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Studies on rat and rabbit Achilles tendons

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Contents

List of Papers

This thesis is based on the following papers:

I. Carina Forslund and Per Aspenberg. OP-1 has more effect than mechanical signals in the control of tissue differentiation in healing rat tendons. Acta Orthop Scand 1998; 69 (6): 622–26.

II. Per Aspenberg and Carina Forslund. Enhanced tendon haling with GDF 5 and 6. Acta Orthop Scand 1999; 70 (1): 51–54.

III. Carina Forslund and Per Aspenberg. Tendon healing stimulated by injected CDMP-2. Med Sci Sports Exerc 2001 May; 33 (5): 685–87.

IV. Carina Forslund and Per Aspenberg. CDMP-2 induces bone or tendon-like tissue depending on the mechanical situation. In press, J Orthop Res.

V. Carina Forslund and Per Aspenberg. Improved healing of transected rabbit Achilles tendon after a single injection of CDMP-2. Revised and resubmitted, Am J Sports Med.

VI. Carina Forslund and Per Aspenberg. A comparative dose-response study of CDMP-1, 2 and 3 for tendon healing in a rat model. Conditionally accepted, J Orthop Res.

Abbreviations

BMP	Bone Morphogenetic Protein
CDMP	Cartilage Derived Morphogenetic Protein
EGF	Epidermal Growth Factor
GDF	Growth and Differentiation Factor
IGF	Insulin-like Growth Factor
OP	Osteogenic Protein
PDGF BB	Platelet Derived Growth Factor BB
TGFß	Transforming Growth Factor beta
Vg-1	Vegetal 1
Vgr-1	Vegetal related 1

Introduction

Achilles tendon healing is a time consuming process with a long period of immobilization and rehabilitation for the patients. Any method that can shorten this time period will be of value for the patients and for society. Attempts have been made with ultrasound, mechanical stimulation and growth factors. This thesis is based on a series of experiments using growth and differentiation factors for augmenting tendon repair in animal models.

Tendon biology

It seems a general rule that long tendons pass through narrow regions—notably in the wrist and ankle. Tendons often contain local regions of fibrocartilage where they traverse joints and wrap around bones (Koob and Vogel 1987, Merrilees and Flint 1980, Okuda et al. 1987).

Synovial sheets surround certain tendons, notably flexors in the hand and foot. They allow the tendons to glide freely and produce synovial fluid, which contributes to tendon nutrition. Some tendons, e.g. the Achilles, that lack true synovial sheets have a false sheath or paratenon which develops simply as a membranous thickening of the surrounding connective tissue. They receive no synovial nutrition. Their blood supply comes from the intrinsic vascular systems at the muscle-tendon junction and the tendon-bone insertion, and from the extrinsic segmental vascular system through the paratenon along the axis of the tendons (Lundborg et al. 1980, Lundborg et al. 1977). The central third of the Achilles tendon in rabbits receives approximately 35% of its blood supply from the extrinsic vascular system (Naito and Ogata 1983). Blood flow in tendons is surprisingly high, approximately 0.10 mL/g/min in rabbit tendons compared with 0.27 mL/g/min for resting muscles (White et al. 1964). The total fraction of the extra-cellular matrix occupied by vessels in the medial collateral ligament is 1.5% (Bray et al. 1996). Blood supply increases in tendons and ligaments with exercise and during healing (Backman et al. 1991). Vessels are most conspicuous on the surface where they form an anastomotic network in the epiligament (Hergenroeder et al. 1982, Kolts et al. 1994, Lundborg et al. 1977). Not all parts of a tendon have blood supply. Regions subject to friction, compression or torsion are often avascular (Hergenroeder et al. 1982, Kolts et al. 1994, Lundborg et al. 1977). Such areas are especially prone to tearing and calcification.

The intact tendon is composed of 3 main constituents: collagen, other ground substance and cells. In degenerative conditions the amount of structural glycoproteins with high water bonding capacity increases, leading to oedema. Conversely, the amount of collagen decreases and the fibers being the most important component for mechanical strength, lose their parallel course.

Histological structure

The majority of the cells in tendons and ligaments are fibroblasts. The tendon fibroblasts have an elaborate spindle-like shape (Figure 1). They lie in longitudinal rows and have numerous sheet-like cell processes that extend into the extra-cellular matrix. Cells within the same row, as well as those in adjacent rows are linked to each other by gap junctions (Benjamin and Ralphs 1997). It seems like the cells form a 3 dimensional communicating

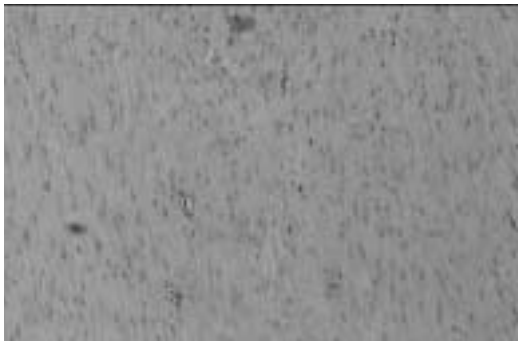


Figure 1. Tendon callus 14 days after Achilles tendon transsection in the rat. The tissue is still rich in cells and blood vessels, but fibers are beginning to orient in the direction of traction (vertical).

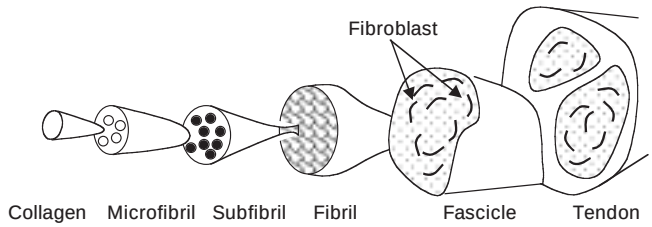


Figure 2. Schematic drawing of tendon structure.

network that extends throughout the tendon and can form the basis of a load-sensing system that allows a tendon or ligament to modulate the composition of its extra-cellular matrix in response to changes in loading pattern. Fibrocartilage cells are present where tendons wrap around bony pulleys. Chondrocytes are also present where the tendon attaches to the bone. Mast cells, endothelial cells and axons are generally thought to be present as well (Hart et al. 1985), although in a recent study Ackermann (2001) presented that nerve fibers are only found in the tendon in the initial phase after a rupture, and then disappear during tissue maturation.

Collagen fibrils are grouped into fibers that can be seen by light-microscopy. In turn, the fibers are collected into fiber bundles, and the bundles into fascicles. A collection of fascicles forms the whole tendon or ligament, and is wrapped up in a surface connective tissue layer called the epitenon or epiligament (Chowdhury et al. 1991)(Figure 2). The fiber bundles and fascicles are enclosed in endotendon which allows them to slide relative to one another and which contributes to overall flexibility. Most human tendons are multifascicular and the fascicles frequently spiral along their length, e.g. the Achilles tendon. As the Achilles tendon descends, it spirals about 90° so that the fibers that were originally posterior become lateral, and anterior fibers become medial. This rotation produces a region of concentrated hydrostatic stress in the middle of the tendon (Kannus and Natri 1997).

Biochemical composition

70–80% of the dry weight of tendons and ligaments is collagen, having a half-life of 300–500 days (Neuberger and Slack 1953). Most collagen is type I, the principal tensile-resistant fiber, but smaller quantities of types III, V and VI are also present (Waggett et al. 1996). Water accounts for 65–75% of the wet weight of a healthy tendon in adult humans and much of this is probably associated with proteoglycans in the extra-cellular matrix (Akeson et al. 1984). Tendons are not uniform compositions along their length. There are regional variations in water, collagen and glycoaminoglycan content that are likely to be reflected in biomechanical differences as well (Merrilees and Flint 1980). Where tendons wrap around bony pulleys, the content of type II collagen (which is typical for cartilage) (Vogel 1995) and glycoaminoglycan is considerably higher. Much of the glycoaminoglycan is chondroitin sulphate associated with aggrecan (Vogel 1995). This is a large aggregating proteoglycan that allows articular cartilage to withstand compression and accounts for the stiffness of tendons in their wrap-around regions.

Several biochemical changes have been observed as tendons degenerate with age. Collagen content increases, but elastin and proteoglycans decrease, resulting in less elasticity. Related to this, water content declines from 80% at birth to approximately 30% in old age (Hess et al. 1989, Jozsa et al. 1989).

