Biomolecular Aspects of Flexor Tendon Healing

MARIA BERGLUND
Dissertation presented at Uppsala University to be publicly examined in Robergsalen, Ing. 40, Akademiska sjukhuset, Uppsala, Thursday, May 6, 2010 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Flexor tendon injuries in zone II of the hand (i.e. between the distal volar crease and the distal interphalangeal joint) can be costly for both the afflicted individual and society because of the high cost of a long rehabilitation period, complicated by tendon ruptures or scarring with adhesion formation, causing impaired range of motion. The aim of the present thesis was to characterize more fully the deep flexor tendon, the tendon sheath and their response to injury in a rabbit model in order to find potential targets to improve the outcome of repair.

The intrasynovial rabbit deep flexor tendon differed from the extrasynovial peroneus tendon in the expression of collagens and transforming growth factor-β1 gene expression. Differences were also found in collagen III and proteoglycans between regions of the flexor tendon subjected to either compressive or tensile load.

After laceration and subsequent repair of the flexor tendon, a shift in collagen gene expression from type I to type III occurred. Proteoglycans were generally increased with the notable exception of decorin, a potential inhibitor of the profibrotic transforming growth factor-β1 which was markedly increased during the first two weeks after repair in tendon tissue but remained unaltered in the sheaths. Both vascular endothelial growth factor and basic fibroblast growth factor mRNA levels remained essentially unaltered, whereas insulin-like growth factor-1 increased later in the healing process, suggesting potential beneficial effects of exogenous addition, increasing tendon strength through stimulating tenocyte proliferation and collagen synthesis.

Matrix metalloproteinase-13 mRNA levels increased and remained high in both tendon and sheath, whereas there was only a transient increase of matrix metalloproteinase-3 mRNA in tendon. We could also demonstrate a significant increase of the proportion of myofibroblasts, mast cells and neuropeptide containing nerve fibers in the healing tendon tissue, all components of the profibrotic myofibroblast-mast cell-neuropeptide pathway.

Keywords: Flexor tendon healing, Growth factor, Metalloproteinase, Collagen, Proteoglycan, Myofibroblast, Hyaluronan synthase, Mast cell

Maria Berglund, Hand Surgery, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

© Maria Berglund 2010

ISSN 1651-6206
urn:nbn:se:uu:diva-120304 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-120304)
To my family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Journal of Hand Surgery*, 2004; 29B:165-169


*Journal of Hand Surgery*, 2006; 31A:1279-1287


IV Berglund M, Hart DA, Reno C, Wiig M. Growth factor and protease expression during different phases of healing after rabbit deep flexor tendon repair.

Submitted.

V Berglund M, Hildebrand KA, Zhang M, Hart DA, Wiig M. Neuropeptide, mast cell and myofibroblast expression after rabbit deep flexor tendon repair.

Submitted.

Reprints were made with permission from the respective publishers.

Cover by Yvonne van den Berg and Michel Danckaarts.
## Contents

Introduction ................................................................................................... 11

Anatomy and biology of flexor tendons .................................................. 12
- The flexor tendon in the human hand .................................................... 12
- Vasculature and nutrition of flexor tendons ........................................ 13
- Components of tendon tissue ............................................................. 14
- Tendon and load .................................................................................. 16
- Phases of the healing process ............................................................. 16
- Extrinsic and intrinsic healing ............................................................. 17

Current clinical practice ........................................................................... 18
- Flexor tendon rupture versus adhesion formation ............................... 18
- Flexor tendon repair ........................................................................... 19
- Rehabilitation protocols .................................................................... 20

Factors affecting tendon healing .............................................................. 21

Experimental models ................................................................................ 24
- Cell culture versus animal model ........................................................ 24
- Choice of species ................................................................................. 24
- The clinical trial .................................................................................. 24

Aims .............................................................................................................. 25

Methods ........................................................................................................ 26
- Animals .................................................................................................. 26
- Intra- and extrasynovial tendons ............................................................ 26
- Flexor tendon injury model ................................................................. 27
- Extraction of RNA ................................................................................ 28
- Semiquantification of mRNA ............................................................... 29
- Immunohistochemistry ....................................................................... 30
- Statistical analysis ............................................................................... 31

Results and discussion ................................................................................ 32
- Study I. Differences between intra- and extrasynovial tendons .......... 32
- Study II. Matrix molecules and growth factors after tendon repair ....... 33
- Study III. Inflammatory molecules and hyaluronan synthases after tendon repair ............................................................. 36
- Study IV. Growth factors and proteases in healing flexor tendon and sheath ............................................................. 38
Study V. The myofibroblast-mast cell-neuropeptide fibrosis pathway in flexor tendon healing.................................................................41

Conclusions........................................................................................................44

Future prospects.................................................................................................45

Summary in Swedish – sammanfattning på svenska ......................................46
   Molekylärbiologiska aspekter på böjsenläkning .........................................46

Acknowledgements............................................................................................48

References.........................................................................................................49
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DIP</td>
<td>Distal interphalangeal joint</td>
</tr>
<tr>
<td>FDP</td>
<td>Flexor digitorum profundus</td>
</tr>
<tr>
<td>FDS</td>
<td>Flexor digitorum superficialis</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthase</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>PIP</td>
<td>Proximal interphalangeal joint</td>
</tr>
<tr>
<td>ROM</td>
<td>Range of motion</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SLRPs</td>
<td>Small leucine-rich proteoglycans</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β1</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VBP</td>
<td>Vinculum brevis profundus</td>
</tr>
<tr>
<td>VBS</td>
<td>Vinculum brevis superficialis</td>
</tr>
<tr>
<td>VLP</td>
<td>Vinculum longus profundus</td>
</tr>
<tr>
<td>VLS</td>
<td>Vinculum longus superficialis</td>
</tr>
</tbody>
</table>
Introduction

Although less than 1% of hand trauma in the emergency unit is primarily flexor tendon injuries, the age and activity level of the affected patients and the time needed for rehabilitation lead to high costs for both society and the afflicted individual. The potential severity of the injury is well illustrated by the view on flexor tendon injuries only a few decades ago. The injured skin, subcutaneous tissues and tendons were regarded as one continuous scar, in which nothing could be gained by primary (direct) repair of the severed tendon. Zone II (Figure 2) was referred to as “no man’s land”. The results of primary repair were so poor that primary complete removal of the injured tendon followed by a later secondary procedure with free tendon grafting was general practice. The first reports of successful primary repair are from the 1960s. Today, with improved surgical techniques, rehabilitation protocols and new insights into the healing capacity of flexor tendon tissue, the results and prognosis are all vastly improved. However, despite these improvements, a deep flexor tendon injury may still lead to impaired function with a decreased range of motion (ROM) because of adhesion formation (scarring between bone, tendon and tendon sheath) or tendon ruptures.

Figure 1. Knife injury to the volar aspect of the hand, with laceration of the deep and superficial flexor tendons of the index and little finger.
Anatomy and biology of flexor tendons

The flexor tendon in the human hand

The human hand is capable of finely coordinated movements. Flexion of the fingers is motored by the deep and superficial flexor muscles, innervated by the median nerve (superficial flexor muscle, and deep flexor to index and middle finger) and the ulnar nerve (deep flexor to ring and little finger). The muscles power the deep (flexor digitorum profundus, FDP) and superficial (flexor digitorum superficialis, FDS) tendons, of which the deep flexor tendon is the most critical to the ROM for the finger, attaching at the base of the distal phalanx and flexing both the proximal (PIP) and distal (DIP) interphalangeal joints. The superficial tendon is divided into two halves at the level of the proximal phalanx, attaching separately at the proximal part of the middle phalanx, flexing the PIP joint at activation. The thumb, having only
one interphalangeal joint (IP), has one flexor muscle and tendon – the pollicis longus.

Between the muscle/tendon interface and the FDP insertion at the distal phalanx, the surroundings of the FDP change and have been divided into five anatomical zones (Figure 2). In zone I and II, the tendon is surrounded by a fibro-osseous digital sheath, creating a gliding surface for the excursion of the tendons (visceral paratenon on the tendons and parietal paratenon on the inner side of the sheath). The sheath is reinforced by a pulley system (Figure 3) with annular and cruciform pulleys. The annular pulleys give stability and prevent bowstringing of the flexor tendon.

**Figure 3.** The tendon sheath with the annular (A1-A4) and cruciform (C1-C3) ligaments. (With permission from Elsevier.)

