *Gene expression levels of (A) COL1 and (B) COL3 in tenocytes cultured with or without PPCR or PRCR. Cells were harvested at day 4, 7, and 14. Gene expression was normalized to 18SrRNA and expressed as n-fold difference from control cultures at the corresponding timepoint. Control is set at 1 and results represent mean ± SD (n=6). * p<0.05 as compared to control; # p<0.05 comparison between two specified groups.*





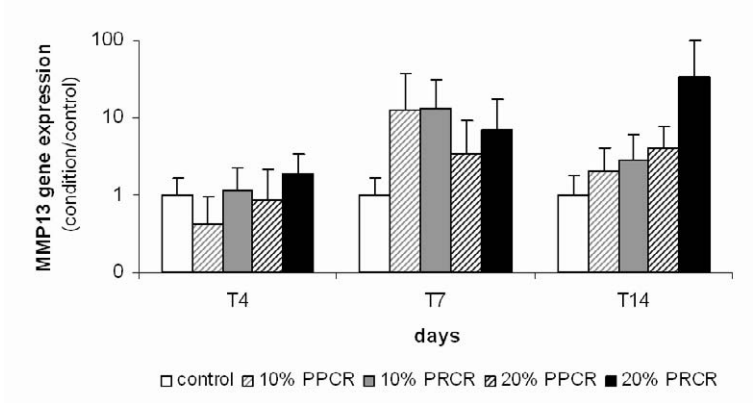


Fig. 6.5 *Gene expression levels of (A) MMP1, (B) MMP3 and (C) MMP13 in tenocytes cultured with or without PPCR or PRCR. Cells were harvested at day 4, 7, and 14. Gene expression was normalized to 18SrRNA and expressed as n-fold difference from control cultures at the corresponding timepoint. Control is set at 1 and results represent mean ± SD (n=6). * p<0.05 as compared to control; # p<0.05 comparison between two specified groups.*

decreased COL3 gene expression at all points in time with exception of 20% PRCR at day 4 (Figure 6.4B). The apparent difference between PRCR and PPCR treated groups at days 4 and 7 did not reach significance. COL3/COL1 ratio did not significantly change in any condition.

PRCR significantly upregulated MMP1 expression at day 7 and 14 in the 20% condition (Figure 6.5A). The apparent dose-related respons in MMP1 expression on day 7 and 14 reached significance only at day 14. PRCR also increased MMP3 gene expression (significant at day 4, Figure 6.5B). No significant differences in MMP1 and MMP3 gene expression were found with PPCR-treatment. Neither PRCR nor PPCR affected MMP13 gene expression significantly (Figure 6.5C).

PRCR highly increased VEGF-A gene expression at all time points (significantly for the 10% PRCR condition at day 7, for the 20% PRCR condition at day 4 and 14). This increase reached up to 30 ±14 times the control level at day 14 in the 20% PRCR condition (Figure 6.6A, note the logarithmic scale). VEGF-A expression in PRCR-treated cells appeared higher than in PPCR-treated cells but this difference did not reach significance. TGFβ1 gene expression was significantly increased only at day 4 in the 20% PRCR condition (Figure 6.6B).

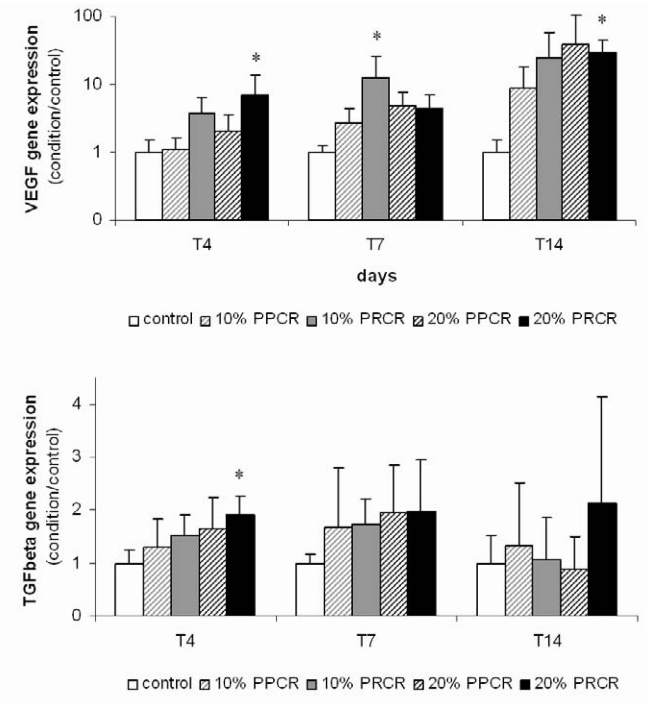


Fig. 6.6 *Gene expression levels of (A) VEGF-A and (B) TGFβ1 in tenocytes cultured with or without PPCR or PRCR. Cells were harvested at day 4, 7, and 14. Gene expression was normalized to 18SrRNA and expressed as n-fold difference from control cultures at the corresponding timepoint. Control is set at 1 and results represent the mean ± SD (n=6). VEGF expression in figure 6.5A is presented on a logarithmic scale. * p<0.05 as compared to control; # p<0.05 comparison between two specified groups.*

DISCUSSION

In this in-vitro study we tested our hypothesis that the releasate from platelet-rich plasma, PRCR, has a positive effect on proliferation and matrix metabolism of human tendon cells to enhance tendon repair. Our results show that PRCR, but also PPCR, enhances cell proliferation and total collagen production by human tendon cells in culture, despite a possible decrease of collagen production *per cell*. PRCR, but not PPCR, slightly increases the expression of matrix degrading enzymes and endogenous growth factors. In vivo these effects of platelet-rich plasma, but also of platelet-poor plasma to a certain extent, on tenocyte behavior might accelerate the catabolic demarcation of traumatically injured tendon matrix and promote angiogenesis and the formation of a fibro-vascular callus. Whether these working mechanisms will also be beneficial in case of degenerative tendinopathies remains to be elucidated.

To examine the effects of PRCR and PPCR on human tendon cells in culture provides an interesting model to study the cooperative effects of a mixture of growth factors. We applied PRCR and PPCR to our cultures in two concentrations that are most frequently used in in-vitro studies with PRP [1, 4, 5], namely 10% and 20% (vol/vol), allowing comparison of our results with literature [4]. We did not apply a 100% concentration of the plasma product to our cultures [30] because we feel that this might be less comparable to the concentration of PRP reached during in-vivo administration. Upon injection of 100% PRP into a tendon in vivo, it is unlikely that tendon cells are exposed for more than several minutes to a 100% PRP concentration, because the PRP will be diluted in extracellular fluids immediately after injection. However, a major problem might be the fact that not only the platelets counts but even more so the growth factor concentrations in the respective releasates, depending on the platelet activation, can not be standardized, rendering comparison of experimental results rather complicated.

The only *in-vitro* study with human tendon cells reported that, in contrast to un-clotted PPP, both 20% PRCR and 20% PPCR stimulated tendon cell proliferation [4]. In line with their results, we also found that PRCR and PPCR increased cell number as well as total amount of collagen, the latter probably being a direct consequence of increased cell numbers. COL1 and COL3 transcripts decreased with both treatments similarly, suggesting a decrease in collagen production per cell. The COL3 versus COL1 ratio of the tendon cells is known to shift towards COL3 in case of tendinosis, in early stages of tendon repair, and in tendon scarring [13, 22]. No significant changes were found in COL3/COL1 ratio in this study, suggesting no negative side effects of PRCR and PPCR on this ratio.

Expression levels of MMP1 and MMP3 were upregulated by PRCR in some conditions, while no significant differences were found with PPCR treatment. Neither PRCR nor PPCR changed MMP13 expression. Gene expression of MMP1 shows no change in chronic Achilles tendinosis [16, 18], but increases in ruptured Achilles and supraspinatus tendons [18, 19]. While expression levels of MMP3 decreases in both degenerative and ruptured Achilles tendon [2, 16, 18, 29], in most studies the gene expression of MMP13 doesn't change significantly. However, conclusions

on MMP activity based on gene expression only must be drawn with utmost precaution. With current knowledge, it is difficult to state whether or not increased gene expression levels of MMP1 and MMP3, as found in our experiment, will be of benefit in degenerative or ruptured tendons. The secretion of MMPs facilitates the ingrowth of neovessels by dissolution of the extracellular matrix [7]. Angiogenesis contributes on one hand to the repair and remodeling of the injured tendon, but, on the other hand the proteolysis of the extracellular matrix by invading endothelial cells results in impaired mechanical stability [26]. Therefore, the application of PRP in already degenerative tendons needs further investigation as progressive weakening of the matrix might predispose for spontaneous rupture [19].

The amount of growth factors VEGF and HGF synthesized by the tendon cells is significantly higher with PRCR than with PPCR treatment, as demonstrated by Anitua *et al.* [4]. In addition to this, we found that PRCR highly increased VEGF-A gene expression by tendon cells. VEGF is active after inflammation, most notably during proliferation and remodeling phases where it has been shown to be a powerful stimulator of angiogenesis [25]. Increased VEGF expression and concentration could be an intrinsic mechanism for inducing angiogenesis as part of a tissue repair process [33]. Furthermore, TGFβ1 expression increased by PRCR at day 4. TGFβ1 is thought to play an important role in the initial inflammatory response to tissue damage having a positive effect on collagen production and viability of tendon cells [25].