Mechanical influence on tendon tissue

Ploetz (1938) and Gillard (Gillard et al. 1979) showed the influence of mechanical factors on the modulation of extracellular matrix constituents in the tendon. They used the rabbit posterior limb digital flexor tendon. It normally runs behind the tibial malleolus and is therefore exposed not only to pulling, but also to pressure and tearing. By dislocating the tendon anterior to the malleolus it was no longer transversely loaded and the fibro-cartilaginous region in the tendon lost its cartilaginous character. Finite element analysis has shown that the region of increased development of cartilaginous matrix in tendons that wrap around bone corresponds to the region in which the tendon cells are

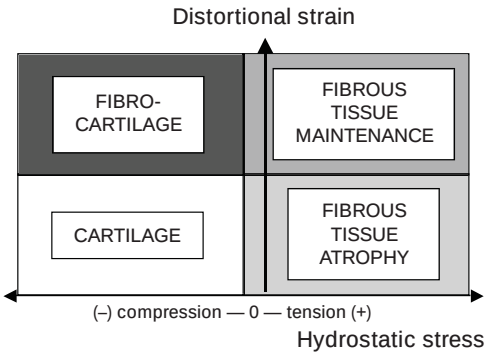


Figure 3. Schematic representation of the mechanical theory of tissue differentiation, adapted from Giori (Giori et al. 1993). The axes denote some function of distortional strain and hydrostatic stress over time. Negative, or compressive, hydrostatic stress equals hydrostatic pressure. A distortional strain causes changes in the fibroblasts shape and stimulates the production of a fibrous extra-cellular matrix. Compressive hydrostatic stress history stimulates the production of a cartilaginous extra-cellular matrix.

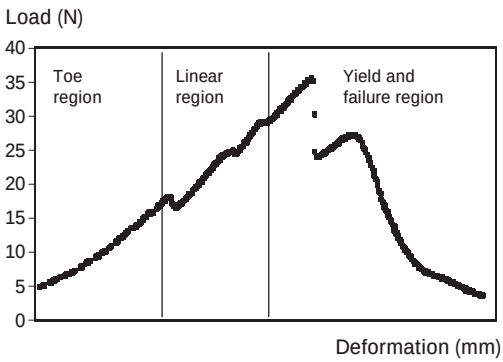


Figure 4. Basic load-deformation curve for tendon.

subjected to higher hydrostatic pressure (Giori et al. 1993) (Figure 3).

Biomechanics—tensile properties

Tendons and ligaments possess the highest tensile strength of any soft tissue in the body, both because collagen is the strongest of fibrous proteins and because these fibers are arranged parallel to the direction of tensile force. The material properties of tendons depend mainly on the mechanical properties and architecture of the collagen fibers, elastin fibers, and proteoglycans.

The material properties of a tendon—its stress-strain relationship—are similar to those of other collageneous soft tissues such as ligament and skin. The stress-strain curve begins with a toe region, in which the tendon stretches (strains) easily, without much force (Figure 4). This behavior has been attributed to the straightening of the crimped fibrils and the orienting of the fibers in the direction of loading. The toe region is rather small in tendon because the collagen fibers are nearly parallel with the long axis of the tendon, and less realignment is required. The toe region decreases with age because the amount of crimp decreases with age.

As strains are increased, the toe region is followed by a fairly linear region. The slope of the line in this region has been used to represent the elastic modulus of the tendon. The slope of the linear region (the elastic modulus), the maximum (ultimate) stress and strain, and the area under the curve (the strain energy density to failure) are required to fully describe the stress-strain curve.

The elastic strain energy recovered when a tendon is unloaded is 90–96% per cycle at physiologically relevant strain rates, indicating that tendons waste only small amounts of energy during activity.

Exercise

Exercise was shown to have a positive long-term effect on the structural and mechanical properties of swine tendons (Woo et al. 1980). The stiffness, ultimate tensile strength, and weight of the tendons increase as a result of long-term training. Crimp angle and crimp length were also influenced by exercise. Other research groups did not see any effect of exercise upon intact (Messner et al. 1999) or healing tendons (Murrell et al. 1998). The inconsistence of these findings may be due to differences in the magnitude of loading applied to various structures during general exercise programs (Tipton et al. 1986). A theoretical computerized model based upon experimental data, showed that the exercise stimulations predict increases of approximately 14% in the tendon cross-sectional area, modulus and strength (Wren et al. 2000) for both immature and mature cases.

Achilles tendon ruptures

A spontaneous tendon rupture may be defined as a rupture that occurs during movements and activities that should not—and usually do not—damage

the involved musculotendinous units (Kannus and Jozsa 1991). Achilles tendon ruptures are common sports injuries in men with a maximum incidence at 35–40 years. As many as 59% of Achilles tendon ruptures are sustained during sports activities, in contrast to only 2% of other tendon injuries (Jozsa et al. 1989). The patients seldom have a history of problems with the Achilles tendon before being injured. The patient mostly feels a sudden "pop" or "snap" in the calf and sometimes hears a sharp sound. On many occasions, the patient believes someone kicked him.

An immediate pain that soon resolves is typical after an Achilles tendon rupture. A persistent weakness, poor balance and changed walking capability are common. Achilles tendon rupture is a clinical diagnosis. During the first 48 hours, a gap is palpable in the tendon at the site of the rupture. The typical clinical investigation is the calf squeeze test described by Thompson (1962), which is simple and reliable. With the patient prone, the calf muscles are squeezed from side to side. If there is a subsequental plantar flexion of the foot, the test is negative and the Achilles tendon is intact. If the plantar flexion movement is absent despite adequate calf squeezing, the test is positive and indicates a completely ruptured Achilles tendon.

Achilles tendon ruptures commonly occur in the mid-substance of the tendon, usually 2–6 cm proximal to the calcaneal insertion. After a few days the tendon gap is filled with a fibrous hematoma and it may be difficult to detect by palpation. Aids like ultrasonography or magnetic resonance imaging can be used for later diagnosis. However, since the frayed tendon ends tend to overlap each other and may give a false impression of a partial rupture, ultrasonography could entail a missed diagnosis.

Etiology of Achilles tendon rupture

The etiology of Achilles tendon ruptures is largely unknown. However, apart from systemic diseases such as rheumatoid arthritis, SLE and gout, 2 different etiologies of Achilles tendon ruptures are mentioned most:

1. Overload due to malfunction of the normal inhibitory mechanism of the musculotendonous junction.
2. Chronic degeneration of the tendon that leads to a rupture without excessive loads being applied. Repetitive micro-trauma and hypovascularity of part of the tendon are suspected as predisposing factors (Ahmed et al. 1998, Carr and Norris 1989, Kannus and Jozsa 1991).

The closed tendon rupture caused by indirect forces like a sudden foot push-off or an unexpected dorsiflexion of the ankle is the dominant immediate etiological factor for Achilles tendon rupture, but there are other possible causes. Most theories are based on mechanical and degenerative factors. Degeneration of tendon tissue is a consistent finding (Kvist et al. 1992). When a tendon ruptures, various pre-existing degenerative changes may be found, including hypoxic degeneration, lipomatotis, mucoid degeneration, calcification and occasionally necrosis. A common belief is that tendon changes are due mainly to impaired vascularisation caused either by changes in the vessel wall such as medial hypertrophy, or by a reduced number of capillaries per tissue volume resulting in an increased distance for oxygen to diffuse. A quantitative assessment of intravascular volume of the human Achilles tendon was done in 10 legs of fresh frozen cadavers, which were injected with a solution of Tc-99m, indiaink and gelatin (Stein et al. 2000). The study shows that the mid-part of the Achilles tendon possesses only half of the vascularity of the proximal and distal part.

Kannus (1991) reported a histological study of 891 ruptured tendons and 445 healthy age-matched control tendons (from individuals killed in accidents, and with no known disease before the accident) that no healthy structures were seen in any of the spontaneously ruptured tendons, but in two-thirds of the control tendons ($p<0.001$). Most (97%) of the pathological changes were degenerative. They included hypoxic degenerative tendinopathy, mucoid degeneration, tendolipomatosis, and calcifying tendinopathy, either alone or in combination. Kannus concluded that degenerative changes may be common in people over the age of 35 years, and it seems likely that these changes predispose to rupture.

Tendinosis

Tendinosis is common among athletes, and mostly occurs after abrupt changes in training schedules where over-ambitious training and competitions can start the process. An inflammatory reaction in

the paratenon, degenerative changes in the tendon or repeated microruptures are often seen. The treatment is rest and unloading of the tendon. Too quick a return to activity often leads to chronic tendinosis, which demands a much longer rehabilitation. Surgery is performed to excise pathological parts of the tendon or paratenon only when conservative treatment has failed.