**Vasculature and nutrition of flexor tendons**

The arteries supplying part of the nutrition to the flexor tendons are the vinculum longus superficialis (VLS) at the proximal phalanx, and brevis superficialis (VBS) and longus profundus (VLP) at the PIP joint. Vinculum brevis profundus (VBP) is the most distal (Figure 4). The flexor tendons are poorly vascularized, especially between the A2 and A4 pulleys in the volar segments of the tendon, earlier even deemed avascular but now known to have a low vascular density. In these areas, they are more dependent on diffusion, i.e. their second source of nutrition. Diffusion of nutrients is supplied via the parietal paratenon in the tendon sheath.
Components of tendon tissue

Collagens
Tendons are hypocellular dense connective tissues that are composed mainly of collagen (65-80% of tendon dry weight). Soluble tropocollagen molecules are connected to each other by cross-links, becoming insoluble and aggregating into microfibrils, and then fibrils. A collagen fiber, the smallest collagen structure visible with light microscopy and constituting the basic unit of the tendon, is a bundle of collagen fibrils. The fibers are bound together by endotenon, a thin connective tissue layer containing nerves and blood vessels, creating primary fiber bundles (subfascicles) that aggregate into secondary bundles (fascicles). Tertiary bundles are groups of collagen fascicles, that in their turn aggregate into the tendon, which is surrounded by the epitenon (Figure 5). The collagen in adult tendons is mainly type I. Apart from the collagen fibers, whose linear arrangement creates a resistance to external forces, the tendon tissue also, to a varying extent, contains elastin fibers (1-2% of tendon dry weight) that contribute to the tendons elastic properties.¹⁰
Figure 5. Tendon anatomy with the structure of collagen fibers, as illustrated by Kannus 70.

**Extracellular matrix with proteoglycans**

The collagen and elastin fibers are surrounded by the hydrophilic gel-like ground substance, together forming the extracellular matrix. The ground substance, consisting mainly of water (60-80%), also contains glycosaminoglycans (GAGs), proteoglycans and glycoproteins. GAGs and proteoglycans contribute to the resistance of tendons to compressive forces through their high water-binding capacity, adding to the elastic properties of the tissue 70.

There are two types of proteoglycans in tendon tissue: small leucine-rich proteoglycans (SLRPs) and large proteoglycans (also called modular proteoglycans or lecticans) 181. The SLRPs (decorin, biglycan, fibromodulin and lumican) regulate the assembly of collagen, i.e. the fibrillogenesis 123. Disturbances in the SLRP distribution can lead to changes in the structure of connective tissues and cause impaired function 37, 67. Some SLRPs can also act as modulators of certain growth factors by binding to them 132. Aggrecan, a large proteoglycan able to resist compressive forces, is more abundant in articular cartilage, but can also be found in tensile regions of tendons. The large proteoglycan versican influences cell proliferation and adhesion, is increased in patellar tendinosis 137 and may have additional functions in the extracellular matrix that are not yet known 172.

**Tenocytes**

Although the tendon is hypocellular, it is a living tissue containing fibroblasts with a phenotype specifically adapted to their surroundings – tenocytes and tenoblasts. Tenocytes and tenoblasts constitute 90-95% of the cellular
elements of tendons, the remainder being mainly vascular cells and chondrocytes at pressure and insertion sites. In the growing tendon, the majority of cells are tenoblasts that are active during growth and development, with a high synthesis rate of collagens, glycoproteins, proteoglycans and elastic fibers. The tenoblasts transform to tenocytes in the adult tendon, becoming elongated with long cellular processes to maintain contact between cells and matrix components in the aging tissue with a decreasing cell-to-matrix ratio. The lower metabolic rate contributes to the endurance of tendon tissues to periods of tensile load with a lower energy supply. Tenocytes are believed to play a role in chronic tendon diseases, exhibiting matrix degeneration before the onset of clinical symptoms.

Tendon and load

Tendon tissue does not have set characteristics: matrix composition and turnover vary in different tendons that are subjected to varying loads. The tenocytes can coordinate their response to load, communicating via gap junctions on the cytoplasmatic extensions that regulate their synthesis and adapt the tendon tissue to the forces it is subjected to.

In the same tendon, there can be regions with different characteristics. When a tendon passes through a pulley or is wrapped around bone, there is, instead of the usual tensile force, an increase in compressive and shear forces. To protect the integrity of the tendon (preventing wear and tear) the composition of the tendon tissue is altered in the exposed region. The segment adopts a more fibrocartilaginous phenotype, giving it a higher resistance to compression. The tenocytes change their appearance, adopting a more chondrocyte-like rounded shape, collagen II increases and the architecture of the collagen fibers becomes less linear. There is a shift in proteoglycan production, with higher amounts of the large proteoglycan aggrecan, which can bind water and give more stiffness to the tissue. Aggrecan can also be found in smaller amounts in the tensile regions of the tendon, but then appears to have a different structure.

The tendon response to load is dynamic. Increased compressive force will stimulate a fibrocartilaginous phenotype with more aggrecan and an elimination of those forces will give the opposite effect. A certain amount of load is necessary for the steady state tissue homeostasis in the tendon, which, if deprived of tensile force, will begin to degrade.

Phases of the healing process

The healing process in tendons is divided into three overlapping phases (inflammatory, proliferative and remodeling phase).
The inflammatory phase
The mechanical trauma with transection of the tendon and the tendon sheath damages blood vessels in the tendon sheath and the surroundings, causing a hematoma. A number of factors are released at the injury site, stimulating vasodilatation and migration of inflammatory molecules. The number of macrophages, neutrophils, monocytes, erythrocytes and platelets increase in the area. There is an increase of various growth factors and tenocyte proliferation is initiated, leading to production of components of the extracellular matrix (such as collagens and proteoglycans) and stimulating angiogenesis. Apoptosis of tenocytes increases. The inflammatory phase dominates the first week after injury. The strength of the repair site relies only on the sutures and a fibrin clot.

The proliferative phase
The production of collagen and other structural molecules begins a few days after the injury and continues during the first month. Tenocytes increase because of ongoing recruitment as well as proliferation. Collagen III, a large number of cells and high water content, are typical for the newly synthesized extracellular matrix. There is inhibitory signaling against tenocyte apoptosis within two to four weeks after tendon injury. The tendon repair gains strength when the tendon gap is bridged by granulation tissue. However, the tendon callus lacks the properties of normal tendon and is characterized by a random collagen pattern with suboptimal mechanical properties. During the period of two to four weeks after tendon repair, the tendon is weak with a maximal risk of rupture.

The remodeling phase
During the remodeling phase, beginning after the first month of tendon healing and continuing until at least nine months after repair, a shift takes place from collagen III to collagen I production. The total cell number and production of components of the extracellular matrix decrease.

The collagen fibers reorganize into a more structured pattern with cross-linking and longitudinal orientation. The tendon now gains higher stiffness and tensile strength.

Extrinsic and intrinsic healing
Previously it was believed that extrinsic healing with formation of adhesions was a necessary process in flexor tendon healing, providing vital nutrients through the angiogenesis in the adhesions. The tendon was regarded as inert, lacking the ability to synthesize new tissue components for repair. The classic studies by Lundborg, which demonstrated the tendons capacity to...
heal in an avascular synovial environment, completely deprived of adhesion formation, altered the view on tendon healing. The deep flexor tendon is a cellular active tissue in which intrinsic healing with migration and proliferation of tenocytes contributes to the repair of the injury. There is diffusion of nutrients not only from the vascularized adhesions but also from synovial fluid.

We now know that both intrinsic and extrinsic healing mechanisms are needed for optimal tendon healing, and that the balance between them is vital for the final functional result. Excessive extrinsic healing with adhesions will decrease tendon excursion, causing an impaired function. The discovery of intrinsic healing has made it possible to counteract adhesion formation through primary repair with atraumatic surgical techniques and early rehabilitation \[^4,46,48,66,96,99,102,103\].

Current clinical practice

Flexor tendon rupture versus adhesion formation

The goal of repair and rehabilitation after flexor tendon injury is to achieve a mechanically strong tendon and a good ROM in the injured digit. Potential complications after flexor tendon repair are tendon rupture or tendon adhesion. In a review citing outcome reports of primarily repaired flexor tendon injuries, the worldwide rupture rate varied between 4 and 18\% \[^151\]. To avoid these complications, the clinical treatment of flexor tendon injuries is a balance between protecting and allowing excursion of the newly repaired tendon. Whereas too much force will result in tendon rupture, insufficient training or immobilization will promote adhesion formation and decreased ROM.