In our experiment we aimed for a PRCR-product with the following characteristics: 1) containing high platelet numbers before clotting as well as a high level of platelet activation, 2) containing mainly growth factors released from the platelets, 3) absence of leucocytes in order to minimize risk of graft versus host reaction in our cultures (even more for the reason that the platelet-concentrate used in this experiment was heterologous). After the concentration procedure, platelet numbers in PRP were 2.55 times baseline (whole blood), which corresponds well to the platelet concentration in the study of Anitua *et al.*, who also applicated their product in 20% (vol/vol) concentration on human tendon cells in vitro [4]. After clotting, the platelet numbers in PRCR decreased to 0.08 times baseline, which indicates that most platelets were actually trapped in the clot. The higher concentrations of VEGF and PDGF-BB measured in PRCR compared to PPCR indicate that the platelets were not only trapped in the clot but also activated to release their growth factors. A possible release of growth factors from white blood cells scarcely affected our results, because up to 98 % of the white blood cells was already eliminated from our preparations before clotting. This simultaneously minimized the risk of a graft versus host reaction in our experiments.

We found that not only PRCR but also PPCR affected the outcome parameters when compared to the control condition. This might be caused by the many unavoidable handling procedures performed to obtain PPCR and PRCR, like drawing and centrifugating the blood which could theoretically have activated some of the platelets leading to untimely release. In this way also PPCR could contain a small but sufficient amount of growth factors, which might have induced the effects that we found in PPCR conditions. However, other studies examining the difference

in growth factor concentrations between whole blood, PRCR, and PPCR indicated that this is unlikely: concentrations of TGFβ1 and PDGF-BB significantly increased in PRCR compared to PPCR and whole blood, at least 2-fold [4, 30] and 3-fold [4, 30] respectively; in addition, growth factor concentrations in PPCR were not significantly different from whole blood [30]. A more plausible explanation for why both PRCR and PPCR affected the outcome parameters compared with control conditions therefore seems to be the fact that all experimental conditions basically consisted of adding extra serum (in the quality of 10% or 20% clot releasates) compared to the control cultures. The higher serum concentrations could account for the higher cell amounts in the experimental conditions, because cells proliferate faster in higher serum concentrations. Notwithstanding the aforementioned observation that PPCR already exerted a considerable effect on the tendon cells, particularly the effects on MMP and growth factor expression were still more pronounced in PRCR conditions than in their equivalent PPCR conditions. These additive PRCR-effects might be attributed to higher concentrations of growth factors present in PRCR compared to PPCR. This is supported by the results of our growth factor concentration measurements in PPCR and PRCR.

Our tendon donors were of relatively young age, which might be a limitation of our study. Although unlikely, it is not known whether adult tendon tissue might respond very differently from adolescent tendon tissue regarding the effects of platelet-rich plasma on tendon cell behavior. Also, the behavior of explanted and passaged tendon cells in an artificial culture environment cannot be considered identical to the behavior of tendon cells in their natural matrix environment in-vivo. Therefore one should always be cautious about translating culture data directly to the in-vivo situation.

Finally, the platelet-rich plasma in our study was applied heterologously to the tendon cell cultures, although in clinical settings this plasma product is usually prepared from autologous blood. For this reason we aimed at and succeeded in minimizing the number of leucocytes so that no graft versus host reactions occurred in our cultures.

Autologous platelet-rich plasma application appears promising in healing of traumatic tendon injuries and tendinopathies, but how PRP might improve or accelerate the tendon repair process remains to be elucidated. We found that platelet-rich plasma clot releasate stimulates cell proliferation, collagen deposition, and enhances the gene expression of matrix degrading enzymes and endogenous growth factors by human tendon cells in vitro. This suggests that in vivo PRP application could lead to accelerated remodeling and angiogenesis in the injured matrix, which may promote repair of traumatic tendon injuries.

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Chapter 7

General Discussion

INTRODUCTION

Tendon injuries are a common problem in the general population as well as in athletes. The precise aetiopathogenesis of degenerative tendinopathies is largely unknown. An abundance of treatment options are available for tendinopathies, none of which have evidence-based satisfactory clinical results. Patients often require lengthy rehabilitation periods or even surgical intervention and they frequently end up with remaining limitation in their everyday life or their sport activities.

The increasing scientific interest in tendon disorders and the concurrent application of biochemical and molecular techniques has led to rapid developments in the understanding of degenerative tendinopathies in the last decades. Notwithstanding the current knowledge many questions remain unanswered yet. The work in this thesis aimed at improving fundamental knowledge of tendon cell behavior and matrix remodeling in tendinopathy, both during the disease process as well as in reaction to treatment modalities. Thus we hope to contribute to the increasing knowledge that will ultimately help to develop improved mechanism-based strategies for the prevention and treatment of tendinopathy.

CHONDROGENIC DIFFERENTIATION IN TENDINOPATHY

Healthy tendons at sites of compressive loading are more fibrocartilaginous in nature [1, 2]. In degenerative tendon disease it has been recognized that there is an accumulation of glycosaminoglycans (GAGs) as well [3] and this was confirmed in this thesis in chapter 6 in which we found an increased GAG staining on histological samples from Achilles tendinotic tissues compared to biopsies from healthy Achilles tendon tissue. In human tendinopathy in vivo we found that the gene expression profile in mid-portion Achilles lesions shifts towards an early chondrogenic phenotype (chapter 6). Later on this gene expression pattern has been confirmed in horses, describing a fibrochondrogenic differentiation pattern as the specific phenotype in chronic tendon disease [4]. Also, in a rat model for supraspinatus tendon overuse, cDNA microarrays showed seventeen cartilage specific genes upregulated in the supraspinatus tendon following running [5]. Recent analyses corroborated our results as the gene expression pattern of proteoglycans in tendinopathy was more similar to a chondrogenic profile [6] and human Achilles insertional tendinopathies displayed increased collagen type II staining compared to healthy insertion sites [7]. All this raises the question whether tendon cells have the capacity to differentiate to other lineages and whether the fibrocartilaginous matrix composition is thus the effect of chondrogenic differentiation of the cells present in the tendon tissue.

In vitro we were able to induce chondrogenic cell differentiation as well as adipogenic and osteogenic differentiation of human non-degenerative adolescent tendon-derived fibroblasts (chapter 5). This multilineage differentiation potential of tendon-derived cells was confirmed in

adult horses [8]. Equine tendon-derived cells had some degree of multipotent differentiation capacity, though they were less capable than adult equine bone marrow-derived mesenchymal cells. Furthermore, murine tendon tissue has been suggested to contain cells with some degree of multipotent differentiation capability, as specific single cell clones derived from murine tendon tissue were demonstrated to undergo multipotent differentiation with the appropriate stimuli [9].

Because early chondrogenic differentiation takes place in tendinotic lesions and tendon-derived cells showed the capacity to differentiate towards the chondrogenic lineage, we developed an in-vitro model to simulate this differentiation process for the purpose of testing the effect of therapeutical interventions on chondrogenic differentiation of tendon tissue (chapter 6). The model in its current state may slightly overstimulate the cells towards a hypertrophic chondrogenic lineage compared to the in-vivo situation. However, even in the stage of hypertrophic differentiation all upregulated chondrogenic markers (including type X) could still be significantly downregulated by removing the chondrogenic stimulus. Importantly, we also showed that chondrogenic differentiation could be modulated for instance by adding platelet-rich plasma or triamcinolone. This model can therefore be used to test substances for their potentially therapeutic role in controlling chondrogenic differentiation in tendinopathy (chapter 6).

These studies implicate that chondrogenic differentiation might be an interesting target for drug treatment of tendinotic lesions. However, we have to bear in mind that these differentiation processes are most likely part of the pathological events but might simultaneously be an adaptive protective mechanism to prevent the tissue from further microdamage.

Biomechanical, biophysical, and biochemical stimuli can contribute to the chondrogenic differentiation in tendinopathy. Compressive forces have already been linked to chondrogenic differentiation [5, 10]. It would be very interesenting to increase our understanding of the molecular pathways that control cellular differentiation in tendinopathy and to investigate which signals influence these pathways. Such signals might arise during wound healing processes, mechanical (compressional) loading, neovascularisation, or inflammatory processes surrounding the tendon tissue.

CELLULAR DIVERSITY IN TENDINOPATHY

The repair of injured and degenerated tendons remains a great challenge, largely owing to a lack of in-depth characterisation of the tendon cells. In healthy adult tendon tissue the tenocytes are sparsely but homogenously distributed within the collagen bundles and the cells look morphologically alike having slender spindle-shaped nuclei. Many studies suggest that these are relatively quiescent native cells functioning at low metabolic activity level to maintain the

tendon matrix [11-13]. The endotenon separating the large tendon bundles consists of a thin reticular network containing fibroblasts and occasional vasculoneural units.