Tendon repair

Primary healing of most soft tissues requires 7–10 days, but because in addition to vascularisation and basic cell proliferation, large quantities of collagen must be synthesized and adequately remodeled to withstand the forces generated by muscles (Enwemeka 1992), tendon healing takes several weeks.

Tendon healing appears in three steps (Enwemeka et al. 1988):

1. The cellular reaction phase with inflammatory cells (until day 5).
2. The fibrous protein synthesis phase with fibroblast proliferation
3. The remodeling phase with collagen fibril synthesis and alignment of fibrils with the longitudinal axis of the tendon.

After the paratenon and the tendon are incised, the wound fills with a haematoma of inflammatory products, nuclear debris, and fibrin. This tissue has no tensile strength. During the first week, proliferating tissue from the paratenon penetrates the gap between the tendon stumps and fills it with undifferentiated and disorganized fibroblasts.

During healing after an Achilles tendon rupture, tendon width increases, regardless of treatment (Möller 2001), and is still increased after 2 years. There appear to be significant differences between surgically and non-surgically treated tendons.

A study including biomechanical, biochemical and electron microscopical investigations was done comparing ruptured and intact tendons in rabbits (Reddy et al. 1999). The rabbits had an Achilles tendon transsection: the tendons were sutured, immobilized for five days in a plaster and the rabbits killed after 15 days. By then the transsected tendons had regained 48% of their normal tensile strength and 20% of the tensile stress. Although there were large differences in biomechanical properties between healing and intact tendons, biochemical analysis showed a collagen content per dry weight in transsected tendons amounting to 80% of the controls and collagen cross-linking (measured by the hydroxypyridinium content in the tendons) to 60% of the intact tendon controls.

Achilles tendon ruptures in rats heal with spontaneous endochondral formation (Rooney et al. 1992). In an Achilles tendon-transsection model, several small cartilage nodules were present from 4 weeks, and were replaced by bone via endochondral ossification from approximately 5–6 weeks. The bone had a marrow cavity, which appeared to contain normal bone-marrow cells. These ossicles could be clearly observed on radiographs.

Treatment

Surgical treatment

Open or percutaneous methods can be used for treatment of Achilles tendon rupture. The primary goal of surgical intervention is the apposition of torn tendon ends, which can be accomplished by a simple end to end suture. The paratenon is closed at the end of the operation. Surgery is followed by immobilization in plaster, or by early motion in a brace.

Surgical repair has been performed under local anesthesia with good results (Andersen and Hvass 1986, Cetti et al. 1981, Keller and Bak 1989, Sejberg et al. 1991). In the early 1990s, percutaneous techniques showed an increased risk of sural nerve injury, and repair was usually weaker than when using open repairs (Aracil et al. 1992). Later studies often favour the percutaneus technique, showing a low rate of reruptures and injuries to the sural nerve (Webb and Bannister 1999).

In a clinical study of surgically treated patients suffering from an Achilles tendon rupture, Wredmark (Wredmark and Carlstedt 1992) found no elongation (radiographically measured with wires inserted at the proximal and distal part of the healing tendon) of the Achilles tendon during the healing period and no change in the range of motion. Interestingly, he found a difference in healing between the dominant and non-dominant side, the non-dominant side seemingly more difficult to restore to full function.

Tendon repair can also be done by use of artificial tendon implants or transplanted tendons in a tendon transfer procedure to reinforce the Achilles tendon.

Non-surgical treatment

Non-surgical treatment includes no treatment at all, immobilization in plaster or functional rehabilitation with early mobilization without previous surgical repair. 8 weeks of immobilization in plaster is most common, first in a plantar flexion then, after 3–5 weeks, in a reduced plantar flexion. Functional non-surgical treatment can also consist of a brace with gradually decreasing heel height.

An editorial in Lancet 1973 says "In view of the excellent results obtainable by conservative treatment it is doubtful whether surgical repair in closed rupture of the Achilles tendon can still be justified". Randomized studies of surgical versus non-surgical treatment were presented by Nistor (1981), Cetti (1993) and Möller (2001). Nistor favors non-surgical treatment, and Cetti and Möller favour surgical treatment. Nistor (1981) studied 105 patients and concluded that non-surgical treatment offers advantages over surgical treatment. The results of both surgical and non-surgical treatment were satisfactory. There were minor differences between the results in the two groups, but the period of morbidity was shorter, complaints fewer and no hospital stays were needed in the non-surgically treated group (Nistor 1981). Post-surgical and non-surgical treatment results were compared in a recent study (Möller 2001). 112 patients suffering from acute Achilles tendon rupture were randomized to surgical or non-surgical treatment. Surgical treatment consisted of an end-to-end suture, postoperative plaster treatment for two weeks, and finally by functional rehabilitation in a brace for 6 weeks. Non-surgical treatment consisted of 4 weeks of plaster in plantar flexion, followed by 4 weeks in a neutral position. Möller concluded that surgical treatment followed by early functional rehabilitation is safe and reliable. The non-surgical treatment of Achilles tendon rupture led to a rerupture in 21% of the patients, and the author concluded that non-surgical treatment can not be regarded as acceptable for healthy, active persons under the age of 65 years. The large difference in rehabilitation treatments after the injury between the surgically and non-surgically treated patients could be of major importance for the outcome of the study, and it is not clear that conclusions can be drawn regarding the effect of surgical repair in itself. However, at the end of the study Möller sustains that "if reruptures are avoided, surgical treatment followed by early functional rehabilitation and non-surgical treatment with a plaster appear to produce equally good results after an Achilles tendon rupture".

McComis (McComis et al. 1997) presented a study where acute Achilles tendon rupture was treated non-surgically with 2 weeks in plaster, followed by 4 weeks in a brace with a heel-lift restricting dorsiflexion, still allowing free plantar flexion. Dorsiflexion was increased by 10° per week, and the patients were allowed to carry approximately 20% body-weight. After 8 weeks, the patients were allowed to walk without crutches carrying full body-weight. After 26 weeks all treated tendons had regained at least 70% of the capacity of the contralateral tendon regarding isokinetic testing. There was one rerupture in 15 patients (patient slipped). The patient was started on the program again with good results. This appears to be a good alternative to surgical treatment. Saleh (Saleh et al. 1992) compared non-operated patients treated with 3 weeks in plaster followed by early controlled mobilization or continued immobilization. The mobilization group used a splint, which holds the ankle in 15° of plantar flexion, but allows some movement over the metatarsophalangeal joint. The immobilized group was treated with 4 weeks in a full-leg cast with the ankle in full equinus, followed by 2 weeks in a below the knee cast with the ankle in mid-equinus, and finally 2 weeks with the ankle in neutral position and weight bearing allowed. Both groups regularly attended physiotherapy. The time until comfortable walking was reduced to almost half for the splint-group compared to the control group ($p < 0.001$). Both groups had one rerupture in 20 patients.

Cetti (Cetti et al. 1994) and Mortensen (Mortensen et al., 1999) also studied early rehabilitation versus immobilization after surgery. They found a faster recovery in the group treated with early rehabilitation using a brace.

Mobilization

Immobilization for 6–8 weeks leads to muscle atrophy, joint stiffness, with risk of osteoarthritis, skin necrosis, infection, tendocutaneous adhesion, rerupture and thrombophlebitis (Enwemeka 1992).

Clinical studies by Armbrecht (Armbrecht et al. 1993) and Sölveborn (Solveborn and Moberg, 1994) showed that immediate ankle mobilization after Achilles tendon ruptures surgery does not pose a significant risk for rerupture.

After the initial protection of a tendon repair site, mechanical stress caused orientation of collagen fibrils (Peacock 1965). Stress promotes the remodeling into mature collagen (Viidik et al. 1982), and passive mobilization increased the tensile strength of healing flexor tendons (Woo et al. 1981). It is therefore to be expected that some stress stimulation will benefit tendon healing.