During the past few years, a number of clinical trials and animal models have investigated the strength of different repair techniques (number of suture strands, braided or unbraided suture, suture purchase of tendon tissue, type of peripheral suture, etc.), as well as the impact of different rehabilitation protocols (immobilization, passive or active training) on rupture rate and ROM \[^4,146\]. There is a trend toward using a stronger four-strand suture method in combination with active rehabilitation protocols \[^151\]. Early rehabilitation within the limits of the strength of the suture stimulates the healing of the repair site as well as the gliding surface, inhibiting the formation of adhesions \[^4\].
Flexor tendon repair

An atraumatic technique is essential when repairing a flexor tendon injury in order not to unnecessarily provoke adhesion formation. To quote Jin Bo Tang\textsuperscript{151}:

> The outcomes of tendon surgery, particularly primary tendon repairs in the digital sheath area, depend upon the expertise of the surgeons. Therefore, another problem, even in countries with a sufficient number of hand surgeons, is that these difficult procedures are often thought of as simply “sewing the ends together” by trainees or inexpert surgeons. Primary operation by an inexperienced surgeon can result in destruction of the tendon, sheath, and pulley structures and may render the delayed primary or secondary repairs ineffective, even when performed by an experienced hand surgeon.

Apart from not causing additional unnecessary trauma, compromising remaining circulation and risking adhesions, optimal sutures with sufficient strength are needed to avoid ruptures. The time frame within which a direct repair of the injured tendon is feasible is not unlimited. When more than two weeks have passed since the injury, a two-stage procedure with tendon grafting may be considered. In an animal model, optimal results with immediate repair compared to repair after one to three weeks has been demonstrated\textsuperscript{51}.

**The core suture**

A strong core suture is necessary to allow active rehabilitation. Many techniques are currently in use, with two-, four- or six-strand sutures\textsuperscript{19, 130, 146, 149}. Therefore, a general recommendation is to reach a strength of 40-50 N (4-5 kg force) to ultimate failure (i.e. the amount of load at which the tendon reruptures)\textsuperscript{4}. The repair strength also depends on the tendon purchase, i.e. the amount of tendon tissue encompassed by the suture, which should be 7-10 mm on either side of the laceration\textsuperscript{152}. The effect of the purchase may not only be due to the amount of tissue in the suture area and the resulting grip on the tendon ends but also to the length of the suture material itself\textsuperscript{76}. However, when attempting to achieve optimal suture strength through multiple strand sutures with extra tendon purchase, allowing a more active rehabilitation, it is important to be aware of the potentially negative impact the foreign material may have on the tendon. Specifically, this negative impact increases the work of flexion\textsuperscript{5} and causes a degradation of tendon tissue with subsequent impaired mechanical properties, counteracting the desired effect\textsuperscript{106, 176}. The recognition of the superior strength of intact tendon tissue compared with suture material, as well as the risk of additionally weakening the tissue through the added material, motivates the recommendation of only repairing tendon lacerations that sever 50\% or more of the tendons cross-sectional area. A limited repair may be indicated if a smaller
laceration causes mechanical hindrance (triggering) of the tendon during excursion. Triggering may also occur if the annular pulleys are too tight for the excursion of repaired flexor tendon, decreasing the strength of the tendon repair, a complication that may be avoided by preoperatively venting the pulleys.

The circumferential suture
The circumferential suture has two functions. First, it reduces the bulk of the tendon repair, allowing for easier tendon excursion through the tendon sheath and pulleys with less friction and resistance. Second, it can contribute to the strength of the tendon suture: biomechanical studies show enhanced maximal failure strength, especially with interlocking sutures and a suture purchase of 4 mm.

Gap formation
Gap formation, an increase in the distance between the sutured flexor tendon stumps during the first weeks of healing, may have an impact on the outcome of tendon repair. Previously, it was believed that even minimal gap formation had a negative influence on healing with increased adhesion formation. Studies that are more recent have shown no such negative impact through gap formation less than 3 mm in length. Tendons with gaps greater than 3 mm, however, lack the increase in tensile strength at the repair site that usually occurs between four and six weeks postoperatively. Gap formation of 2 mm changes the gliding resistance of the tendon, even risking triggering on the annular pulleys if as large as 3 mm. Thus, it is advisable to aim for a strong, carefully adapted repair to minimize gap formation.

Rehabilitation protocols
Post-surgical rehabilitation is a well-motivated health care investment. The increased complication rate that accompanies immobilization after flexor tendon repair causes higher cost for both society and the individual. A variety of protocols have been suggested, with different degrees of movement range that is either passive or to some extent active. It is important to avoid simultaneous extension at wrist and digits since the combined strain may exceed the strength of the core suture. Digital extension should therefore be combined with synergistic wrist flexion, and vice versa.

Earlier studies supported a tendon excursion of 3 to 5 mm during rehabilitation to counteract adhesion formation, but more recent experimental findings from animal models indicate that as little as 1.7 mm may be sufficient. Although there is now a trend toward more active rehabilitation protocols, it is not necessarily true that this promotes an increase in the strength of
the healing tendon tissue. The protocols currently in practice range from partially passive to active. However, as of yet, there are no randomized clinical trials with sufficient evidence to support a general recommendation to mainly use active protocols.

Factors affecting tendon healing

Great advances in flexor tendon healing have been achieved through developing suture techniques and rehabilitation protocols, but it is likely that progress has been maximized. Consequently, investigators now aim to influence more directly the healing process on a biomolecular level.

Growth factors

**Transforming growth factor-β1** (TGF-β1) contributes to the initial scar tissue formation that is essential for wound healing. However, at later stages in the healing process an increased TGF-β1 activity may lead to excessive scarring, with subsequent fibrosis and adhesion formation. TGF-β1 stimulates collagen III production in flexor tendon and tendon sheath tissue and inhibition of TGF-β1 after deep flexor tendon injury has reduced adhesions in animal models.

**Connective tissue growth factor** (CTGF, CCN2) is active in angiogenesis, tissue repair and fibrosis. It mediates some of the profibrotic effects of TGF-β1 and is active in fibrotic processes in diverse types of tissue. Increased amounts of CTGF have also been seen in load-induced tendinopathy models. Targeting CTGF has been suggested as one way of modulating the potential fibrotic effects of TGF-β1 in connective tissue.

**Basic fibroblast growth factor** (bFGF, FGF-2) is usually increased in acute connective tissue injuries, but down regulated in chronic wounds, such as pressure ulcers. It is involved in tissue remodeling, the formation of granulation tissue and reepithelialization. Tenocytes increase their collagen production and proliferation in vitro when stimulated with bFGF and treatment of flexor tendon injuries with bFGF in animal models has increased the biomechanical strength of the healing tendon. Aside from potentially decreasing the risk of rupture of the healing tendon through improved biomechanical properties, bFGF has been shown to increase gene expression of lubricin and hyaluronan synthase in vitro, which may have positive effects on tendon gliding.

**Insulin-like growth factor-1** (IGF-1) has dose-dependent effects on matrix synthesis, including collagens, is a protector from apoptosis and stimulates cell proliferation and migration. In animal models of tendinitis, treatment with IGF-1 has improved the outcome. **Vascular endothelial growth factor** (VEGF) stimulates collagen deposition and angiogenesis, promoting the proliferation and migration of endo-
Increased amounts of VEGF have been detected after cyclic loading of tendons, in tendinitis and in ruptured tendons. Treatment with VEGF has been positive in hypoxic environments (e.g. diabetic wounds). Attempts to optimize Achilles tendon healing with VEGF improved the tensile strength in the early healing period, but in vitro VEGF stimulation of canine tenocytes did not lead to an increase in collagen synthesis or cell proliferation.

Nerve growth factor (NGF), a neurotrophin previously thought to act only on the peripheral nervous system, has been shown to improve wound healing, stimulating angiogenesis and reinnervation. When applied to healing medial collateral ligaments in the knees of rats, it improved the biomechanical properties and increased the vascularity and nerve density of the connective tissue.

Bone morphogenetic proteins (BMPs) stimulate synthesis of tendon tissue components and increase the biomechanical properties of repaired Achilles and patellar tendons in animal models.

Platelet derived growth factor (PDGF-BB) stimulates tenocyte proliferation, synthesis of ECM components and has shown some positive effects on flexor tendon healing in canine models.

Cyclooxygenase-2
Inhibition of cyclooxygenase-2 (COX-2) has been found to limit the inflammatory response after flexor tendon injury and subsequently diminished adhesion formation in animal models. Healing rat Achilles tendon lacerations have been shown to exhibit a smaller transverse area with undiminished failure load and increased tensile stress at failure after treatment with COX-2 inhibitors. However, they have also been shown to decrease the migration and proliferation of tenocytes. Furthermore, lacerated rat Achilles tendons treated with parecoxib or indomethacin in another study healed with decreased strength and stiffness. It has been suggested that COX-2 inhibitors may have negative effects on the tendon tissue during the first phases of healing, but improve biomechanical properties at a later remodeling stage, inhibiting prolonged inflammatory responses.