In contrast, degenerative tendon tissue shows a range of histological appearances concerning cellular morphology and distribution within the tendon bundles [14-16]. One can distinguish areas of hypercellularity and of hypocellularity. In some areas the cell nuclei are more rounded as opposed to the spindle-shaped 'healthy' cell nuclei, possibly reflecting changes in metabolic activity. For example, a high metabolic cellular activity is suggested in chapter 3 and a change in cellular differentiation is suggested in chapters 5 and 6. Some cells are aligned like trains in a longitudinal direction in certain areas whereas in other areas they are still single and randomly distributed. The remarkable morphological variation between the cells raises objection against the conventional presumption that there is only one tendon cell phenotype. In contrast, the cellular diversity probably reflects functional differences between different cells during the disease process.

In our differentiation experiments on tendon-derived fibroblasts in vitro, not all cells had the same differentiation potential in culture, which argues for a diversity in cellular phenotype within the tissue (chapter 5). This was also concluded from the differentiation experiments with single cell clones derived from rat tendons [9]. Certain tendon cell populations appear to have the capacity to differentiate towards other lineages whereas others might be highly specialized tenocytes that lost their multilineage potential. Even specific quiescent stem cell niches might exist in tendon tissue which might become active during degeneration and repair processes, quite similar to other connective tissues [17]. To date one recent study has addressed this issue and may have identified tendon stem cell niches in tendon tissue [18]. It should be mentioned in this context that the absence of specific tenogenic markers remains an important limitation in tendon cell differentiation research. Collagen type I is the main collagen type in tendon tissue, but it is also produced by other fibroblasts, for example in fibrocartilaginous tissue, skin, and bone. Tenascin C and Cartilage oligomeric matrix protein are not only present in tendon but also in (fibro)cartilage. Scleraxis and Tenomodulin might be the most appropriate tenogenic markers at this moment as the specificity of these molecules for tendon cells and tendon tissue is quite well-defined. However, at the time of our own differentiation studies the antibodies and the primers for these markers were not available yet for analyses of human tissues.

Although cells with multilineage differentiation potential appear to exist in tendon tissue, it cannot be excluded that cells from the periphery also contribute to the phenotypical cell diversity in tendinotic tissue. Cell tracking studies with Green Fluorescent Protein (GFP) chimeric rats for instance have shown that circulation-derived cells invade injured patellar tendons within 24 hours [19].

All this suggests that a variety of phenotypically different cells contribute to tendinopathy. However, this issue has barely been addressed in tendon literature yet. Specific markers to discriminate between different tendon fibroblast subtypes are only starting to be used. Efforts on defining specific cellular phenotypes, functional differences in metabolic behavior

and contribution to tendon degeneration could provide us with crucial information on the pathophysiology of tendinopathies. Furthermore, certain cell phenotypes might be interesting targets for treatment by either stimulating or ceasing their specific metabolic activities. Suggestions for investigation involve either in situ hybridisation [2] or laser capture microscopy followed by gene expression profiling of tendon cell subtypes in vivo. Sequential enzymatic digestion protocols could be developed to acquire different tendon cell populations from peritenon, endotenon, and tendon bundles to study their phenotypic characteristics separately or in co-cultures in vitro [20].

COLLAGENOUS MATRIX REMODELING IN TENDINOPATHY

The extracellular matrix (ECM) in tendons is in a state of dynamic equilibrium between synthesis and degradation. This balance is disturbed in tendinopathy. In healthy adult tendons the collagen remodeling rate is considered very low. Little collagen turnover was seen in healthy biceps brachii tendons, showing a linear accumulation of spontaneous pentosidine cross links with increasing age [21]. In contrast, supraspinatus tendons obtained from asymptomatic shoulders showed relatively high levels of collagen turnover, with lower levels of pentosidine than expected for the age of the tissue, and there were correspondingly high levels of matrix metalloproteinase (MMP) activity. It was suggested that this high remodeling rate in supraspinatus tendons compared to biceps tendons represents either a repair function or a maintenance function occurring in normal supraspinatus tendons as a result of the high mechanical loads placed upon this tendon (as compared to the biceps tendon). It may also be associated with an underlying degenerative process which was common in the asymptomic supraspinatus tendon biopsies. Supporting this latter explanation, in chapter 2 we found in microscopically degenerated mid-portion Achilles tendons an increased collagen remodeling rate compared to less affected tissue biopsies from the same tendon, adjacent to the macroscopically lesional area.

Although a lot is already known about the differences in collagenous matrix composition and turnover in tendinopathies, a very important question remains unanswered: to what extent the changes in collagenous matrix remodeling in tendinopathy represent a limited repair response to microscopic fiber damage or an adaptive response to changes in loading patterns? The answer is of crucial importance for our understanding of the tendinopathy and the development of mechanism-based treatment strategies to prevent or treat tendinotic lesions. For example, as shown in chapter 5 and 6, the in-vitro effects of platelet-rich plasma on human tendon cells or explants in culture can be studied and they exist of increased tendon cell proliferation, increased expression of collagens, MMPs, VEGF and TGFβ, as well as a modulatory effect on chondrogenic differentiation. Therefore, in-vivo use of PRP in tendon injuries might for instance accelerate the catabolic demarcation of traumatically injured tendon matrix and

promote angiogenesis and formation of a fibro-vascular callus. This sounds very promising, but whether it will also be beneficial for degenerative tendinopathies remains to be elucidated.

Tendons consist predominantly of collagen, but there are many other important matrix constituents, including proteoglycans. In this thesis the emphasis was on collagenous matrix composition and turnover. Very little is known about the changes in proteoglycan composition in tendinopathy. The proteoglycan content might be considered as an indicator of the 'mechanical history' of the tendon, with regions exposed to predominantly tensional loading displaying a different proteoglycan composition than regions exposed to compressional loading or shear forces. Since levels of proteoglycans are increased in tendon degeneration (see also the increased GAG-staining in tendinotic Achilles mid-portion biopsies chapter 2) and the proteoglycan composition is also changed [1, 3, 6], it will be very interesting to determine whether this is caused by an increase in proteoglycan synthesis or a decrease in proteoglycan degradation or both. Also, the question to what extent these alterions are an adaptive response to changes in loading patterns in tendinopathy should be elucidated in future studies.

THOUGHTS ON THE VULNERABILITY OF THE ACHILLES TENDON MID-PORTION

The focus in this work was on mid-portion Achilles tendinopathy. The Achilles tendon is the strongest tendon in the body but at the same time this tendon is frequently subject to overuse pathology [22, 23]. Two distinct locations are specifically at risk. Firstly, enthesional pathology is seen at the insertion of the tendon into the calcaneus. In this area the collagen fibers are mineralized and integrated into bone tissue. The area is subjected to both tensional and compressional forces, but not perfectly equipped for either one. Additionally Haglund deformities of the calcaneus can contribute to insertional pathology as well. The insertional area can be considered a weak spot in the Achilles tendon design because of the transition in tissue composition and tissue function. Thus, the occurrence of insertional pathology is considered 'understandable'. A completely different story is told by the mid-portion of the Achilles tendon. Why would this specific anatomical area 4 to 6 centimetres proximal of the insertion be so frequently subject to pathology? Some thoughts on this are embedded deep in the scientists' general view on mid-portion Achilles tendinopathy:

(1) Firstly the mid-portion has the smallest diameter of the whole tendon. This implies that the tensional stress experienced per cross-sectional area is largest in the mid-portion, making specifically this region vulnerable for microdamage following non-physiological mechanical loading.

(2) Concurrently the mid-portion area has very limited blood supply, even for the norms of tendon tissue [24]. Whether this is due to extrinsic factors such as tissue compression or to a simple lack of blood vessels because the mid-portion is essentially more avascular than

other areas, remains an interesting and important question to be answered. The result of a lack of blood supply either way would be a lack of oxygen and nutrients resulting in an impaired tissue repair capacity. To repair the microdamage following non-physiological overloading the tenocytes have to increase their metabolic activity and accelerate tissue turnover rate. We demonstrated this adaptation of tendon cell performance to increase matrix remodeling rate in chapter 3. The tendon cells need extra energy to upgrade their performance. This could account for the high lactate levels in mid-portion tendinopathy indicating anaerobic conditions [25], and it may be a triggers for the cells to increase VEGF production [26] to stimulate neovascularisation. In this context a curative effect of platelet-rich plasma might be expected, because these platelet concentrates have high levels of VEGF and platelet-rich plasma is also able to increase VEGF gene expression of tendon cells in culture (chapter 7).

(3) Mid-portion tendinopathy has historically been attributed to tensional overload, but arguments have risen for increased compressional loading in this area as well. The unique anatomical structure of the triceps surae muscle-tendon unit, being a fusion of several muscles bodies into one tendon, might implicate a role for compressional overload in two ways. Firstly, the tertiary bundles in the Achilles tendon twist approximately 90 degrees around the tendon's longitudinal axis, the largest part of this twist taking place in the mid-portion area [27]. During tensional loading, the mid-portion may experience torsional compressive and shearing forces (similar to wringing out a towel) [27, 28]. Secondly, individual force contributions of the triceps surae components cause non-uniform stress in the Achilles tendon. This may result in frictional and compressive forces between tendon fibres [29]. Lastly, the frequent co-morbidity of a locally thickened paratenon/peritendineum, might act as a constrictor increasing compressional forces within the tendon body. These unique features of anatomy are subject to individual variations. More insight in the anatomical variations concurrent with individual differences in loading duration and intensity might help to explain why some individuals will easily and some will never develop mid-portion Achilles tendinopathy. The presumed compressional forces as well as the lack of blood supply (low oxygen levels) might contribute to the chondrocyte-like cell response showed in chapter 6 in vivo in mid-portion Achilles tendinopathy.