Experimental studies

The effect of early rehabilitation and mobilization after Achilles tendon rupture has been studied by many groups. Functional loading augments the tensile strength and energy absorption capacity of experimentally tenotomized rabbit tendons without promoting rerupture (Enwemeka 1992). The effect was significant for the first weeks, but after 3 weeks the control group had caught up, which suggests that Achilles tendon ruptures should be carefully loaded during the early, rather than later, stages of healing. Murrell (Murrell et al. 1994) showed that immobilization by external fixation in a model of Achilles tendon ruptures in rats had a strong negative effect on the functional and mechanical recovery of the tendon. Immobilization of the leg after tendon transsection in rats was also studied by Rantanen (Rantanen et al. 1999). The leg was immobilized in one of two positions intended to produce a long or a short defect, and the cast was removed after either 5 or 10 days. They found no difference in tendon healing between the modes of immobilization, and the final end-to-end distance did not differ. The muscles in the short defect group had a higher degree of atrophy than the muscles in the long defect group. The results indicate that the degree of inevitable muscle atrophy during immobilization can be decreased by maintaining a certain level of tension in the immobilized muscle tendon. Applying controlled motion and tensile stress across a healing tendon also promotes fast recovery by enhancing the formation of cross-links between collagen fibrils (Enwemeka 1989, Enwemeka 1992, Ketchum 1977)

Tendon repair with growth factors

Several groups have tried to increase tendon repair with different growth factors (Table 1). Via a possible anti-inflammatory mechanism in rats, Insulin-like growth factor 1 (IGF 1) decreased the time to functional recovery (foot-print measurements) after Achilles tendon injury (Kurtz et al. 1999). Biomechanical tests however, revealed no significant difference between the groups. In a rat medial collateral ligament injury model, Platelet Derived Growth Factor (PDGF) on a collagen carrier increased ultimate load after 12 days if administrated within 24 hours (Batten et al. 1996). If the PDGF was administrated 48 hours after the injury, however, it tended to decrease the ultimate load (27% lower than the controls). Treatment with PDGF BB on a fibrin-sealant delivery vehicle in the ruptured medial collateral ligament in rabbits afforded a higher ultimate load, energy absorbed to failure and less ultimate elongation than control groups after 6 weeks (Hildebrand et al., 1998). Addition of Transforming Growth Factor beta (TGFß) did not lead to any further increase. In rabbits, Epidermal Growth Factor (EGF) combined with TGFß1 improved the biomechanical properties of the healed medial collateral ligament 6 weeks after injury (Woo et al. 1998). FGF-2 was used in an in vitro model with patellar tendon cells to study its effect in a wound closure healing model (Chan et al., 1997). After a “wound” was produced, FGF-2 was added in a medium. A 4-fold increase in cell proliferation with all doses (2–50 ng/mL) was seen after 24 hours of incubation. 10 ng FGF-2/mL had a significantly greater wound closure than the other groups.

Bone Morphogenetic Proteins (BMPs)

Hippocrates (c. 460–370 BC), while contemplating on the Greek island of Kos, was reportedly awe-struck by the fact that among the many tissues in human body, bone has considerable potential for

Table 1. Papers on applying growth factors to healing tendon or ligament.

Growth factor	Doses	Model	Evaluation	Time	Results	Study
IGF-1	25 µg	AT-transsection in rats	F, B	15 d	IGF-1 reduced maximum functional deficit and accelerated recovery after AT injury	a
PDGF	0.5, 1, 5 µg	CL injury model in rats	B	12 d	Dose-dependent beneficial effect if administrated within 24 hours	b
PDGF-BB	400 ng, 20 µg	MCL in rabbits	B, H	6 w	Improved ultimate load, energy absorbed to failure and ultimate elongation	c
PDGF-BB + TGFβ1	400ng + 4ng, 20 µg + 200 ng	MCL in rabbits	B, H	6 w	Same results as with PDGF-BB alone. TGFβ1 did not lead to additional improvements in the MCL	c
EGF + TGFβ	100ng + 4 ng, 50 µg + 2 µg	MCL in rabbits	B, H	6 w	EGF combined with TGFβ1 improved the biomechanical properties of the healed MCL	d
FGF-2	2, 10 and 50 ng/mL	In vitro wound closure model	W	12 h	10 ng bFGF/mL had a significantly greater wound-closure than the other groups	e

CL – collateral ligament
MCL – medial collateral ligament
F – functionally
B – biomechanically
H – histologically
W – wound-width measurements

a – Kurtz et al., 1999
b – Batten et al., 1996
c – Hildebrand et al., 1998
d – Woo et al., 1998
e – Chan et al., 1997

repair. Then, as described by Reddi (1997) "There was a lull in the research activity for over 23 centuries until Senn …described the utility of antiseptic decalcified bone implants in the treatment of osteomyletis and certain bone deformities". In 1938, Levander found ectopic bone formation after injections of alcoholic extracts of bone into the rectus muscle in rabbits. He then suggested the existence of "… a substance having the power to activate the non-specific mesenchymal tissue into the formation of bone tissue…" (Levander 1938). The substance he was aiming at was later to be named BMP. The history of BMP began with Urist (Urist 1965), who made the key discovery that demineralized, lyophilized segments of bone induced new bone formation when implanted in muscle pouches in rabbits. There are now several related BMPs known that initiate bone through endochondral as well as intramembraneous bone formation pathways (Wozney 1998) (Table 2).

Attempts at purifying BMPs from bone started with rat bone. Rough estimates revealed however that only a microgram of active protein was present in a kilogram of bone. So, instead of rat bone, over a ton of bovine bone was processed to isolate a few micrograms of active osteogenic fractions by heparin affinity chromatography (Luyten et al. 1989). In 1988, one group reported having purified BMP-1, BMP-2A and BMP-3 from guanidinium chloride extracts of demineralized bone, which induced bone formation greater than 300,000-fold at ectopic implantation sites. 50 ng of highly purified protein was active in an in vivo cartilage and bone-formation assay (Wozney et al. 1988). OP-1 was purified from bovine bone with similar methods (Sampath et al. 1990).

BMPs belong to the TGFß protein family. They initiate, promote and maintain chondrogenesis and osteogenesis, and are also involved in the morphogenesis of organs other than bone.

Members of the BMP family have been determined to be key signalling molecules in embryogenesis, in species ranging from Drosophila to humans (Wozney 1998). Studies of the expression pattern of different BMPs as well as the analysis of spontaneously mutated or genetically depleted mice have demonstrated a broad range of functions apart from bone induction. These activities are mainly located at sites of epithelial-mesenchymal interaction, including, but not restricted to, the skeleton (Ducy and Karsenty 2000). Functions of BMPs include cell proliferation and differentiation, apoptosis, morphogenesis, patterning of various organs including the skeleton and organogenesis (Hogan

Table 2. Bone morphogenetic proteins (Reddi 1997).

Subfamily	Members
BMP 2/4	BMP 2 BMP 4
OP-1/BMP-7	OP-1/BMP-7 OP-2/BMP-8 BMP-8b (mouse) BMP-5 BMP-6/Vgr 1
GDF-5/CDMP-1	GDF-5/CDMP-1/BMP-14 GDF-6/CDMP-2/BMP-13 GDF-7/CDMP-3/BMP-12
BMP-3/osteogenin	BMP-3/osteogenin GDF-10/BMP-3b
GDFs	GDF-1 GDF-3/Vgr-2GDF-12 GDF-8 GDF-9 GDF-11/BMP-11 GDF-14
Other sub-families/members	BMP-9/GDF-2 BMP-10 Dorsalin-1 (chicken) BMP-15 Screw (Drosophilia) Nodal (mouse) Vg-1 (Xenopus) Univin (sea urchin)

1996, Graff 1997, Ebendal et al. 1998, Wozney 1998, Tsumaki et al. 1999). Localization studies in both human and mouse tissue have demonstrated high levels of mRNA expression and protein synthesis for various BMPs in kidney (BMP-3, -4, -7), lung (BMP-3, -4, -5, -6), small intestine (BMP-2, -7), heart (BMP-2, -4, -6, -7), limb bud (BMP-2, -4, -5 and -7), and teeth (BMP –3, -4 and -7). Rat embryos cultured with anti OP-1 antibodies consistently exhibited a number of abnormalities, including over-all embryo size reduction, reduced facial size and smaller hearts. The majority of these embryos lacked eyes (Solursh et al. 1996). At least some members of the BMP-family are required for proper patterning in the vertebrate limb and in other skeletal structures. BMP-4 (together with Vgr 1) plays a key role in the initial stages of neurogenesis and organogenesis during murine development (Jones et al. 1991).