Hyaluronan synthases
There are three known hyaluronan synthases (HAS) in mammalian species, of which HAS2 synthesizes high molecular weight and HAS3 low molecular weight hyaluronan. Hyaluronan, an endogenous high molecular weight polysaccharide, is active in wound healing, angiogenesis, tumorigenesis and organization of the extracellular matrix. Attempts have been made to improve flexor tendon healing and reduce adhesion formation by adding hyaluronan. However, both in vitro and in vivo studies (animal models and clinical studies) have shown contradictory results that
may be due to different methods of delivery, varying concentrations and high versus low molecular weight hyaluronan.

**Matrix metalloproteinases and their inhibitors**

The matrix metalloproteinases is a family of enzymes, designed to degrade components of the extracellular matrix, such as collagens and proteoglycans. The intricate balance between matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) is essential for the quality of connective tissues. Excessive amounts of MMPs may cause degradative conditions and a dominance of TIMPS can lead to fibrotic processes. When MMP inhibitors have been used in cancer treatment trials, an increased incidence of frozen shoulder and other musculoskeletal conditions have been noted. Moreover, in animal studies, systemic treatment with the MMP inhibitor doxycycline has weakened the rat Achilles tendon, emphasizing the importance of balance.

It is, however, tempting to manipulate the balance to improve the outcome of healing. After surgical tendon repair, there is commonly degradation and weakening of the tissue surrounding the suture material, possibly caused by MMPs. Sutures coated in the MMP inhibitor doxycycline have been shown to improve the suture-holding capacity of rat Achilles tendons.

**The myofibroblast-mast cell-neuropeptide pathway**

Lately, a new pathway in fibrosis has been suggested. After connective tissue injury, mast cells increase in the area. The mast cells, reacting to the physical trauma and also being directly stimulated to degranulate by neuropeptides, release a number of factors that are part of the first inflammatory stage of the healing process but also factors with potential profibrotic effects (e.g. TGF-β). Thus, the mast cells are believed to induce a fibrotic response, activating fibroblasts and myofibroblasts in the connective tissue. The myofibroblast is a specialized fibroblast, recognizable by its expression of α-smooth muscle actin (α-SMA). The myofibroblast contains fibers allowing it to generate greater amounts of contractile force, altering the character of the connective tissue. This is a necessary feature of wound contraction, but can cause morbidity when excessive, such as in Dupuytren’s contracture.

Increased amounts of myofibroblasts, mast cells and neuropeptides have been found both in skin wound healing models and in joint contractures. Attempts to modulate this suggested profibrotic myofibroblast-mast cell-neuropeptide fibrosis axis with a mast cell stabilizer (such as ketotifen) have shown some promising results in a model of hypertrophic skin wound healing.
Experimental models

Cell culture versus animal model

Cell cultures as a model to study tendons have been very valuable as a starting point for investigating the response of tenocytes to interventions (e.g. different mechanical forces and pressures or addition of growth factors). It is, however, not practically possible to create a model of flexor tendon injury using cell cultures. To better mimic the clinical situation, animal models have been necessary.

Choice of species

Many species have been utilized in the study of flexor tendon injury and healing. These include chickens, mice, rabbits, canines and primates. In addition to the ethical issues that call for choosing the lowest possible species, there are some practical considerations. A mouse model of flexor tendon adhesion has lately been advocated, but to create a model that is similar to the repair of human deep flexor tendons in clinical practice, the tendon has to be of a certain size to allow for similar suturing techniques and biomechanical testing. Rabbit models have been used in a multitude of studies, fulfilling the requirements of the lowest possible species that still has a relevant resemblance to human anatomy and a deep flexor tendon size that makes it possible to design an injury and subsequent repair mimicking the clinical situation.

The clinical trial

Clinical trials of flexor tendon healing have been, and are still, difficult to design. The heterogeneity of the group of patients and individual variations in mechanisms of trauma and associated injuries contribute to confounding factors in the interpretation of the data. To minimize variation, it is desirable to have the same surgeon repairing all tendon injuries included in a study, which, however, is rarely possible in the clinical situation and organization. Moreover, the results of the tendon repair largely depend on successful rehabilitation, which heavily relies on patient compliance.

All these factors contribute to the difficulties of clinical prospective studies of the impact of different interventions on the outcome of flexor tendon repair. There is also a limitation in the instruments available to measure the results. Only tendon rupture rates, ROM, strength and different questionnaires can be utilized: there is no method of investigating the potential positive and negative consequences of the intervention on a biomolecular level. It is therefore essential to gather that information in other types of models before implementing a new intervention in a clinical situation.
Aims

The general aim of the thesis was to more fully characterize the deep flexor tendon and its response to injury and repair. The outcome of flexor tendon healing can be compromised by tendon rupture or adhesions. Therefore, we focused on factors believed to contribute to tendon strength or extrinsic healing with potential adhesion formation, studying the flexor tendon healing process in a rabbit model, to find potential targets to influence the healing process and improve the outcome.

The specific aims of the study were to:

- compare the tissue composition between the intrasynovial deep flexor tendon, extrasynovial tendon and the tendon sheath.
- describe changes in gene expression of basic components of tendon and tendon sheath tissue after injury.
- study alterations in the inflammatory response in the flexor tendon and tendon sheath during healing.
- determine the pattern of gene expression for VEGF and IGF-1 in the tendon and tendon sheath after injury.
- further describe the temporal pattern of MMP and TIMP gene expression after tendon injury.
- determine whether the factors of the hypothesized myofibroblast-mast cell-neuropeptide fibrosis axis are increased in flexor tendon healing.
Methods

Animals
In all the presented studies, skeletally mature female New Zealand White (NZW) rabbits were used with the approval of the local ethics review board. The rabbits, weighing 3 kg (± 0.3 kg), were allowed unrestricted cage activity and provided with a standard laboratory diet and water ad libitum. After one to two weeks of acclimatization to the new laboratory environment, surgery was performed under sterile conditions in an animal operation facility, using microsurgical instruments and magnification. The rabbits were anesthetized with intramuscular injections of fentanyl-fluanisone (Hypnorm®, Janssen, Beerse, Belgium; 0.3 ml/kg body weight) and midazolam (Dormicum®, Roche, Basel, Switzerland; 2 mg/kg body weight). To prevent postsurgical infections, a single intravenous dose of the antibiotic cefuroxime (100 mg; Zinacef®, GlaxoSmithKline, London, UK) was administered immediately before surgery.

At certain time points after surgery or, in the case of the animals in the first study and control animals for the later studies, after one to two weeks acclimatization, the rabbits were euthanized by a lethal dose of either Pentobarbitalnatrium® (Apoteket, Uppsala, Sweden) after first having received a sedative dose of midazolam (Dormicum®, Roche) or fentanyl-fluanisone.

Intra- and extrasynovial tendons
In healthy, uninjured rabbits, intra- and extrasynovial tendons were harvested. The peroneus tendon from the lateral compartment of the lower hind leg was chosen to represent extrasynovial tendons. Two segments of the intrasynovial deep flexor tendons were harvested – one proximal segment at the level of the first annular ligament, where the tendon is subjected to compressive force, and one intermediate segment between the first and second annular ligament, with tensile forces acting on the tendon tissue (Figure 6). With the deep flexor tendon, the tendon sheath was also excised and analyzed.
Figure 6. The intrasynovial deep flexor tendon of the rabbit paw with the proximal and the intermediate segments indicated.

Flexor tendon injury model

The rabbit deep flexor tendon injury model has been designed to mimic deep flexor tendon injury and repair with partially active rehabilitation protocols in clinical practice. The rabbit paw anatomy resembles the human hand, with a superficial and a deep flexor tendon surrounded by a tendon sheath, reinforced by pulleys (Figure 6).