CHALLENGES AND LIMITATIONS OF IN-VITRO TENDON RESEARCH

For a considerable part of this research project we performed in-vitro studies both in monolayer cultures as well as explant cultures. However when interpreting experimental in-vitro data one should always be careful to directly extrapolate the findings on cellular behavior to the in-vivo situation. The following paragraphs describe the challenges and limitations of in-vitro tendon research due to the artificial handling procedures and culture environment which can markedly affect the behavior of the cells.

Culturing in monolayer in an appropriate medium for fibroblasts excludes the presence of other cell types like endothelial cells and monocytes, simply because those cells will not survive under the fibroblast culture conditions. However in the case of tendon-derived fibroblast cultures we cannot make a distinction between the various tendon cell subtypes. For example fibroblasts derived from the endotenon and fibroblasts derived from within the tendon bundles are cultured and analysed collectively although they probably have a different behavior and function in vivo. It would be a very interesting challenge to develop aforementioned sequential digestion methods or develop cell surface markers for discriminating between the different cell types present in tendon tissue. This will create the opportunity to study their specific behavior under controlled circumstances either separately or in co-culture systems.

The two most frequently used cellular extraction methods are explantation of the cells and enzymatic digestion of the ECM. Both methods have the disadvantage of strongly selecting for certain cell populations. Using the explantation method a relatively small number of cells is able to crawl out of the tissue explants in culture. The majority of the cells simply remain in the discarded tissue. During the outgrowth period another selection is made in favor of those cells that achieve to adhere to the culture well and start proliferating. In contrast to explantation, enzymatic digestion has the advantage of every cell ending up in the suspension after the ECM is completely dissolved by the enzymes. However in both methods the cells that adhere to the culture well and proliferate fastest are selected for during subsequent culturing. In chapter 5 for instance, it cannot be concluded that specifically the adhering and fastly proliferating cells are the ones that have the capacity to differentiate and thus the percentage of cells in tendon tissue that have multilineage differentiation potential is overestimated.

Apart from the practically unavoidable selection for fastly proliferating cells, another disadvantage of monolayer expansion cultures is the phenotypic drift described in tendon-derived cells cultured in monolayer [30]. Both rapid proliferation as well as the non-physiological mechanical and biochemical environment markedly influence the cells behavior. This might be partially overcome by using explant cultures like we did in chapters 4 and 6. In such an explant culture system the cells are not attached to the culture well in monolayer fashion but instead remain surrounded by their natural tendon ECM. Also an expansion period is not needed to acquire enough cells for the experiments.

Nonetheless, even in explant cultures the cells are still subject to a non-physiological biochemical, biophysical, and biomechanical environment. Studying tendon cell behavior and matrix remodeling under physiological mechanical loading conditions in-vitro, as well as under more physiological oxygen tension, pH levels, and metabolic activity are important future experimental directions. For the purpose of mimicking the low metabolic activity of tendon cells in vivo, experiments tend to shift towards culturing in the presence of 5% foetal calf serum (FCS) or less as opposed to 10% FCS. The exact temperature, pH, and oxygen level in healthy tendon tissue in vivo is not known, nor the changes in these parameters in tendinotic tissues, although these factors most probably contribute to the development of tendinotic lesions,

considering for instance the neovessel formation and high lactate levels found in tendinopathy [25, 31].

These and other challenges deserve attention in future research because of the many advantages of in-vitro research over animal studies or human trials. For in-vitro experiments tendon material from surgery, post-mortem material, or slaughterhouses is often sufficient. Generally a smaller number of humans or animals is needed to perform the research, and the studies do not involve any suffering of testsubjects. In-vitro the effect of changing one or two parameters can be studied under controlled circumstances, i.e. with all other parameters unchanged. Also, it is easier to study basal cellular and molecular mechanisms under controlled culture conditions than in animal experiments. Finally, biological variation and systemic influences can be avoided or minimized in culture experiments.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

Tendinopathies are a common clinical problem. They tend to heal slowly rarely attaining the structural integrity and mechanical strength of normal tendons. The development of new treatment options for degenerated tendons has been hampered by a limited understanding of basic tendon biology. The work in this thesis contributed to the current understanding of cellular behavior and matrix remodeling in tendinopathies and some of the biochemical signals that can influence these processes, both during the disease process as well as in reaction to treatment modalities. Suggestions for future research directions include (1) studying and influencing the causes, the presence, and the effects of chondrogenic differentiation in tendinotic lesions; (2) characterizing the tendon cell subtypes in the tendon as well as the peritendinous structures; and (3) studying the effects of mechanical loading, oxygen tension, and pH on tendon cell behavior and matrix composition in vivo and in vitro. This will gain more insight in the function and regulation of the cells in the tendon bundles and the connective tissues surrounding the tendon bundles during the development, maintenance, and repair of tendinopathic lesions. This should ultimately lead to the development of more successful mechanism-based treatment and prevention options for tendinopathies.

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Chapter 8

Summary

Tendon injuries are a common problem in the general population athletes as well as in athletes. Despite the high prevalence, there are still many unsolved questions and differences of opinion concerning pathology and etiology of degenerative tendinopathies. A significant amount of knowledge is available on the clinical presentation and pathological changes associated with tendinopathy, but the precise aetiopathogenesis is not yet elucidated. Resulting from this, the currently available treatment options are merely aimed at symptom relief. Tendinopathy often responds poorly to conservative treatments requiring lengthy periods of rehabilitation or even surgical intervention. With the work in this thesis we hope to contribute to the increasing fundamental knowledge of tendon pathology that will ultimately help to develop improved mechanism-based strategies for the prevention and treatment of tendinopathy.

An overview of the composition, structure, and function of healthy tendon tissue as well as the pathological alterations in tendinopathy is provided in **chapter 1**. This chapter also includes the presently most important hypotheses on the pathogenesis of tendinopathy, reflecting the research directions at this moment. An overview of the current and promising future treatment options is given. This is followed by the general aims and outline of the thesis. This work aimed at increasing fundamental knowledge of tendon cell behavior and matrix remodeling in tendinopathy, both during the disease process as well as in reaction to treatment modalities. The focus in this work was on mid-portion Achilles tendinopathy.

Describing the biochemical and structural changes of the extracellular matrix in Achilles tendinopathy is an important first step for understanding the pathology of tendinopathies. Assumptions on the biochemical collagen compositional changes in Achilles tendinopathy could thus far only be extrapolated from supraspinatus tendinopathy studies. Therefore we focused in **chapter 2** on mid-portion Achilles tendinopathy, purely describing the changes we observed in cell behavior, biochemical composition, and collagen turnover rate in vivo. We collected tendinotic biopsies and biopsies from macroscopically healthy tendon tissue adjacent to the lesion from patients undergoing surgery for Achilles tendinopathy. Furthermore, healthy tendon biopsies were collected from donors with asymptomatic Achilles tendons. The mildly and moderately severe tendinotic samples appeared to be metabolically more active with a higher collagen turnover rate and the presence of an early fibrotic repair process was suggested. A clinically relevant finding was that the degenerative changes anatomically extend further than macroscopically is visible for the surgeon by visual inspection of the tendon tissue during surgery.

We formulated a number of hypotheses regarding pathogenic processes that might play a role in the development and persistence of tendinopathy. In the following chapters these processes were explored in vivo and in vitro for their potential contribution to tendinopathy. Firstly, we focused on the catabolic processes in tendinopathy and asked ourselves the question whether Toll-like receptors are involved in these processes, similarly to cartilage degeneration in osteoarthritis. Toll-like receptors (TLRs) are cell surface receptors that have been shown to re-appear on the surface of chondrocytes during inflammation of the joint and degeneration

of the cartilage. Exploring the similarities between cartilage and tendon overuse pathology, we hypothesized in **chapter 3** a role for TLRs in tendinopathy, possibly quite similar to the role of TLRs in osteoarthritis and their even greater role in rheumatoid and inflammatory arthritis. The most likely TLR candidates TLR2 and TLR4 could be up regulated in vitro by inflammatory cytokines TNFα or IL-1β. However there was no up regulation on transcriptional level and no immunohistochemical staining of TLR2 and TLR4 in the tendon cells in tendinopathic samples. Therefore the catabolic processes in tendinopathy cannot be attributed to regulation of TLR2 and TLR4 by tenocytes.