Histological and biochemical analyses showed that cartilage appears 5–10 days after subcutaneous implantation of active BMP-containing demineralized bone matrix, which mineralizes by day 7–14 and is subsequently replaced by bone (Sampath and Reddi 1983). After 21 days hematopoetic bone marrow formation can be observed (Reddi et al. 1987).

Cartilage Derived Morphogenetic Proteins (CDMPs)

CDMP-1, -2 and -3 are the human equivalents of Growth and Differentiation Factors (GDFs) 5, 6 and 7 (also called BMP -14, -13 and -12). They are closely related to each other with aminoacid homologies of between 80 and 86% (Reddi, 1994). They are also closely related to BMP -5, -6 and -7 with an aminoacid homology of approximately 50% (Chang et al. 1994). CDMPs are predominantly expressed in cartilage, such as the cartilaginous cores of long bones during human embryogenesis and in the joint cartilage in postnatal life (Chang et al. 1994, Storm et al. 1994, Storm and Kingsley 1996). Studies in mice and humans have shown little expression of CDMP-1 in the axial skeleton such as vertebrae and rib. This restricted spatial expression pattern of the CDMP-1 gene suggests that CDMP-1 plays a crucial role in the pattering of the appendicular skeleton, longitudinal bone growth, and chondrogenesis (Tsumaki et al. 1999). CDMP-1 mutations in humans result in shortened limbs and dysmorphogenesis (Storm et al. 1994, Thomas et al. 1997). Similar phenotypes have been observed in the mutated CDMP-1 (GDF5) deficient brachypodism mice (Mikic et al. 2001).

In vitro studies have shown CDMP-2 to be less osteogenic than CDMP-1 in equivalent doses (Erlacher et al. 1998, Gruber et al. 2000). CDMP-1 and -2 equally stimulate de novo synthesis of aggrecan in vitro in a concentration-dependent manner. This activity was equipotent to OP-1, although CDMPs were significantly less stimulatory than OP-1 in osteogenic differentiation as evaluated by alkaline phosphatase activity and expression levels of bone markers. CDMP-2 was the least osteogenic in these assays. The underlying basis for the differential biological responses between CDMP-1, -2 and OP-1 might be their relative affinities for specific receptor complexes (Erlacher et al. 1998).

Conflicting data on bone versus tendon or ligament induction

Conflicting data have been published regarding in vivo effect of CDMP implants. One group implanted collagen granules (guanidine extracted demineralized bone matrix, 75–250 µm) with CDMP-1, 2 and 3 subcutaneously in 4-week-old male rats (Wolfman et al. 1997). They found no induction of cartilage or bone, but formation of a connective tissue rich in collagen type I fibers which, when studied histologically, displayed a wave-form with regular periodicity resembling embryonic or neonatal tendon and ligament. They failed to observe bone formation with implants containing levels of CDMP-1, -2 or -3 that were 20 times higher than normally needed for bone induction with an osteoinductive protein. Thus, 25 µg CDMP-1, -2 and -3 did not induce any bone or cartilage after 10 days (165 subcutaneous implants altogether), whereas 5 µg BMP-2 induced bone and cartilage in 54 out of 54 subcutaneous implants. Controls did not induce bone or cartilage in any implant. BMP-2 combined with CDMP-3 resulted in implants containing both bone and neotendon or ligament-like connective tissue. After 21 days, maturing implants consisted of a densely packed connective tissue composed of collagen fibers that under polarizing light showed the intensity of birefringence and the regular periodicity which is characteristic of tendons and ligaments. CDMPs produced larger amounts of neotendon or ligament-like tissue after 3 weeks intramuscularly, than an equal amount of protein implanted at a subcutaneous site.

In contrast, another group found dose-dependent de novo cartilage and bone formation in an ectopic implantation assay with CDMP-1, -2 and OP-1 (Erlacher et al. 1998). They implanted subcutaneous collagen implants in the thoracic region of 4–5 weeks old rats. The rats were evaluated after 10 and 21 days with histology and alkaline phosphatase activity. After 10 days the implants contained chondrocytes with ongoing de novo mineralization. At 21 days, bone formation was apparent in all implants. Recently, a study of intramuscular injections of first-generation CDMP-2 adenoviral vector in athymic nude rats was presented (Helm et al. 2001). As early as 2 days after injections of Ad-CDMP-2, progenitor cells were observed infiltrating between the transduced muscle fibers. These cells subsequently proliferated, differentiated and secreted large amounts of collageneous extra-cellular matrix. By 100 days post-injection, the treated tissue displayed the histological and ultrastructural appearance of neotendon or neoligament, which was clearly demarcated from the surrounding muscle. Small foci of bone and fibrocartilage were also seen within the treated tissue, whereas the control Ad-beta-gal gene injection sites were found to contain only normal muscle.

Effects of CDMP-1 implants in a wider variety of models and species were published by Spiro (Spiro et al. 2000). Subcutaneous implants in rats on collagen or mineralized collagen carriers lead to a dose-dependently increased alkaline phosphatase activity, indicating increased bone formation. In intramuscular implants, a chondrocytes-like morphology was observed. The ectopic bone formation in response to CDMP-1 was however less than with other BMPs. In baboons they used a 1.5 cm fibular defect model. The animals were treated with four different concentrations of CDMP-1 (0 µg, 22 µg, 220 µg and 2200 µg) on a collagen carrier. After 21 weeks they found bony healing in 4 out of 4 animals treated with the highest dose (compared to 1 out of 4 control animals). In a spinal fusion model in baboons, the animals were treated with CDMP-1 on a mineralized collagen matrix in doses of 5 and 15 mg, or with autograft. CDMP-1 had limited effect in this model. Spines treated with the highest dose showed less fusion than those treated with the lowest dose of CDMP-1 (500 µg) or animals treated with autograft.

So far, CDMP-3 is the least investigated protein of the CDMPs. Recombinant adenovirus mediated CDMP-3 gene transfer, has however been shown to induce tendon- and cartilage-like tissue formation when injected intramuscularly into the thigh muscle in nude mice (Lou et al. 1999). There was no change in alkaline phosphatase activity, indicating no ongoing cell differentiation into the osteoblastic phenotype. The same group has also reported a two-fold increase of tensile strength and stiffness of repaired tendons after a CDMP-3 gene transfer into a complete tendon laceration model in chicken (Lou et al. 2001).

Aims

The major aim of our studies was to improve Achilles tendon healing. The specific aims were:

1. To find out if OP-1 could be used for improving tendon repair.
2. To find out if CDMPs could be used for improving tendon repair.
3. To find an appropriate dose of CDMPs in rats
4. To find out if CDMPs could be delivered by local injections with retained improvement of repair.
5. To describe the extent of cartilage and bone formation induced by CDMPs in repairing tendons.
6. To find out if the amount of cartilage and bone formation was influenced by the mechanical environment
7. To compare the 3 CDMPs as regards potency to improve tendon repair and tendency to cause bone formation.
8. To test the optimal treatment methods developed in rats also in a rabbit model.

Methods

200 g female Sprague Dawley rats were used in all rat experiments. They were intraperitonelly anesthetized with chloral hydrate (4 mg/kg bodyweight).

Rat tendon model (Papers 1–4, 6)

The rat tendon model was adopted and further developed from the rat Achilles tendon model described by Murrell et al. (1994). The Achilles tendon complex was dissected free from other tissues (Figure 5). The paratenon was split longitudinally, the plantaris tendon removed and the Achilles tendon transsected before skin closure. In the original model, Achilles tendon healing was studied in one loaded and one unloaded version. In the unloaded version, the ankle of the leg with a transsected tendon was immobilized by external fixation, which we found traumatic and difficult. The insertion of pins for the external fixation, influenced tendon healing dramatically, even when not fixated (Murrell et al. 1994). Instead we tried to unload the healing tendon by denervation of the calf muscle by tibia nerve transsection. This yields loss of muscle traction on the Achilles tendon, with retention of passive motion. For comparison, we also reduced this muscle force by fore-foot amputation. Rats included in papers 3, 4 and 6 got a larger defect by removing 3 mm of the Achilles tendon in order to delay the healing and thereby increase the possibility of showing an effect of treatment.

Unloading of the rat tendon

Unloading of the Achilles tendon was done using two different methods:

1. Unloading through denervation of the tibia nerve (Papers 1, 2) leaving the peroneal nerve intact.
2. Unloading through fore-foot amputation (Papers 1, 4) (Figure 6).