After the hind paws had been prepared with shaving and sterile draping, the flexor tendons were partially divided above the ankle at the site of the tendon-muscular junction. The division partially unloaded the phalangeal segment of the flexor tendons to allow continued activity without immobilization of the operated paws in casts and to mimic the either passive or protected unloaded active rehabilitation protocols in current clinical practice, without sustaining an unacceptable rupture rate. Similar methods of unloading have been used before in flexor tendon studies. The partial unloading preserves some tension, protecting the tendon from the degradation, which has been shown to occur with complete unloading and which may confound later assessments of the tendon tissue. The zone II segments of the flexor tendons were accessed through a longitudinal incision on the volar side of the proximal phalanx of the third digit. The superficial flexor tendons were resected after carefully opening the flexor tendon sheath between the first and second pulleys and the deep flexor tendons were sharply and transversally divided at the intermediate segment (Figure 5). The deep flexor tendons were repaired with a modified Kessler core suture (non-resorbable 5-0 Prolene®, Ethicon, Johnson & Johnson, Sollentuna, Sweden) and a run-
ning circumferential peripheral suture (resorbable 6-0 or 7-0 PDS®, Ethicon). The tendon sheaths were closed with a running 6-0 or 7-0 PDS® suture and the skin with a running non-resorbable suture (5-0 Ethilon®, Ethicon). A single dose of local analgesic was injected into the hind paws for postoperative pain release (Marcain®, AstraZeneca, Södertälje, Sweden). After surgery, the animals were allowed unrestricted cage activity.

At the end point (ranging from three days to six weeks), tendons and tendon sheaths were harvested in 8 mm segments (4 mm proximal and distal to the repair site, respectively). After rinsing in physiological saline, the tendons and tendon sheaths were immediately frozen in liquid nitrogen and stored at -80°C until further analysis (for mRNA measurements) or fixed in formalin (for immunohistochemistry).

![Rabbit paw with the third digit incised three weeks after suturing the deep flexor tendon. The tendon sheath has been divided and the repaired tendon lies beneath.](image)

**Figure 7.** Rabbit paw with the third digit incised three weeks after suturing the deep flexor tendon. The tendon sheath has been divided and the repaired tendon lies beneath.

**Extraction of RNA**

RNA extraction from the tissue samples was performed using the TRIspin method 124, which combines the Trizol method with the RNeasy® Total RNA Kit (Qiagen, MERCK Eurolab, Stockholm, Sweden) and is a reliable method for extracting RNA from hypocellular, dense connective tissues, such as tendon and ligament 21, 93, 94. The frozen tendon tissues were placed in liquid nitrogen-cooled custom-made vials and pulverized using a Braun Mikro-Dismembrator (B. Braun Biotech International, Melsungen, Germany) at 2600 rpm for 30 seconds. The frozen powdered tissue was thawed in 1 ml TRIzol™ (Gibco Life Technologies, Carlsbad, CA, USA) and rotated for five minutes. After addition of 0.3 ml chloroform and centrifugation for 15
minutes at 12000 rpm, ethanol (0.6 ml 70%) was added to the upper aqueous phase and total RNA isolated from the samples using the RNeasy® Total RNA Kit. DNase (RNase-Free DNase Set, Qiagen) was added during the procedure to eliminate potential DNA contamination (this was confirmed using no RT controls in a subsequent PCR analysis). The isolated RNA was quantified using the Sybrgreen II reagent (BioWhittaker Application, Invitro, Stockholm, Sweden). Samples of total RNA were stored at -80°C until further analysis.

Semiquantification of mRNA

Reverse transcription (Omniscript RT Kit; Qiagen) was performed with 1 μg of total RNA with random primers. All samples in a given experiment were subjected to reverse transcription at the same time to avoid potential variation. Similarly, all cDNA samples in a given experiment were assessed by PCR at the same time to avoid any potential variation in efficiency and detection of the amplicons. Aliquots (15 μl) of cDNA were amplified in a total volume of 50 μl containing 1x polymerase chain reaction buffer: 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphate mixture, 0.5 μM of each primer and 1 U Taq DNA polymerase. Known rabbit sequences, which have been validated in previous studies, were used to design the primers (Table 1). To optimize the number of cycles and the temperature during the annealing phase of the PCR, the samples were analyzed at 18 to 30 cycles. The PCR protocol was developed such that product yields were within the linear range of the PCR amplification. Aliquots (20 μl) of the PCR products were analyzed by separation in a 2% agarose gel at 60 V/cm in 1x Tris-Acetate-EDTA buffer. The gel was then stained with ethidium bromide and photographed. The photographs were analyzed by densitometry (MasterScan Interpretive Densitometer and RFLPscan, Scanalytics, Billerica, MA, USA) and values determined to be within the linear range of the image analysis system. Values for each gene were then normalized to the housekeeping gene β-actin (Paper I-III) or 18S (Paper IV-V) for each sample.

For some factors in study IV and V, real-time PCR measurements were performed in addition to the semiquantitative method with similar results (TGF-β1, α-SMA and MMP-13). However, semiquantitative PCR was mainly used in the studies since we do not yet have access to a full range of validated primers for real-time PCR. The methodology of semiquantitative PCR is highly reproducible and the results found to be comparable to those obtained by qPCR using an iCycler.
Table 1. Primers used for PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>gtc ccc caa ctt ctt a</td>
<td>cac cta cgg aaa cct tgt tac</td>
</tr>
<tr>
<td>α-SMA</td>
<td>gtg tga gga aga gga cag ca</td>
<td>tac gtc cag agg cat aga gg</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>gag gag agt gag ggt gag gtc ttt</td>
<td>ctt cgc ctt tgt agc aga tg</td>
</tr>
<tr>
<td>β-actin</td>
<td>tgc ttc tag ggc gac tgt ta</td>
<td>cgt cac atg gca tct cac ga</td>
</tr>
<tr>
<td>Biglycan</td>
<td>gat ggc ctt cag ctc aa</td>
<td>ggt tgt tga aga ggc tg</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>agc tgg gga gaa agc tgg gga aac aag g</td>
<td>agg cac aaa caa atg ggg aaa cca aac a</td>
</tr>
<tr>
<td>Collagen I</td>
<td>gat gcc ttc cag tgt cag ga</td>
<td>ggt ctt cgc gtt gtc tgt ta</td>
</tr>
<tr>
<td>Collagen III</td>
<td>tta taa acc aac ctc ttc ct</td>
<td>tat tat agc acc att gag ac</td>
</tr>
<tr>
<td>COX-2</td>
<td>tca gcc aag cag caa atc ct</td>
<td>gtt atc tgg atg tca gca cg</td>
</tr>
<tr>
<td>CTGF</td>
<td>tgt tag ctc atg ctt ctc ac</td>
<td>cca cta aaa agg tgc aaa cat gta a</td>
</tr>
<tr>
<td>Decorin</td>
<td>tgt gga caa tgt ttc ctc tg</td>
<td>cca cat tgc agt taa ggt tcc</td>
</tr>
<tr>
<td>bFGF</td>
<td>tac aac ttc aag cag aag ag</td>
<td>cag ctc tta gca gac att gg</td>
</tr>
<tr>
<td>HAS2</td>
<td>ggc cgg tcg tct caa att ca</td>
<td>cca ccc cat ttt tgc atg at</td>
</tr>
<tr>
<td>HAS3</td>
<td>aag tgc ctc aca gag acc cc</td>
<td>aag atc atc tgt gca tgt cc</td>
</tr>
<tr>
<td>IGF-1</td>
<td>gca tcc tgt ctc ctc cgc at</td>
<td>gtc tgt ggc atg tgc tgt tg</td>
</tr>
<tr>
<td>IL-1β</td>
<td>tac aac aag agc ttc cgg ca</td>
<td>ggc cac agg tat ctt gtc gt</td>
</tr>
<tr>
<td>iNOS</td>
<td>cgc cct tcc gca gtc tgt</td>
<td>tcc agg agg aca tgc agg ac</td>
</tr>
<tr>
<td>Lumican</td>
<td>ctt cag tgt ctc atc cta</td>
<td>gag ctc cag gta atc gtt</td>
</tr>
<tr>
<td>MMP-3</td>
<td>gcc aag aga tgg tgt tga tg</td>
<td>agg tgt tgt gaa cgc tgt ta</td>
</tr>
<tr>
<td>MMP-13</td>
<td>ttc gcc tta gag tgt aga gg</td>
<td>act ctt gcc ggt gta ggt gt</td>
</tr>
<tr>
<td>NGF</td>
<td>ggt gca tag tga tgt gtc ca</td>
<td>tgt ctc cgg tgt gtc tgg tt</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>cgg cag ctc tgt att gag tt</td>
<td>agc gca cga tca tgt tgg ac</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>gca act ccc acc tgt tca tc</td>
<td>agc gta ggt ctt gtt gaa gc</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>ctc gca act ccc aca tgc tg</td>
<td>cgg atg cag ggc tag tgt t</td>
</tr>
<tr>
<td>TNF-α</td>
<td>agc cca ctt aag agc aca cca</td>
<td>tgt tgt gca gaa agg agg tgt a</td>
</tr>
<tr>
<td>VEGF</td>
<td>gga gta ccc ctt tga gat cga</td>
<td>tgt tgt cta ctc atc aaa ttt gt</td>
</tr>
<tr>
<td>Versican</td>
<td>gat tgt tgt tgt tgg gat ca</td>
<td>cat cca atc tgt cag gga</td>
</tr>
</tbody>
</table>