Tendinotic lesions display an increased amount of glycosaminoglycans and may also contain calcifications and lipid accumulations. We therefore hypothesized that alterations in cellular differentiation stage might contribute to the development of tendinopathy. In **chapter 4** we studied whether a population of cells with intrinsic differentiation potential is present in tendon tissue. In culture experiments with cells explanted from non-degenerative adolescent human tendon tissue, we tried to differentiate these tendon-derived fibroblasts towards cells with more chondrogenic, adipogenic, or osteogenic characteristics. This study explicitly does not describe the behavior of cells that invade from the periphery into a lesional area. We specifically studied the cells resident in the tendon tissue itself and we showed on protein level and gene expression level that a proportion of these explanted tendon-derived fibroblasts had an intrinsic differentiation potential in vitro, especially towards the chondrogenic phenotype.

Following up on this in **chapter 5**, we confirmed the presence of a chondrogenic differentiation pattern in mid-portion Achilles tendinopathy in vivo. Considering excessive or inappropriate fibrocartilaginous matrix production in tendons as part of the pathological process, removing or opposing the stimulus that causes the metaplasia might help the tenocytes to return to their normal tendon matrix production. Therefore, we developed a tendon explant culture model in which we were able to induce a chondrogenic differentiation quite similar to the in-vivo situation in tendinopathy. By studying the effects of adding triamcinolone or platelet-rich plasma (PRP) to the model, we demonstrated that this model can be a useful tool to investigate early chondrogenic differentiation as a possible target for drug treatment of tendinotic lesions.

In the present literature local injection of the aforementioned PRP is considered clinically as a promising treatment intervention for tendinopathy although the exact effects on tendon cell behavior are largely unknown. The effects of PRP in the previous culture model inspired us to explore the metabolic reaction of tendon cells to this treatment modality in more detail. In **chapter 6** platelet-rich plasma was added to explanted non-degenerative adolescent human tendon cells for 7 days. We found that PRP, but also the control condition platelet-poor plasma (PPP) to a certain extent, stimulates cell proliferation and collagen deposition, and enhances the gene expression of matrix degrading enzymes and endogenous growth factors by these human tendon-derived cells in vitro. This suggests that in-vivo use of PRP, but also of PPP to a certain extent, might accelerate the catabolic demarcation of injured tendon matrix and

promote angiogenesis and formation of a fibro-vascular callus. Whether this will ultimately be beneficial for degenerative tendinopathies remains to be elucidated.

In **chapter 7** the main results described in this thesis are discussed in relation to each other and in the context of current scientific knowledge. Also, certain challenges and limitations of in-vitro tendon research are reflected upon. Furthermore, suggestions for follow-up experiments and future research directions are given throughout the general discussion section. Suggestions for future research directions include (1) studying and influencing the causes, the presence, and the effects of chondrogenic differentiation in tendinotic lesions; (2) characterizing the tendon cell subtypes in the tendon as well as the peritendinous structures; and (3) studying the effects of mechanical loading, oxygen tension, and pH on tendon cell behavior and matrix composition in vivo and in vitro. This will gain more insight in the function and regulation of the cells during the development, maintenance, and repair of tendinopathic lesions and it should ultimately lead to the development of more successful mechanism-based treatment and prevention options for tendinopathies.

Appendices

NEDERLANDSE SAMENVATTING

Peesletsels zijn een veel voorkomend probleem zowel in de algemene populatie als onder atleten. Ondanks de hoge prevalentie zijn er nog altijd veel vragen onbeantwoord en verschillen de meningen wat de pathologie en de etiologie van degeneratieve tendinopathieën betreft. Er is een aanzienlijke hoeveelheid kennis met betrekking tot de klinische presentatie en de pathologische veranderingen die worden geassocieerd met tendinopathie, maar de precieze etiopathogenese is nog niet opgehelderd. Als gevolg hiervan zijn de huidige behandelmogelijkheden vooral gericht op symptoomverlichting. Tendinopathieën reageren vaak matig op conservatieve therapie, waarbij langdurige revalidatie en uiteindelijk soms zelfs een chirurgische interventie nodig kan zijn. Met dit proefschrift hopen we bij te dragen aan de toenemende fundamentele kennis omtrent peespathologie die uiteindelijk zal leiden tot de ontwikkeling van effectievere methoden voor preventie en behandeling van tendinopathie, omdat zij gericht zullen zijn op het ontstaansmechanisme van de afwijking.

In **hoofdstuk 1** van dit proefschrift treft u een overzicht van de opbouw, de structuur en de functie van gezond peesweefsel, alsmede van de pathologische veranderingen die kenmerkend zijn voor een tendinopathie. In dit hoofdstuk komen tevens de belangrijkste hypotheses van dit moment omtrent de pathogenese van tendinopathie aan de orde, hypotheses die een afspiegeling vormen van de actuele richtingen van het biomedische onderzoek naar tendinopathie. Daarnaast wordt een overzicht gegeven van de huidige en van veelbelovende toekomstige behandelmogelijkheden. Het hoofdstuk sluit af met een formulering van het specifieke doel van dit proefschrift en een omschrijving van de inhoud van het proefschrift: dit werk heeft tot doel de fundamentele kennis te vergroten van het gedrag van peescellen en van de samenstelling en veranderingen van de extracellulaire peesmatrix in het geval van tendinopathie. Dit betreft de peescellen en de peesmatrix zowel tijdens de ontwikkeling van de pathologie als in het kader van de reactie op diverse behandelingen. In dit proefschrift ligt het focus van de onderzoeken op de tendinopathie van de mid-portion van de achillespees.

Het beschrijven van de biochemische en structurele veranderingen van de extracellulaire matrix bij achillespeestendinopathie is een belangrijke eerste stap naar het begrip van het ziektebeeld. Tot nu toe was in het geval van achillespeestendinopathieën slechts mogelijk om aannames ten aanzien van de biochemische veranderingen in collageenopbouw te extrapoleren vanuit onderzoek naar tendinopathie van de schouderpees, de supraspinatus-tendinopathie. In **hoofdstuk 2** van dit proefschrift leest u over een in vivo observationeel onderzoek naar mid-portion achillespeestendinopathie. Hierin worden de veranderingen beschreven die gevonden zijn in het celgedrag, de biochemische samenstelling en de snelheid van de ombouw van het zieke achillespeesweefsel. We hebben hiertoe peroperatief peesbiopten genomen en op deze wijze materiaal verzameld van tendinotische haarden en van macroscopisch gezond

ogend peesweefsel aangrenzend aan de tendinotische haard. Het betrof dan patiënten die een chirurgische behandeling ondergingen voor hun achillespeestendinopathie. Tevens werden peesbiopten afgenomen van gezonde donoren met asymptomatische achillespezen, patiënten die om een andere reden een operatie aan het been ondergingen. De milde en matig ernstige tendinotische weefselmonsters bleken metabool actiever dan gezond peesweefsel, met een hogere ombouwsnelheid van het collageen. Tevens vonden wij aanwijzingen voor de aanwezigheid van een vroeg reparatieproces met fibrosevorming, zoals ook kenmerkend voor littekengenezing. Een klinisch relevante bevinding vormde het feit dat de degeneratieve veranderingen anatomisch verder uitgebreid zijn dan macroscopisch zichtbaar is voor de chirurg bij visuele inspectie van het peesweefsel tijdens de operatie.

Vervolgens hebben wij een aantal hypothesen geformuleerd ten aanzien van de pathogenetische processen die een rol zouden kunnen spelen in de ontwikkeling en het voortduren van een tendinopathie. In opeenvolgende hoofdstukken worden deze processen onder de loep genomen in het kader van hun mogelijke bijdrage aan het ziekteproces.

Allereerst hebben we ons gericht op de katabole processen die een rol spelen bij tendinopathie en ons de vraag gesteld of er bij deze processen Toll-like receptoren betrokken zijn, analoog aan de kraakbeendegeneratie zoals deze aan de orde is in het geval van artrose. Toll-like receptoren (TLRs) zijn receptoren aan het celoppervlak waarvan gebleken is dat deze verschijnen op onder andere het oppervlak van kraakbeencellen bij ontsteking van het gewricht en bij degeneratie van het kraakbeen. De overeenkomsten tussen de pathologie van overbelast kraakbeenweefsel en overbelast peesweefsel explorerend, hebben we in **hoofdstuk 3** de hypothese gevormd dat TLRs een rol zouden kunnen spelen in tendinopathie. Een rol die mogelijk overeenkomstig is met de rol van TLRs in artrose en hun nog grotere aandeel in reumatoïde en inflammatoire artritis. In vitro kon door middel van stimulatie met inflammatoire cytokinen TNFα of IL-1β een toename in genexpressie worden bewerkstelligd van twee TLR-varianten, namelijk TLR2 en TLR 4. Er was echter in vivo geen toename waarneembaar op genexpressieniveau, maar ook niet bij immunohistochemische kleuring: TLR2 en TLR4 kleurden niet aan in de peescellen van tendinopathisch weefsel. Gesteld werd dat de katabole processen van tendinopathie niet kunnen worden toegeschreven aan upregulatie van TLR2 en TLR4 door peescellen.