Administration of growth factors

Administration of growth factors was done using two different methods:

1. *Applying growth factor on a collagen sponge* (Papers 1, 2, 4). 1 × 2.5 × 2.5 mm pieces of collagen (Helistat, Colla-tec, Inc.) were prepared aseptically from larger pieces. The growth factor was dissolved in 6 µL 20 mM Acetate-buffer (OP-1) or sterile water (CDMP) and soaked on the collagen which was then lyophilized before implantation.
2. *Applying the growth factor through a local injection* (Papers 1, 3, 4, 6). Six hours after tendon transsection the growth factor was dissolved in 50 µL 20 mM Acetate buffer and locally administrated into the gap between the tendon ends.

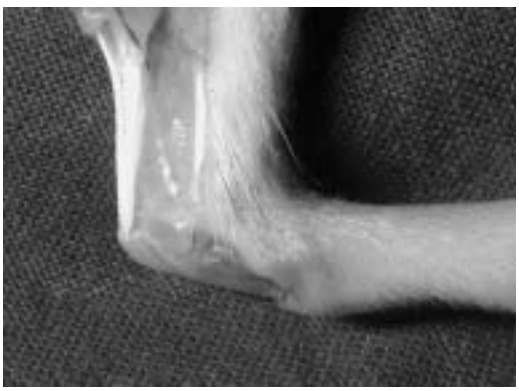


Figure 5. The rat Achilles tendon. Skin has been removed to afford a better view.

Figure 6. A rat 14 days after a fore-foot amputation.

Figure 7. Collagen sponges with 0 or 10 µg CDMP-2 were implanted, subcutaneously on top of the head, and intramuscularly in the abdominal muscle.

Subcutaneous implantation (Paper 4)

After anesthesia, 2 superficial subcutaneous pouches where created at the top of the rats heads. Two collagen sponges, 1 x 1.5 x 1.5 mm prepared as above, were implanted in the pouches and the skin was sutured (Figure 7).

Intramuscular implantation (Paper 4)

Intramuscular implantations were done at the same time in the same rats that received the subcutaneous implants. Bilateral muscle pouches were created by separating two muscle layers. Two collagen sponges of the same size as those subcutaneously implanted were implanted in the muscle pouches before they were closed with a suture (Figure 7).

Rabbit tendon model (Paper 5)

The rabbits were intramuscularly anaesthetized with Hypnorm® (Janssen Pharmaseutica, Beerse, Belgium 0.9 mL /kg bodyweight). The Achilles tendon complex was dissected free from surrounding tissues with the paratenon intact. The paratenon was split longitudinally and carefully loosened from the tendon complex. The Plantaris tendon was removed and the Achilles tendon was transsected. To ensure correct placement of the CDMP-2 injection, a polypropylen tube was placed in the defect (Figure 8). Two hours after the transsection 60 µL of 20 mM acetate buffer, with or without 10 µg CDMP-2, was injected through the tube into the defect. The tube was then removed.

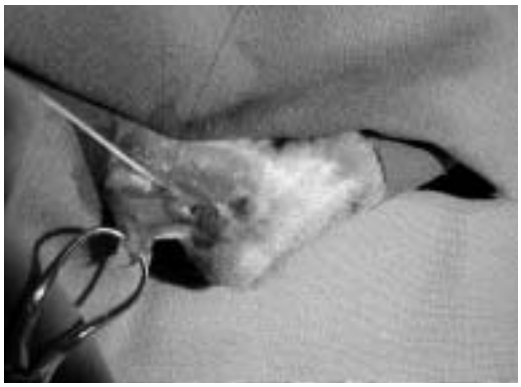


Figure 8. The paratenon is sutured after the polypropylene tube has been inserted in the rabbit Achilles tendon.

Biomechanical testing of the tendons (Papers 1–3, 5, 6)

The tendon was fixed between two metal clamps and pulled at a constant speed of 1 mm/s until failure. The angle between the calcaneus and the Achilles tendon during testing corresponded to 30° dorsiflexion of the foot (Figure 9). Mechanical testing was performed using a materials testing machine (100 R, DDL Inc. Eden Praire, Mn, USA) (Papers 5 and 6). Peak force, stiffness and energy uptake until failure were recorded. A testing

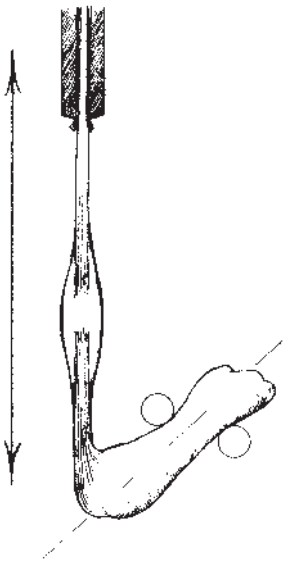


Figure 9. Set-up for tensile strength measurement.

Figure 10. The "home-made" materials testing machine used in the first 3 studies.

machine built for this study was used for the first 3 papers (Figure 10).

Histological evaluation (Papers 1, 2, 4–6)

Specimens taken for histology were fixed in 4% phosphate buffered formaline, decalcified in Parengy's solution (chrometrioxide 1.5 g, nitric acid 5 mol/L 160 g, ethanol 270 g/L) for 3 weeks and finally paraffin embedded. For tendons, 6 µm sections where taken every 0.5 mm through-out the specimens, and the subcutaneous and intramuscular specimens were serially cut all through the specimen. The slides where stained with Heamatoxylin and Eosin and blindly examined in a microscope (Olympus BX 50, Olympus, Japan) using 10 x magnification.

Summary of Papers

Background to own experiments

Our studies were derived from bone research (Wang and Aspenberg, 1993). We have improved bone formation with FGF-2 in a bone chamber model. FGF-2 also greatly increased the amount of fibrous tissue in the chamber. This latter finding led us to believe that FGF-2 could be beneficial for tendon healing. We adopted and modified an Achilles tendon transsection model in rats (Murrell et al. 1994) and started applying FGF-2. We executed a successful experiment, which improved tendon strength by 43% for tendons treated with FGF-2 at a low dose ($p = 0.01$), but were then never able to repeat the results. After many attempts with a variety of doses and regimens, we finally gave up. After FGF-2 we tried TGFß at several doses, which did not work, and finally, we got access to OP-1.

In the first study, OP-1 was added to the healing tendon both on a collagen carrier and as a local injection. The chemical signals from OP-1 overtook the mechanical signals of the tendon environment, and OP-1 lead to bone-formation at the expense of tensile strength in the healing tendon. Although this was not the result we were hoping to achieve, the effect of OP-1 was clear and easy to detect biomechanically as well as histologically. This encouraged us to continue the experiments with a less osteogenic BMP than OP-1. The results of the OP-1 study made it possible for us to carry out experiments with the rather new members of the BMP-family, the CDMPs. Since some studies had shown CDMP-1, -2 and -3 to induce tendon and ligament-like tissue, we were hoping to improve tendon healing with a CDMP.

Paper 1. OP-1 has more effect than mechanical signals in the control of tissue differentiation in healing rat tendons

Does a mechanical signal known to stimulate the differentiation and organization of healing tendon also direct BMP-induced differentiation towards formation of tendon tissue?

92 rats had an Achilles tendon transsection (Table 3). 41 of the rats had the tendon unloaded by denervation and 9 by forefoot-amputation. 76 rats were given 0 or 10 µg OP-1 on a collagen sponge in the tendon defect. The remaining 16 rats were locally injected with 100 µg OP-1, 6 hours after denervation and tendon transsection. 14 days after the operation, the rats were killed and the tendons were biomechanically tested and histologically evaluated for bone or cartilage content.

Treatment with OP-1 decreased tensile strength by 39% ($p = 0.01$) no matter if the tendon was unloaded or not. Unloading through denervation decreased tensile strength by 61% ($p = 0.0001$) independent of OP-1. Unloading through fore-foot amputation decreased tensile strength by 42% ($p = 0.04$). Histolgically, large bony ossicles with a marrow cavity could be seen in the OP-1 treated specimens (Figure 11).

Table 3. Number of animals allocated to the treatment groups in Paper 1. All groups had transsection of the Achilles tendon

Model	Method	Treatment	Count
Transsection only	Collagen	10 µg OP-1	21
Transsection only	Collagen	Control	21
Denervation	Collagen	10 µg OP-1	12
Denervation	Collagen	Control	13
Denervation	Injection	100µg OP-1	8
Denervation	Injection	Control	8
Forefoot amputation	Injection	Control	9

Paper 2. Enhanced tendon healing with GDF 5 and 6

Does a tendon and ligament inductive morphogen such as CDMP-1 or -2 improve tendon healing in an Achilles tendon transsection-model in rats?