Immunohistochemistry

Tissue samples intended for immunohistochemistry were, after fixation in formalin, embedded in paraffin blocks and sagitally cut in 6-μm sections. Five sections from each sample were mounted on glass slides, deparaffinized and rehydrated. Myofibroblasts, mast cells and neuropeptide-containing nerve fibers were measured with a triple labeling protocol. Markers used were α-SMA (myofibroblasts), tryptase (mast cells) and SP (neuropeptide-containing nerve fibers). After treatment of the sections with a blocking agent (10% goat serum in PBS for 20 minutes), the primary monoclonal α-SMA antibody (clone 1A4, Sigma-Aldrich, St Louis, MO, USA) was applied. The samples were incubated in 37°C for 60 minutes, washed and treated with the secondary antibody (sheep antimouse IgG HRPO (horseradish peroxidase conjugate) in 1:1000 dilution, Röche Molecular Biochemicals, Laval, Quebec, Canada) for another 60 minutes at room temperature.
After washing, DAB (3,3-diaminobenzidine solution)/peroxide substrate (1:10, Röche) was applied, followed by washing and treatment with polyclonal tryptase goat antibodies (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and the secondary anti-goat antibody conjugated with Cy3 (1:1000, Peninsula Laboratories, Houghton, MI, USA) was applied for 60 minutes at room temperature. Polyclonal guinea pig antibodies to SP (1:200, Peninsula Laboratories) were added and incubated at room temperature for two hours. The SP antibodies were followed by application of an anti guinea pig secondary antibody (1:1000) conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) at room temperature for 60 minutes. Slides were then treated with DAPI (4’-6-diamidine-2-phenyl indole, Vector Laboratories, Burlington, Ontario, Canada) and viewed under a Zeiss light microscope (Zeiss, Axioskop 2 plus, Toronto, Ontario, Canada). A digital camera (Zeiss, Axiocam) was used to photograph five areas from each section. The photographs were analyzed with the program Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). For each image, blinded counts of the total cell number as well as myofibroblasts (α-SMA), mast cells (tryptase) and neuropeptide-containing nerve fiber (SP) were repeated three times and averaged. This procedure was followed by determining the average for each section and, consequently, each sample.

Statistical analysis

All calculations were performed using the Statistica software package 7 (StatSoft, Tulsa, OK, USA). Group data were subjected to standard descriptive statistics (mean, standard error and deviation and percentage).

Differences between groups (different tendons and tendon sheaths) at the same time points were determined with Student’s t-test. To detect significant changes within a group over time, analysis of variance (ANOVA) followed by Tukey’s HSD post-hoc test were used. Only differences or changes with a p-value ≤ 0.05 were considered significant.

For a number of variations in expression, significant differences could not be confirmed. The small number of animals in the groups (n=3-7), giving a low power, requires larger differences between groups to lead to significant results.
Results and discussion

Study I. Differences between intra- and extrasynovial tendons

The aim of study I was to investigate differences in mRNA expression for a number of relevant molecules in the extrasynovial peroneus tendon, two regions of the intrasynovial deep flexor tendon subjected to compressive or tensile loading and the sheath of the flexor tendon. Six NZW rabbits were euthanized and the tissues mentioned above harvested. Measurements of mRNA from extracted RNA were performed using semiquantitative RT-PCR.

The collected data for the pattern of mRNA expression supported previous findings achieved with histological or biomechanical methods. The proximal segment of the intrasynovial rabbit deep flexor tendon is subjected to compressive forces over the metatarsophalangeal joint and subsequently has a distinctive fibrocartilaginous phenotype, separating it from the intermediate segment which is subjected to tensile forces. Accordingly, the proximal segment contained significantly higher levels of mRNA for molecules associated with fibrocartilaginous tissue, such as aggrecan, biglycan and collagen III (Figure 8). The tendon sheath had more total RNA, reflecting a higher cellularity for its role as supplier of nutrition to the tendon tissue, but significantly lower levels of proteoglycan and collagen mRNA as a consequence of a lower load. The growth factor TGF-β1 was higher in the intrasynovial flexor tendon tissue than in the extrasynovial peroneus tendon, mirroring the pattern of collagen I expression it has been shown to stimulate. It can also increase the expression of aggrecan and biglycan.
The differences in the patterns of mRNA expression between the regions and types of tissue underline the importance of designing a model of deep flexor tendon injury relevant to the clinical situation. In an injured and repaired deep flexor tendon, different mechanical forces acting on the tendon tissue in separate regions will have an impact on the biomolecular phenotype and influence the results of the study. There are also differences in the composition of intra- and extrasynovial tendons. Further, data from studies of extrasynovial tendons (such as the Achilles tendon) cannot automatically be presumed true for the intrasynovial flexor tendon. In the clinical setting, this has implications for the use of extrasynovial tendons (usually the palmaris longus or the plantaris tendon) as grafts in delayed repair of flexor tendon injuries. Toe flexors have been suggested as alternative grafts to preserve the character of the tendon tissue suited to the local environment and improve tendon excursion, but are generally not utilized. This lack of utilization of toe flexors may be due to practical considerations or a cautiousness for donor site morbidity, although studies claim good results.

Study II. Matrix molecules and growth factors after tendon repair

In our second study, we investigated the effect a deep flexor tendon injury with subsequent repair has on the mRNA expression of some of the base
components (collagens and proteoglycans) of tendon tissue in the tendon and the surrounding tendon sheath. Three growth factors implicated in healing and fibrotic processes in connective tissues were also included. In a rabbit model of deep flexor tendon injury (five groups of NZW rabbits including controls, with $n=6$ for each group), mRNA levels were analyzed by semi-quantitative RT-PCR at different time points (3, 6, 12 and 24 days) after injury and repair.

After flexor tendon injury, there was a shift in collagen mRNA expression with an increase of collagen type III in both tendon and tendon sheath. In contrast, collagen type I remained unaltered in tendon (Figure 9). This change in expression is in accordance with studies of proliferation and remodeling of connective tissue (e.g. tendon and ligament) $^{114, 150}$ and has been hypothesized to decrease the strength of the tendon tissue $^{170}$.

![Figure 9. Collagens and TGF-β1 mRNA expression in tendon tissue. Significant differences compared with controls are indicated by an *.](image)

Proteoglycans (decorin, biglycan and lumican) participate in the regulation of collagen fibrillogenesis through binding to collagen fibrils $^{132}$. In our study, all proteoglycans except decorin were increased in both tendon tissue and tendon sheath after injury, decorin mRNA instead being markedly down-regulated (Figure 10). Biglycan is believed to be of importance to the composition of flexor tendons, with knockout mice exhibiting flexor tendons with decreased biomechanical properties $^{128}$. However, the high levels of biglycan mRNA detected in tendon tissue may have a negative impact on the healing process, biglycan being associated with fibrotic processes $^{45, 144}$ and known to accumulate for long periods after other connective tissue injuries.
Decorin levels may be related to the biglycan expression. During development, decorin knockout mice have increased amounts of biglycan, the SLRPs to some extent being able to compensate for the lack of the other. It is possible that they have somewhat different functions in collagen composition, biglycan being the more dominant in rabbit deep flexor tendons during the first month after injury in our study, resembling the down-regulation seen in rabbit medial collateral ligaments. This posttraumatic balance is not necessarily an ideal healing environment. One study has claimed improved results of tendon healing with decreased adhesion formation after treating sutured rabbit deep flexor tendons with topically administered decorin. The potential beneficial effect of decorin can be hypothesized to be both an effect of decreasing the amount of biglycan and of inhibiting TGF-β1.

![Figure 10. SLRPs mRNA expression in tendon tissue after injury and repair. Significant differences compared with controls are indicated by an *](image)

We could show that mRNA levels for the large aggregating proteoglycans aggrecan and versican, mediating fibroblast cell proliferation and inflammatory cell migration, increase after injury. An overexpression of aggrecan and versican could be hypothesized to prolong an inflammatory response and increase adhesion formation, but mRNA expression for both were approaching normal levels at the last time point assessed (24 days).

TGF-β1, a regulator of wound repair and implicated in fibrotic processes, exhibited similar patterns of mRNA expression as collagen III in tendon tissue in our study (Figure 8), which is in accordance with the known stimulatory effect of TGF-β1 on collagen III production.