Tendinosehaarden vertonen een toegenomen hoeveelheid glycosaminoglycanen en kunnen ook calcificaties en ophopingen van lipiden bevatten. We veronderstelden daarom dat veranderingen in celdifferentiatie zouden kunnen bijdragen aan de ontwikkeling van tendinopathie. In **hoofdstuk 4** onderzochten we of er een celpopulatie met een intrinsiek differentiatiepotentieel aanwezig is in peesweefsel. In kweekexperimenten met cellen, fibroblasten, die werden geoogst uit niet-degeneratief humaan peesweefsel afkomstig van adolescenten, hebben we getracht om deze fibroblasten afkomstig uit het peesweefsel in hun differentiatie te sturen

in de richting van cellen met meer chondrogene, adipogene of osteogene eigenschappen. Deze studie beschrijft uitdrukkelijk niet het gedrag van cellen die vanuit de periferie een lesie invaderen. We hebben specifiek cellen bestudeerd uit het peesweefsel zelf en we hebben op het niveau van zowel eiwitexpressie als genexpressie aangetoond dat een deel van deze fibroblasten afkomstig uit het peesweefsel in kweek een intrinsiek differentiatiepotentieel hebben, in het bijzonder in de richting van het chondrogene (kraakbeen) phenotype.

Hierop doorgaande hebben we in **hoofdstuk 5** in vivo de aanwezigheid van een chondrogeen differentiatiepatroon in mid-portion achillespees-tendinopathie bevestigd. Indien wij de overmatige of ongepaste productie van fibrocartilagineuze matrix in pezen beschouwen als een onderdeel van het pathologische proces, dan zou het opheffen of tegengaan van de stimulus die deze metaplasie veroorzaakt ervoor kunnen zorgen dat de peescellen teruggaan naar hun normale peesmatrixproductie. Volgens deze gedachte hebben wij een kweekmodel ontwikkeld waarbij we in stukjes peesweefsel chondrogene differentiatie konden induceren die zeer veel leek op de in vivo chondrogene differentiatie situatie bij tendinopathie. Met een onderzoek naar de effecten van het toevoegen van triamcinolon of plaatjes-rijk plasma (PRP) aan het model, hebben we aangetoond dat dit model een functioneel instrument kan zijn om vroege chondrogene differentiatie te onderzoeken. Dit niet alleen om meer kennis te verkijgen over dit aspect van het ziekteproces, maar ook in het kader van een mogelijk aangrijpingspunt voor medicamenteuze behandeling van tendinotische lesies.

In de huidige literatuur wordt de locale injectie van PRP in klinische setting beschouwd als een veelbelovende behandeling van tendinopathie, alhoewel de precieze effecten op het gedrag van peescellen nog grotendeels onbekend zijn. De effecten van PRP in het bovengenoemde kweekmodel brachten ons ertoe om de metabole reactie van peescellen op deze behandelmethode uitgebreider te bestuderen. In **hoofdstuk 6** werd plaatjes-rijk plasma gedurende 7 dagen toegevoegd aan niet-deneratieve humane peescellen afkomstig van adolescenten. We ontdekten dat het PRP (maar ook tot op zekere hoogte het plaatjesarme plasma (PPP) uit de controlegroep) de proliferatie van cellen en de depositie van collageen stimuleert en de genexpressie van matrixafbrekende enzymen en endogene groeifactoren doet toenemen. Dit doet vermoeden dat het in vivo gebruik van PRP (maar ook van PPP, zij het in mindere mate) de katabole demarcatie van een beschadigde peesmatrix zou kunnen versnellen en angiogenese en de vorming van een fibro-vasculaire callus zou kunnen bevorderen. Of dit mechanisme kan bijdragen aan genezing van degeneratieve tendinopathieën, moet nog blijken uit klinische vervolgstudies.

Tot slot worden in de discussie van **hoofdstuk 7** de belangrijkste resultaten van dit proefschrift besproken in relatie tot elkaar en in het kader van de actuele wetenschappelijke kennis over dit onderwerp. Daarnaast wordt aandacht besteed aan de uitdagingen en beperkingen van

peesonderzoek in vitro en worden suggesties gedaan ten aanzien van hierop aansluitende experimenten en mogelijke richtingen voor het vervolg van het tendinopathie-onderzoek. Mogelijkheden voor toekomstig onderzoek zijn (1) het bestuderen en beïnvloeden van de oorzaken, de aanwezigheid en de gevolgen van chondrogene differentiatie in tendinotische lesies; (2) het identificeren van subtypes van cellen zowel binnen de pees alsook in de peritendineuze structuren; en (3) het bestuderen van de effecten van mechanische belasting en veranderingen in zuurstofspanning op het gedrag van peescellen en matrixcompositie in vivo en in vitro. Kennis hieromtrent zal meer inzicht verschaffen in de functie en regulatie van de cellen tijdens de ontwikkeling, de instandhouding en het herstel van tendinopathische lesies, en zal uiteindelijk leiden tot de ontwikkeling van effectievere behandel- en preventiemethoden, juist omdat deze direct zullen aangrijpen op het ontstaansmechanisme van tendinopathie.

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CURRICULUM VITAE

Marieke de Mos is geboren op 19 januari 1980 in het Diaconessenhuis te Voorburg. De lagere school doorliep zij tot en met groep zeven op de Kijkduinschool in Den Haag. Aansluitend doorliep zij de middelbare school op het Gymnasium Haganum. Zij behaalde in 1997 het eindexamen. Het daaropvolgende jaar behaalde zij haar propedeuse Biomedische Wetenschappen aan het Leids Universitair Medisch Centrum. Aan diezelfde faculteit werd zij het jaar daarop ingeloot voor de studie Geneeskunde. In september 2004 legde zij het artsexamen af.

Door Prof.dr. Jan Verhaar werd zij ontvangen op de afdeling Orthopedie van het Erasmus Medisch Centrum te Rotterdam. Aldus werd zij de eerste promovendus op een peesproject (de peesAIO) aan het orthopaedisch laboratorium van Prof.dr.ir. Harrie Weinans. Zij deed haar onderzoek onder de dagelijkse begeleiding van haar co-promotor Dr. Gerjo van Osch, hoofd van de toenmalige kraakbeengroep. Dit heeft na een kleine vier jaar geresulteerd in uitbreiding van het peesproject tot een translationele peesonderzoekslijn in samenwerking met de onderzoeksgroep van veterinair arts Prof.dr. René van Weeren verbonden aan de Hoofdafdeling Gezondheidszorg Paard van de faculteit Diergeneeskunde in Utrecht en de onderzoeksgroep van sportarts Dr. Hans Tol verbonden aan de afdeling Sportgeneeskunde van het Medisch Centrum Haaglanden. Tevens heeft het geleid tot de totstandkoming van dit proefschrift.

Marieke de Mos is op 21 september 2007 getrouwd met Clio Balázs. Op dit moment is Marieke de Mos werkzaam als arts bij Achmea Vitale in Rotterdam.

PHD PORTFOLIO SUMMARY

Name PhD Student:	Marieke de Mos
Erasmus MC Department:	Orthopedic Research Laboratory
Research School:	MUSC
PhD period:	October 2004 - June 2008
Promotor:	Prof.dr. J.A.N. Verhaar
Co-promotor:	Dr. G.J.V.M. van Osch

1. PhD training

In-depth courses

Cursus Basisdidactiek (OECR, Risbo)	2007
Classical methods for data-analysis (NIHES)	2005-2006
Biomedical english writing and communication (EUR)	2005

Conferences and presentations

Podium presentations

NVMB meeting, Lunteren	2008
VSG Wetenschappelijk Sportmedisch Congres, Noordwijkerhout	2007
ISL&T meeting, San Diego, USA	2007
NOV jaarvergadering, Rotterdam	2007
VSG Wetenschappelijk Sportmedisch Congres, Noordwijkerhout	2006
Awarded best abstract price	
NVMB meeting, Lunteren, The Netherlands	2006
ORS meeting, Chicago, USA	2006
NOV jaarvergadering, Amsterdam	2006
NBTE meeting, Lunteren	2005
ETES meeting, Munich, Germany	2005
NVMB meeting, Lunteren	2005
Awarded Pauline van Wachem presentatieprijs	

Posterpresentations

ORS meeting, San Francisco, USA (2 posters)	2008
ICRS World Congress, Warsaw, Poland	2007
NVMB and GCTS joint meeting, Münster, Germany	2007
ORS meeting, San Diego, USA	2007
TERMIS-EU meeting, Rotterdam	2006
NVMB meeting, Lunteren	2006
Regenerate World Congress, Pittsburg, Pennsylvania	2006
ORS meeting, Chicago, USA	2006

2. Teaching activities

Lecturing

"Tendinopathieën - achillespeestendinopathie onder de loep genomen" 2007
College tweedejaars geneeskunde

"Peesweefsel en de gevolgen van overbelasting " 2006
Invited lecture
Nascholingsdag clubartsen betaald voetbal

"De achillespees in vitro - tendinose onder de loep genomen" 2005
Regionale sportrefereeravond

Supervising practicals and review assignments

Tutoraat eerstejaars geneeskunde 2007

"Het gevaar van steroid injecties bij peesaandoeningen" 2007
Review tweedejaars geneeskunde

"De effectiviteit van ESWT op een tenniselleboog" 2005
Review tweedejaars geneeskunde