67 rats had an Achilles tendon transsection and partial unloading of the tendon with denervation of

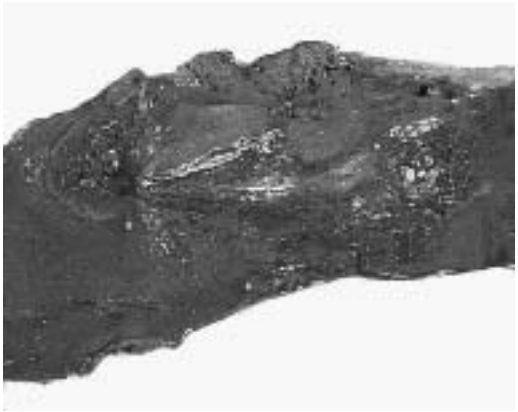
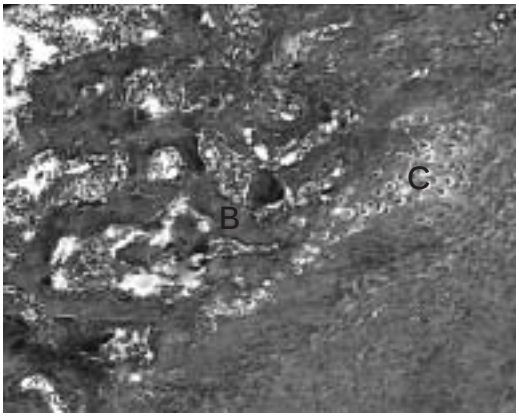


Figure 11. A. Bony ossicle with a marrow cavity in a denervated OP-1 treated specimen.



B. Enlargement of trabecular bone (B) and cartilage (C).

the tibiae nerve. CDMP-1 or -2 in doses 0, 1 or 10 µg was applied on collagen sponges and inserted into the Achilles tendon defect. The rats were killed 14 days after the operation, and the tendons were biomechanically tested and histologically evaluated.

Both CDMP-1 (10 µg) and CDMP-2 (1 and 10 µg) improved tendon healing compared to the control group. The effect appeared to be stronger with CDMP-2, although this was not significant. CDMP-2 improved tendon strength by 47% ($p = 0.02$) in the 10 µg dose. No bone or cartilage was histologically detected.

Paper 3. Tendon healing stimulated by injected CDMP-2

BMPs have been claimed to need some kind of carrier to administrate the protein. Would it be possible to repeat the previous experiment, using a local injection of CDMP-2 instead of a carrier?

50 rats had a tendon transsection with a 3-mm segment resection. 6 hours post operative, the rats were injected with 0, 2, 10 or 50 µg CDMP-2 in an acetate buffer. 8 days after operation the rats were killed and the tendons tested biomechanically.

The tensile strength was improved by 39% ($p = 0.0008$). We found no difference of tensile strength between 2 and 50 µg of CDMP-2 (Figure 12). Possibly the injected CDMP-2 protein could bind to the exposed collagen in the wound similarly to the implanted collagen carrier.

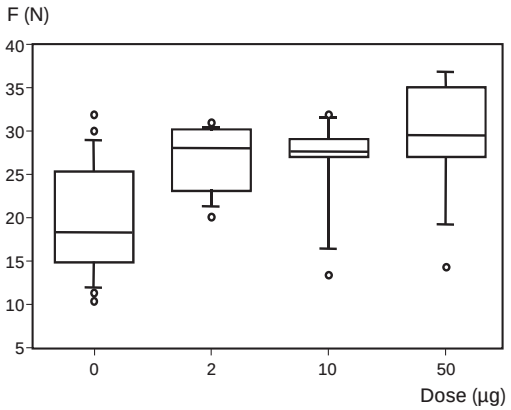


Figure 12. Force at failure (N) 8 days after transsection in tendons injected with buffer or CDMP-2 at doses 2, 10 or 50 µg.

Paper 4. CDMP-2 induces bone or tendon-like tissue depending on the mechanical situation

Does the differentiation of mesenchymal stem cells induced by CDMP-2 depend on the mechanical load at the implantation site?

The hypothesis was that CDMP-2 should cause more bone formation in a less tensile loaded site than in the Achilles tendon. Although different sites in the body have different mechanical load situations, there are also other differences, unrelated to mechanics. The final test of the hypothesis that mechanical load influences the response to CDMP-2 should therefore be to use the same tissue and location, but under different loading

Table 5. Strength, stress, stiffness, energy and transverse area for rabbit tendons treated with or without CDMP-2 after 8 and 14 days (1-way Anova, Scheffe's post hoc test; Paper 5)

Treatment	Days	Strength N mean	SD	Stress MPa mean	SD	Stiffness N/mm mean	SD	Energy Nm mean	SD	Area mm^2 mean	SD
CDMP-2	8	102	18	1.6	0.3	17	2.4	0.58	0.18	63	8
Control	8	85	22	2.6	1.4	16	3.1	0.44	0.18	38	12
p-value		0.6		0.3		1.0		0.2		< 0.0001	
CDMP-2	14	225	30	6.1	1.8	35	9.3	1.26	0.35	41	13
Control	14	167	39	7.8	3.5	26	5.1	1.02	0.25	23	5
p-value		0.008		0.1		0.04		0.05		0.007	

conditions. This was done using one loaded and one partially unloaded version of the Achilles tendon defect model.

67 rats were used for the study. 16 rats received subcutaneous and intramuscular implants with 0 and 10 µg CDMP-2 at both sites. 8 rats were killed after 14 days, and 8 rats after 28 days.

In 60 rats, a tendon transsection with a 3-mm defect was made. 20 of these rats received a collagen implant with 0 or 10 µg CDMP-2. 10 rats were killed after 14 days and 10 rats after 28 days.

The last 40 rats were injected with 0 or 10 µg CDMP-2 6 h after operation and killed after 28 days. 10 rats of each dose had the tendon unloaded via fore-foot amputation. All specimens were histologically examined after the harvest.

Bone-formation was greatest in the subcutaneously implanted CDMP-2 specimens, which was the most unloaded site. This load dependence was confirmed in the tendons, where unloading increased bone and cartilage formation compared to loaded tendons (Table 4).

Table 4. Number of implants with bone or cartilage in the tendon callus per total number of implants with or without CDMP-2 at different sites after 14 and 28 days in Paper 4

Implantation site	Time (days)	CDMP-2 10 µg	Control
Subcutaneous	14	6/7	0/8
	28	7/8	0/8
Intramuscular	14	6/7	1/8
	28	6/7	0/8
Transsected tendon	14	2/5	0/5
	28	2/5	0/5

Paper 5. Improved healing of transsected rabbit Achilles tendon after a single injection of CDMP-2

Is it possible to repeat the results from the rat-tendon study in a larger animal model?

The larger animal would allow us to imitate the patient situation more closely, with the transsected tendon surrounded by the paratenon. Thereby the role of interfering cells from the environment should be limited.

40 rabbits had a tendon transsection. 2 hours after the operation the rabbits were locally injected into the tendon defect with 0 or 10 µg CDMP-2. The rabbits were killed after 8 days (10 from each treatment), 14 days (6 from each treatment) and 56 days (8 CDMP-2 treated). Tendons harvested after 8 and 14 days were biomechanically tested. Tendons harvested after 8 weeks were histologically and radiographically examined to exclude bone and cartilage formation after a longer time.

Tendons treated with CDMP-2 showed improved biomechanical properties (Table 5). No bone or cartilage was seen, neither histologically, nor radiographically (Figure 13) after 8 weeks.

Paper 6. A comparative dose-response study of CDMP-1, -2 and -3 for tendon healing in a rat model

Is there a difference in tendon healing and osteogenesis between the different CDMPs?