Despite CTGFs connection to TGF-β1 and involvement in fibrotic processes and tendinopathies, the mRNA levels were not significantly al-
tered after injury. A possible explanation is the timing of the analysis: a po-
tential rapid transient increase in CTGF could exhibit levels already decreas-
ing toward normal at the first time point assessed. In a later study, an early
increase in CTGF mRNA three days after flexor tendon injury could be
demonstrated in a chicken model, but levels were down-regulated after the
first week, returning to normal or sub-normal levels \(^{27}\). Our findings do not
indicate that the growth factor would be a likely target to decrease adhesion
formation mediated by TGF-\(\beta1\) in deep flexor tendon healing.

Studies by other investigators have shown conflicting results concerning
the endogenous bFGF response to flexor tendon injury. Both increases \(^{25}\) and
decreases or no alterations \(^{27, 73, 159}\) have been reported. We found, in agree-
ment with the majority of studies, a transient increase in bFGF mRNA in
flexor tendon tissue three days after repair, the gene expression remaining at
normal levels at all time points assessed in the tendon sheaths. This finding
supports the hypothesized beneficial effects an exogenous addition of bFGF
may have on the tendon tissue composition, reinforcing the tendons’ re-
response to injury.

Study III. Inflammatory molecules and hyaluronan
synthases after tendon repair

Earlier intervention studies in animal models of tendon healing have demon-
strated some potential for the treatment with anti-inflammatory agents \(^{44, 85,}
147\). In previous studies, IL-1\(\beta\) has been reported to induce COX-2 mRNA
expression in tendon tissue \(^{160}\). We found similar temporal alterations in the
pattern of mRNA expression for IL-1\(\beta\) and COX-2, with the levels reaching
a peak three days after tendon repair, and then decreasing after the first week
(Figure 11). Although our findings indirectly support the effect of IL-1\(\beta\) on
COX-2 expression, the rapid increase in COX-2 with a decline during the
first week after injury suggests that any treatment targeting COX-2 would
only be relevant during the first week after tendon repair. The effects of
COX-2 inhibitors on tendon tissue have mainly been investigated in Achilles
tendons; the results from these studies are not consistent, however \(^{40, 44, 158}\).
Figure 11. IL-1β and COX-2 mRNA expression in tendons and tendon sheaths after injury and repair. Significant differences compared with controls are indicated by an *.

TNF-α, which was undetectable, and IL-1β are both known inducers of collagenases 32, 160. In the tendon tissue, levels of the collagenase MMP-13 mRNA were increased with maximum levels two weeks after tendon repair. They remained at peak levels in the sheath at the last time point assessed, which is in accordance with earlier reports of a higher MMP production in tendon sheath fibroblasts in in vitro studies 74, a possible component of the inflammatory response associated with extrinsic healing. TIMP-1, an endogenous regulator of MMP expression 22, exhibited a similar pattern of mRNA expression to MMP-13 in tendon.

Other than anti-inflammatory therapies, exogenous hyaluronan has been advocated as an inhibitor of adhesion formation 92, 109, 125, 186. However, there is little knowledge of the endogenous hyaluronan response to flexor tendon injury. In the present study, two hyaluronan synthases were assessed (HAS2 and HAS3). We could demonstrate that the mRNA expression was increased, HAS2 reaching levels seven-fold those of the controls in the tendon tissue. HAS3 demonstrated a lesser increase in tendon tissue but also exhibited tendencies to an increase in the sheaths (Figure 12). This observation indicates differences between the regulation of endogenous hyaluronan production in the tendon and the tendon sheath and that the high molecular weight hyaluronan produced by HAS2 may have a greater role than the low molecular product of HAS3 after deep flexor tendon injury.
Study IV. Growth factors and proteases in healing flexor tendon and sheath

This fourth study demonstrated a steady-state expression of IGF-1 mRNA in uninjured tissues that was markedly higher in the tendon sheaths, with increased levels after injury in the tendons reaching the essentially unaltered levels of the sheaths after 42 days (Figure 13). Previous studies have found different patterns of IGF-1 expression depending on the type of pathology affecting the tendon tissue. Chronic conditions (tendinosis induced chemically or by overuse) caused a slow but prolonged increase, whereas acute mechanical injury resulted in an early transient increase in studies monitoring a shorter time. Whether the differences are due to type of animal or tendon, or the time points chosen, remain to be determined. However, our findings could support the concept of IGF-1, a known stimulator of tenocyte cell proliferation and collagen synthesis, as a potential agent to improve the mechanical properties of the tendon, decreasing the risk of tendon rupture without stimulating adhesion formation.
Other investigators have demonstrated a transient increase of the angiogenic growth factor VEGF during the first or second week after tendon injury, but have no data for the later healing phase. In our study, VEGF tended to increase in the inflammatory phase after three days in tendon and tendon sheath, but was significantly decreased at later time points when the poorly vascularized deep flexor tendon has a higher risk of rupture. We could not demonstrate any significant increases, that if pronounced could contribute to the extrinsic healing response with adhesion formation. Thus, exogenous addition of VEGF, which does not seem to be overexpressed in normal flexor tendon healing, may strengthen the compromised healing tendon tissue without stimulating adhesions.

We could also demonstrate that the neuropeptide NGF, addition of which has had positive effects on ligament healing in animal studies, was present in both healing and uninjured tendon and tendon sheath tissues. There were no significant changes in expression after injury to support the
concept of NGF as an active contributor in deep flexor tendon healing, but that does not exclude the possibility that an engineered increase of NGF may have beneficial effects.

In our third study, we found an increase in MMP-13 during the first three weeks after tendon repair. We could now confirm this increase and show that it remains after six weeks, consistent with the continued breakdown of collagens during the remodeling phase. This finding is in contrast to MMP-3 mRNA expression, which is down-regulated already by the end of the first week after injury in the tendon tissue, with no significant increase at all in the sheath. An earlier study by Oshiro et al\textsuperscript{114} demonstrated a more marked and prolonged increase in MMP-3, but involved complete unloading of the tendon tissue, which will have an impact on MMP expression. However, the mRNA levels do not necessarily mirror the levels of active MMPs in the tissue and therefore must be interpreted with caution. TIMP-1 mRNA expression was increased after three weeks, whereas TIMP-3 remained at subnormal levels six weeks after tendon injury in the sheath, with no changes in the tendon tissue (Figure 14). A down-regulation of TIMP-3 over a longer period may have negative effects, being associated with chronic tendinopathies in rotator cuff and Achilles tendons\textsuperscript{69, 94}.

![Figure 14. MMP and TIMP mRNA expression, presented as a normalized ratio to housekeeping gene 18S. Significant differences compared with control values are indicated by an *.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure14.png}
\caption{Figure 14. MMP and TIMP mRNA expression, presented as a normalized ratio to housekeeping gene 18S. Significant differences compared with control values are indicated by an *.}
\end{figure}
Study V. The myofibroblast-mast cell-neuropeptide fibrosis pathway in flexor tendon healing

In our fifth study, we demonstrated significantly increased numbers of myofibroblasts, mast cells and neuropeptide-containing nerve fibers in rabbit deep flexor tendon tissue after injury. In the normal uninjured controls, they constituted less than 10% of the total cell population as compared with levels reaching 33-50% after tendon repair, which is comparable with results from joint capsule contractures in previous studies \(^{59, 60}\). The increase remained at the last included time point (six weeks after surgery) with similar patterns for all factors assessed (Figure 15).

**Figure 15.** Myofibroblasts, mast cells and neuropeptide-containing nerve fibers in tendon tissue – percentage of total cell count in controls and at different time points after tendon repair. Significant differences compared with uninjured controls are indicated by an *. 

![Graph showing the percentage of total cells for myofibroblasts, mast cells, and neuropeptide-containing nerve fibers at different time points post-injury.](image-url)
Mast cells, which we have shown increase in number after deep flexor tendon injury, can stimulate TGF-β1, which up-regulates α-SMA in fibroblasts and induces the myofibroblast phenotype \(^6^2, ^6^8, ^1^6^2\). In the present study, we demonstrated increased mRNA levels of both TGF-β1 and α-SMA in the repaired tendons (Figure 17). In contrast, the mRNA levels of TGF-β1 and α-SMA remained essentially unaltered in the sheaths.