Supervising Master's theses

"The development of an equine real-time RT-PCR primer set, evaluation of the effects of ESWT on healthy equine tendons and the development of an in-vitro tendinosis model"
R. van Binsbergen, biologie-studente
Master program Bioveterinary Sciences, 9 months 2006-2007

"Effecten van PRP- en/of PPP-clot-relysaat op peescellen in vitro"
A.E. van der Windt, geneeskunde-studente
Onderzoeksstage doctoraalfase, 8 months 2006

LIST OF PUBLICATIONS

1. **M. de Mos**, J.L.M. Koevoet, H.T.M. van Schie, N. Kops, H. Jahr, J.A.N. Verhaar, G.J.V.M. van Osch. In-vitro Model To Study Chondrogenic Differentiation In Tendinopathy.
   Manuscript under revision by Am J Sports Med

2. **M. de Mos**, L.A.B. Joosten, B. Oppers-Walgreen, H.T.M. van Schie, H. Jahr, G.J.V.M. van Osch, J.A.N. Verhaar. Tenocytes do not contribute to tendon degeneration by regulation of TLR2 and TLR4.
   Manuscript under revision by J Orthop Res

3. G. Bosch, **M. de Mos**, R. van Binsbergen, H.T.M. van Schie, C. H. A. van de Lest, René van Weeren. Extracorporeal Shock Wave Therapy: effect on collagen matrix and gene expression in sound equine tendons.
   Manuscript accepted in Eq Vet J, 2008

4. **M. de Mos**, A.E. van der Windt, H. Jahr, H.T.M. van Schie, H. Weinans, J.A.N. Verhaar, G.J.V.M. van Osch. Can platelet-rich plasma enhance tendon repair: a cell culture study.
   Am J Sports Med Jun;36(6):1171-8, 2008

5. **M. de Mos**, J.L.M. Koevoet, H. Jahr, M.M.A.Verstegen, M.P. Heijboer, N. Kops, J.P.T.M. van Leeuwen, H. Weinans, J.A.N. Verhaar, G.J.V.M. van Osch. Intrinsic differentiation potential of adolescent human tendon tissue: an in–vitro cell differentiation study.
   BMC Musculoskeletal Disorders: 8:16, 2007

6. **M. de Mos**, B. van El, J. DeGroot, H. Jahr, H.T.M. van Schie, E.R. van Arkel, J.L. Tol, M.P. Heijboer, G.J.V.M. van Osch, J.A.N. Verhaar. Achilles tendinosis: changes in biochemical composition and collagen turnover rate.
   Am J Sports Med Sep;35(9):1549-56, 2007

Color figures

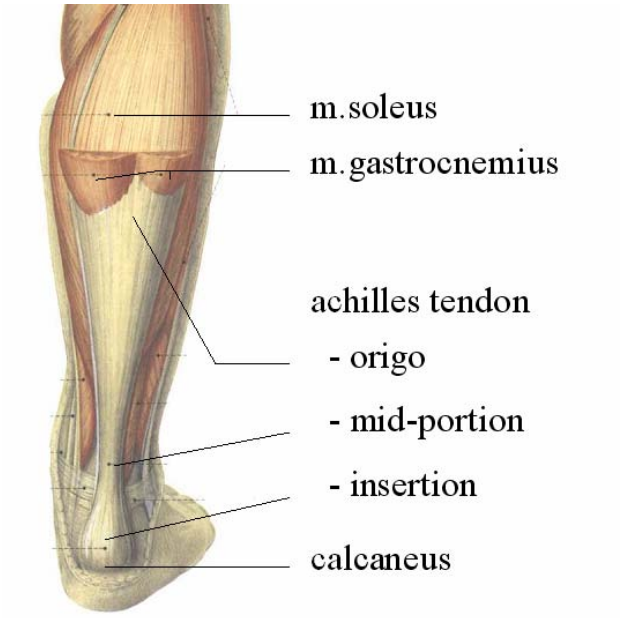

Figure 1.2 *Anatomy of the Achilles tendon (adapted from Wolf-Heidegger's Atlas of Human Anatomy, 4th edition).*

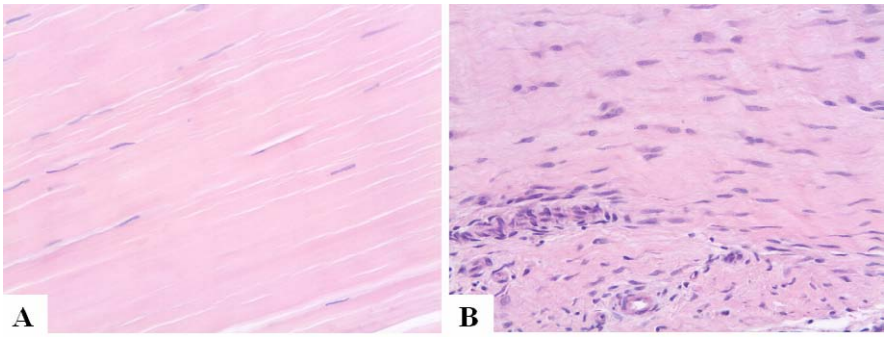

Figure 1.3 *Histological picture of (A) healthy and (B) tendinotic Achilles tendon tissue (Magnification 200x).*

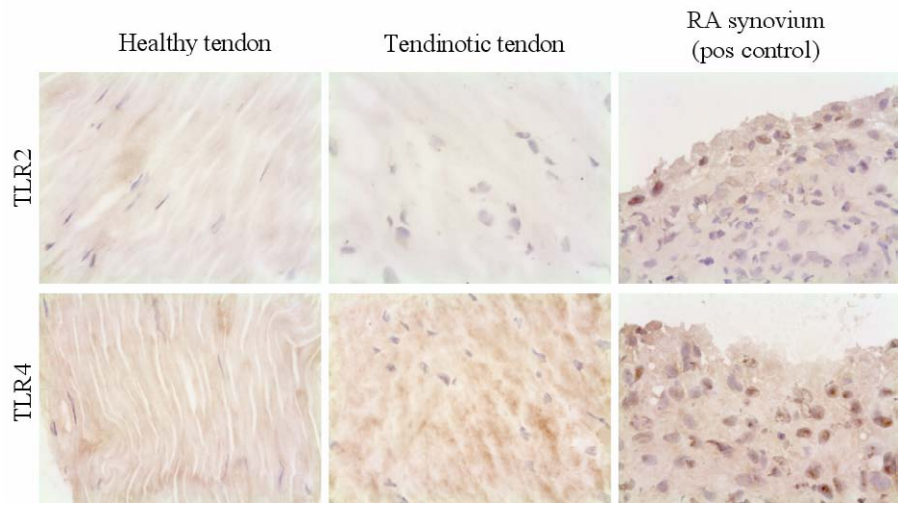

Figure 3.2 *Immunohistochemical staining for TLR2 and TLR4. Representative photomicrographs are shown from frozen longitudinal sections of healthy Achilles tendon samples (n=5) and tendinotic Achilles tendon samples (n=13). Human rheumatoid arthritis (RA) synovium was used as positive control. Magnification 400x.*

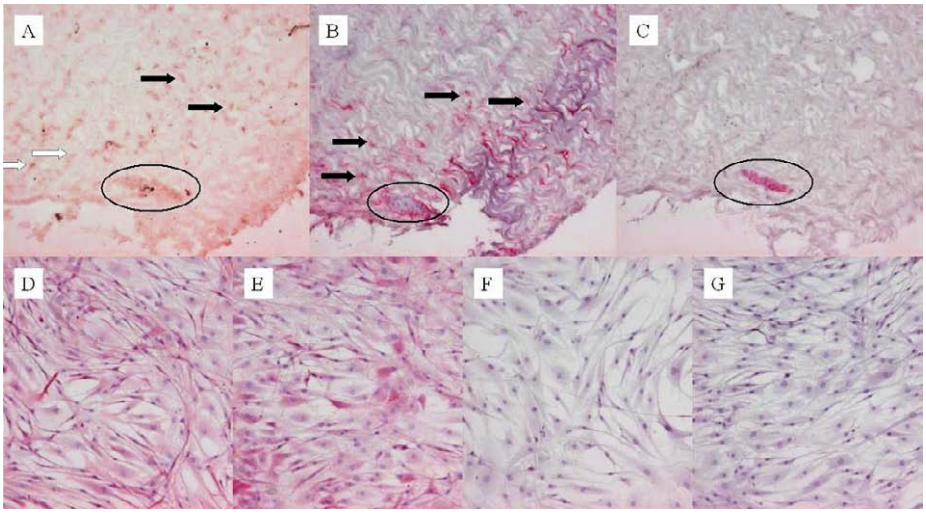

Figure 4.1 *Ki-67, D7-FIB, and α-SMA staining on tendon explants (day 6 of explantation period) and on tendon-derived fibroblasts (TDF) in monolayer culture. Ki-67 positive (proliferating) cells in the explants were located in the tendinous tissue (A, black arrow), in the endotenon (A, white arrow), and in the vascular walls (A, circle). Cells in the tendon tissue and in the endotenon stained positive for fibroblastmarker D7-FIB (B). Cells in the vascular walls remained negative for D7-FIB (B) and instead stained positive for α-SMA, a marker for pericytes and smooth muscle cells (C). All TDFs in monolayer culture stained positive for D7-FIB from passage one (D) to passage four (E) and remained negative for α-SMA from passage one (F) to passage four (G).*

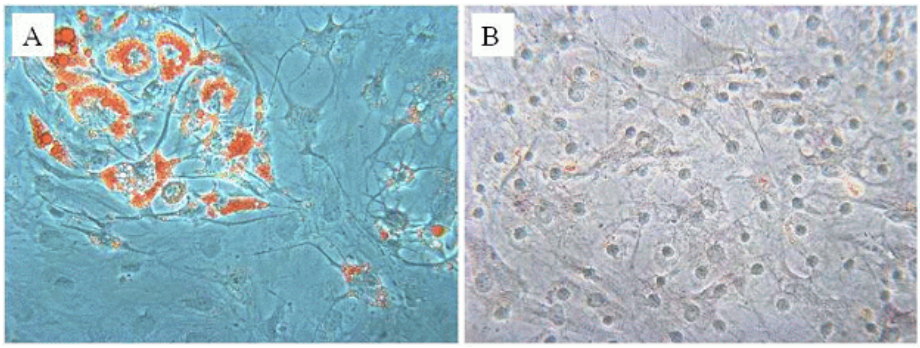

Figure 4.2 *Oil Red O staining on tendon-derived fibroblasts cultured for 21 days in adipogenic medium (A) (note that not all cells but merely clusters of cells formed Oil Red O positive lipid vacuoles inside the cell's main body) or in control medium (B). Like cells in control medium, cells cultured in osteogenic or chondrogenic medium were negative (figures not shown).*

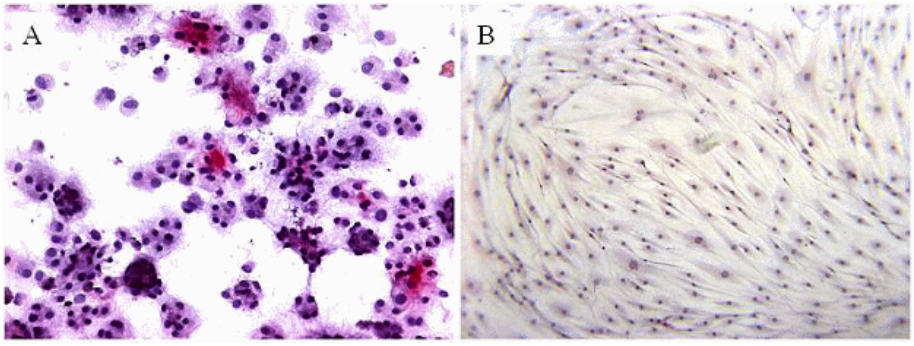

Figure 4.4 *Immunohistochemical staining for collagen type II on tendon-derived fibroblasts. 5% of the cells cultured for 21 days in alginate beads in chondrogenic medium stained positive (A). Cells cultured in monolayer in control medium remained negative (B) as did cells in adipogenic or osteogenic media (figures not shown).*

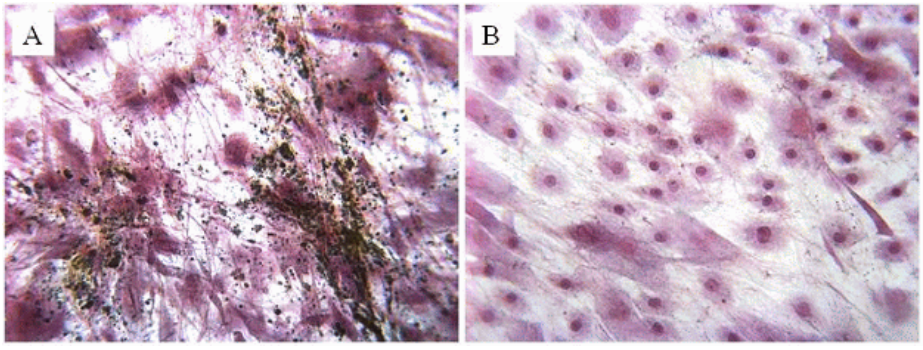

Figure 4.6 *Von Kossa staining on tendon-derived fibroblasts cultured for 21 days in osteogenic (A) or control medium (B). Calcium deposition was seen in osteogenic medium (A), not in control medium (B) or in adipogenic or chondrogenic media (figures not shown).*

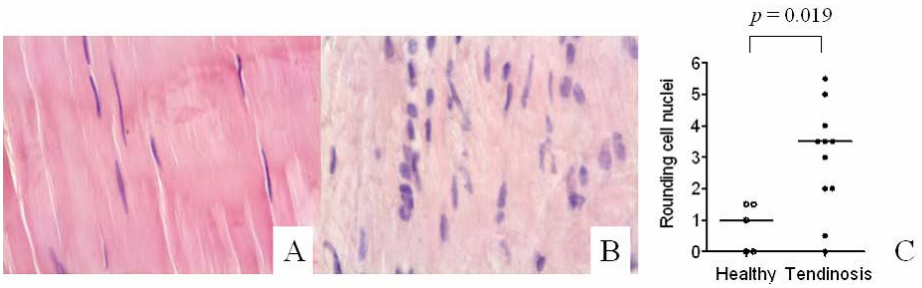

Figure 5.1 *Changes in cell morphology in mid-portion Achilles tendinosis. Hematoxilin and eosin staining of tendon samples. Representative photomicrographs (400x magnification) of healthy Achilles tendon samples (n=5, A) and of Achilles tendinotic samples (n=12, B) are shown; rounding of the cell nuclei was scored for each individual sample; total score can range from 0 (normal) to 6 (maximum deviant), line represents median score (C).*

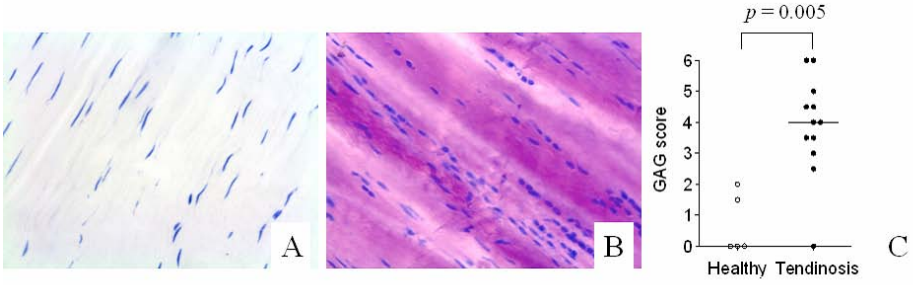

Figure 5.2 *Fibrocartilaginous metaplasia in mid-portion Achilles tendinosis demonstrated by thionin staining for glycosaminoglycans (GAGs). Representative photomicrographs (200x magnification) of healthy Achilles tendon samples (n=5, A), and of Achilles tendinotic samples (n=12, B); GAG stainability was scored for each individual sample, total score can range from 0 (normal) to 6 (maximum GAG staining), line represents median GAG score (C).*

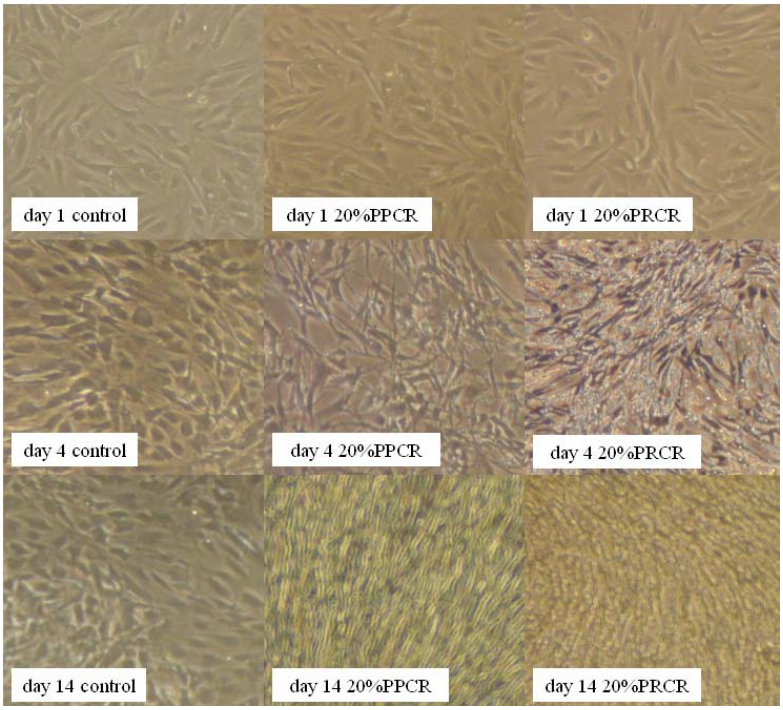

Fig. 6.1 *Photomicropgraphs of tenocyte cultures with or without PRCR or PPCR. Representative photomicrographs (200x magnification) are shown of control, 20% PPCR, and 20% PRCR conditions on day 1, 4, and 14 of the experimental culture period.*