Recently, a profibrotic myofibroblast-mast cell-neuropeptide pathway has been suggested in the development of posttraumatic capsular fibrosis with joint contracture \(^5^3, ^5^9, ^6^0\). In our rabbit model of deep flexor tendon injury and repair, all three factors implicated in the suggested myofibroblast-mast cell-neuropeptide pathway exhibited similar patterns of increase, remaining high at all time points during the study and thus indirectly supporting the theory of interdependence in expression. This finding may have implications for adhesion formation as well as ultimate tendon strength after repair, and raises questions on the possible significance for the homeostasis and profibrotic effects in surrounding tissues, such as the digital interphalangeal joint capsules.
Figure 17. The mRNA expression of α-SMA and TGF-β1 in tendon tissue as percentages of uninjured control values. Significant differences compared with controls are indicated by an *.
Conclusions

- The gene expression for collagens, proteoglycans and TGF-β1 varies between different regions of intrasynovial tendon, extrasynovial tendon and tendon sheath.

- After flexor tendon injury and repair, a shift occurs in collagen gene expression from type I to type III. Most proteoglycans are up-regulated in response to injury, with the notable exception of decorin.

- No increase of CTGF gene expression was observed after tendon injury. CTGF, although involved in fibrotic processes in other types of tissue, may not be a factor in flexor tendon healing.

- Hyaluronan synthase mRNA is present in deep flexor tendons and tendon sheaths. After injury, both HAS2 and HAS3 mRNA increase in the tendon tissues, with the high molecular weight hyaluronan producing HAS2 reaching the highest levels.

- IGF-1 exhibits a late increase and VEGF a down-regulation in gene expression after injury.

- MMP-3, MMP-13, TIMP-1 and TIMP-3 exhibit different temporal gene expression patterns after tendon injury. MMP-13 mRNA remains increased six weeks after injury in both tendon and sheath, suggesting its active role in all phases of the healing process.

- There is an increase of the factors implicated in the profibrotic myofibroblast-mast cell-neuropeptide pathway after deep flexor tendon injury, suggesting its involvement in flexor tendon healing.
The major goal of flexor tendon healing is to achieve a strong tendon (decreasing the risk of rupture) without adhesions (optimizing tendon excursion; ROM). The current challenge is to stimulate certain aspects of the healing process in order to reinforce tendon strength but not simultaneously increase the extrinsic healing mechanisms and subsequent adhesion formation, and vice versa.

Several paths of action can be considered. We can focus on agents that limit the inflammatory response and any potential excessive cell proliferation (counteracting extrinsic healing and adhesion formation), such as anti-inflammatory or anti-proliferative drugs, or antibodies to growth factors, but will they weaken the tendon and increase the risk of rupture? We can add exogenous growth factors, supporting the endogenous response to traumatic injury, but will that cause excessive healing with scarring?

One patent solution that fits all kinds of deep flexor tendon injuries is unlikely. However, the compiled knowledge of the behavior of the flexor tendons after injury steadily increases, and maybe we are not far from a commercially available growth factor cocktail (possibly containing VEGF, bFGF and IGF-1) to add during surgery, that could stimulate a faster and stronger repair of the tendon tissue, facilitate active rehabilitation and decrease the risk of tendon ruptures, even when compliance fails. For patients with crush injuries, concomitant fractures or extensive soft tissue damage, a tendon wrapping material that includes hyaluronan and antifibrotic agents (such as antibodies to TGF-β1) could limit excessive scarring and adhesion formation.

The finding of factors in the profibrotic myofibroblast-mast cell-neuropeptide pathway in tendon tissue after injury introduces some tantalizing questions. Would their increase be even more pronounced in a flexor tendon injury model that provokes adhesion by post-operative immobilization? What is their expression pattern in finger joint capsules and to what extent may the extension defects with stiffness of the PIP and DIP joint after flexor tendon injury be connected to an activation of this pathway? Would mast cell stabilizers have positive effects on ROM after flexor tendon injury where the patients’ compliance or concomitant injuries necessitate immobilization?
Molekylärbiologiska aspekter på böjsenläkning


Trots de framsteg som skett kan en böjsenskada fortfarande innebära stora kostnader för både samhället och för den drabbade individen. Förutom en lång rehabiliteringsperiod kan den ge bestående rörelseinskränkning med nedsatt handfunktion. För att ytterligare kunna optimera resultaten av böjsenkirurgi studeras nu olika aspekter av böjsenan på molekylärbiologisk nivå i djurmodeller.
Denna avhandling är baserad på fem studier med syftet att bidra till kartläggningen av böjsenans uppbyggnad och reaktion på skada. I samtliga studier har en kaninmodell använts.

I den första studien jämförde vi olika delar av den intrasynoviala böjsenan (omgivna av en senskida) med den extrasynoviala peroneussenan (saknar senskida) och senskidevävnad. Vi fann att mRNA uttrycket för vanliga byggstenar i senvävnad (kollagener och proteoglykaner) varierade mellan olika regioner av böjsenan som utsätts för olika typer av belastningar. Komprimerande tryck ger högre nivåer av proteoglykaner och typ III kollagen vilket resulterar i en mer broskliknande vävnad. I de regioner där tensionskrafter dominerar var uttrycket av proteoglykaner lägre. Vi fann även skillnader mellan den intrasynoviala böjsenan och den extrasynoviala peroneussenan, vilket ytterligare stödjer att böjsenan är unik och att kunskap om andra typer av senor inte kan förutsättas gälla även för böjsenan.

Den andra studien fokuserade på förändringar i genuttryck i böjsenan och dess senskida efter att senan skadats och reparerats. Den oskadade böjsenan är uppbyggd av framför allt typ I kollagen men under den första delen av läkningsprocessen dominerade typ III kollagen. Proteoglykaner hade kraftigt ökade mRNA nivåer, med undantaget decorin som istället nedreglerades. Vi fann även att mRNA för tillväxtfaktorn bFGF, som tros kunna förbättra sensans läkning och hållfasthet, endast kortvarigt uppreglerades under den första veckan av läkningsprocessen.

I den tredje studien kunde vi visa att böjsenan ökade uttrycket av mRNA för komponenter i den endogena hyaluronsyreproduceringen efter en skada. Vi fann även en ökning av mRNA för faktorer involverade i det inflammatoriska svaret (COX-2 och IL-1β) men det var kortvarigt med normaliserade nivåer redan efter den första veckan.

Den fjärde studien följde böjsenans läkningsförlopp över en längre tidsperiod och inkluderade början på remodellingsprocessen sex veckor efter skadan. Vi kunde visa att kollagenaset MMP-13 hade höga mRNA nivåer under hela perioden och därmed verkar delta i alla delar av läkningsprocessen, till skillnad mot MMP-3 som nedreglerades redan efter en vecka. Tillväxtfaktorer som kan vara positiva för sensans styrka uppvisade antingen en nedreglering av mRNA (VEGF) eller en sen ökning (IGF-1), vilket ytterligare styrker hypotesen att en tillförsel utifrån av dem skulle kunna optimera böjsenläkning. Vi fann även att tillväxtfaktorn NGF fanns i både oskadad och skadad böjsena, men att dess mRNA uttryck inte påverkades av en skada.

I den avslutande femte studien undersökte vi om faktorer involverade i en hypotetisk fibrosstimulerande cykel ökar även efter böjsenskada. Vi visade att andelen myofibroblaster, mast celler och nervfibrer med neuropeptider blev större i den skadade senvävnaden, en ökning som varade minst sex veckor. Detta kan ha betydelse för både böjsenan och dess omgivande leder.
Acknowledgements

I would like to express my sincere gratitude to everyone that made this doctoral thesis possible. In particular I would like to thank:

Monica Wiig, my supervisor, for introducing me to the world of hand surgery and flexor tendons and for your professional guidance.

David Hart, my co-author and mentor, for never-ending patience, invaluable feedback and support.

Olle Nilsson, my co-supervisor, for your guidance and encouragement.

Carol Reno, for brilliant technical support and patiently answering all my questions.

Britt-Marie Andersson and Mei Zhang, for skilful technical assistance.

Kevin Hildebrand, my co-author, for generously sharing his knowledge.

Stephan Wilbrand, Head of Department of Hand Surgery, for giving me the time and support to complete my thesis.

Everyone at the Department of Hand Surgery, Uppsala University Hospital, for your friendship, support and encouragement. You make work fun!

Bertil Vinnars, for valuable advice and inspiring enthusiasm.

All my friends, with special thanks to Pauline Englund and Dina Giese, for keeping things in perspective, and Kristin Elf, for valuable advice.

My parents Inga-Lill and Bengt, and my brother Mathias, for always being there for me.

And finally, Lukas Brandts, for always supporting me and being an endless source of encouragement. Ik hou van jou!
References


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 544

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)