This should preferably have been done at the very beginning of the studies, but at that time-point CDMP-3 was not available for us. A tendon transsection was done in 110 rats. 6 hours after

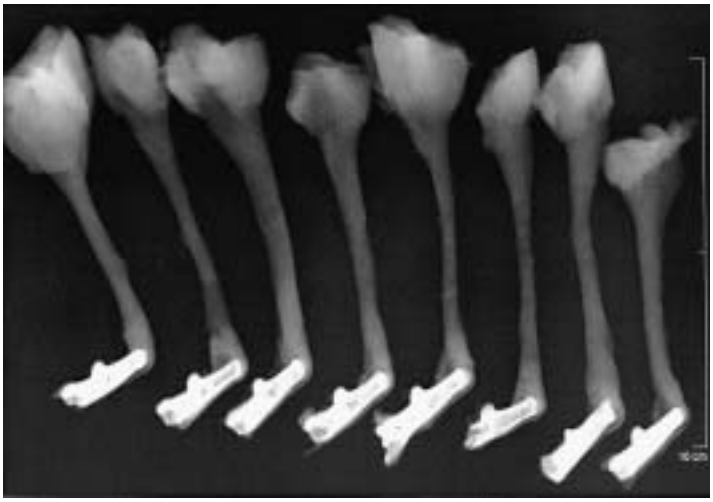


Figure 13. Healed rabbit Achilles tendons treated with CDMP-2, 8 weeks after transsection. Note absence of bone.

operation they were injected with 0, 0.4, 2 or 10 µg CDMP-1, 2 or 3 with 10 rats in each group (one group of 10 rats was not injected at all) and killed after 8 days for biomechanical tests and histological evaluation. Another 50 rats were operated on in the same way and then injected with buffer or 10 µg of CDMP-1, 2, 3 or OP-1. These rats were killed after 28 days and examined histologically to compare osteogenecy.

There was no significant biomechanical difference between the different CDMPs. There was no effect with the lowest dose of any of the CDMPs. After four weeks, specimens treated with OP-1 and CDMP-3 contained most bone. Specimens treated with CDMP-2 contained less bone than CDMP-1 and –3, but differences were not significant.

Results and Discussion

Achilles tendon rupture is a common injury, not only in humans but also in veterinary medicine, where treatment with CDMPs may become a complement to current treatments. Non-operative treatment of Achilles tendon rupture has shown results comparable to surgery, and with the injection of a factor like CDMP-2, it might even become better. CDMP-2 improves tendon callus strength by over a third after 8 days in rats. When considering the difference in metabolic activity, 8 days in rats would correspond to about 4 weeks in humans.

There is of course a big difference between transsected tendons in rats and rabbits, and ruptured tendons in humans. First of all, the tendons in this model are cut off sharply, leaving the tendon stumps with clean endings. The naturally ruptured tendon has frayed tendon ends. The larger exposed area of collagen in frayed tendon ends may actually be beneficial in a possible future treatment of Achilles tendon rupture with CDMP-2, since it may increase the ability to bind CDMP-2 after injections into the ruptured defect. Another difference in the tendons studied in our models, compared to the clinical situation, is the lack of degenerative changes. Healing occurs in healthy tendons with no degenerative history. This in contrast to reported clinical situations, where spontaneously ruptured tendons have pathological changes of degenerative origin (Kannus and Jozsa 1991).

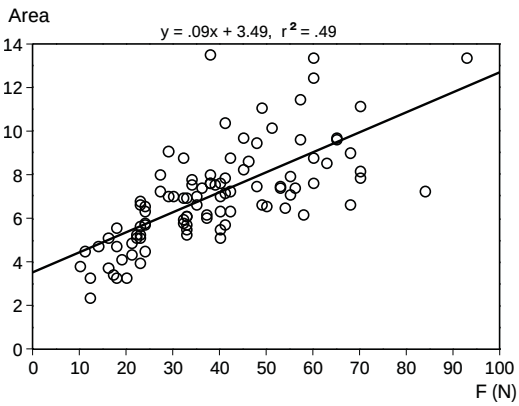


Figure 14. Relation between transverse area and strength.

In the first study with CDMP (Paper 2), we discussed that CDMP could stimulate the differentiation of mesenchymal stem-cells towards tendon tissue. Later results seem more to indicate an increased cell proliferation, leading to a larger callus mass, seen as an increased diameter of the healing tendon after treatment with CDMP, leading to an increase in tensile strength (Figure 14).

Prior to the third study we carried out a time-sequence study with CDMP-2 on a collagen carrier to establish the time-point when the effect of CDMP-2 was the greatest. The tendons were studied histologically at various time-points between 2 and 57 days. The transverse area increased until 8 days after transsection and started thereafter slowly to decrease. Tendons harvested 14 days and later sometimes contained minor cartilage or bone nodules (Table 6). There was no significant difference between CDMP-2 treated tendons and controls regarding bone and cartilage formation, even though there was a tendency for cartilage or bone to be found more often in CDMP-2 treated specimens than in control specimens. The histolog-

Table 6. Number of implants with bone or cartilage in the callus 2–57 days after Achilles tendon transsection.

Treatment	Time	Count	Bone/cartilage
CDMP-2	2	2	0
Control	2	2	0
CDMP-2	5	3	0
Control	5	3	0
CDMP-2	8	3	0
Control	8	3	0
CDMP-2	11	3	0
Control	11	3	0
CDMP-2	14	7	3
Control	14	3	0
CDMP-2	17	3	3
Control	17	3	0
CDMP-2	28	3	2
Control	28	3	0
CDMP-2	42	3	2
Control	42	3	0
CDMP-2	57	5	1
Control	57	5	1

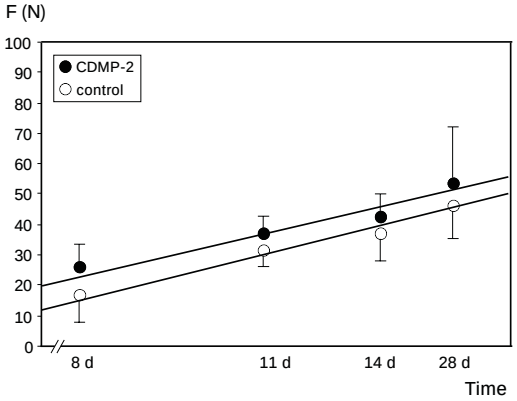


Figure 15. Force at failure in tendons treated with 0 or 10 µg CDMP-2 on a collagen carrier 8, 11, 14 and 28 days after transsection.

ical study was followed by a biomechanical study where the tendons were tested after 8, 11, 14 and 28 days. In the time-sequence study we could see that healing time was reduced by about one third after treatment with CDMP-2. The results of this unpublished study made us shorten harvest time from 14 to 8 days, where the effect of CDMP-2 seemed to be the largest (Figure 15).

Since rats may form bone spontaneously during tendon healing (Rooney et al. 1992), we wanted to evaluate whether the observed tiny nodules of cartilage and bone were a result of CDMP, or just a natural part of the healing process in the Achilles tendon. In Paper 4, implants with CDMP-2 yielded different responses at different implantation sites. To be able to draw the final conclusions on whether the results were due to load or to environment, transsected tendons with a normal high load were compared to partly unloaded transsected tendons. A decrease in load lead to an increase in bone formation, indicating that bone and cartilage formation in tissue exposed to CDMP-2 is a result of the local load, rather than the origin of the surrounding tissues. These findings extend the mechanical differentiation theories by Plötz (1938), Pauwels (1960) and Carter (Carter et al., 1996) saying that an undifferentiated mesenchymal tissue develops into fibrous tissue, cartilage or bone depending on the mechanical loading situation. Not only is the mechanical environment important under normal remodeling and repair, but also to some extent to the response to exogenous growth factors. OP-1, a well known bone-inducer, also induced bone in our model (Forslund and Aspenberg 1998). The response to OP-1 was even stronger than the effect of the mechanical environment since bone was formed in all rats, in a loaded as well as in an unloaded Achilles tendon transsection model. Strangely this was not the case in the last study where 10 rats were injected with OP-1 and bone was found only in 6 of the rats after 4 weeks (Paper 6). In the first study, 10 µg OP-1 was administered on a collagen sponge in the Achilles tendon defect, or 100 µg OP-1 was given as a local injection in the tendon defect. The injected rats received a higher dose than the rats with collagen implants because we had not used the injection technique before and were not certain if the injected solution would stay in the area. The resulting bone-formation in these rats (regardless of way of administration) was always dramatic with large ossicles occupying most of the callus volume. In the last study, the rats were injected with the same dose as the different CDMPs, which was also the same dose as previously used on the collagen implants (10 µg). The lower dose of OP-1 may be the reason for the reduced frequency of bone formation in the last study.

Even though early rehabilitation after an Achilles tendon rupture is often recommended (e.g. Cetti et al. 1994, Mortensen et al. 1999, Möller et al. 2001, Saleh et al. 1992), unloading of the tendon after an Achilles tendon rupture in human patients is still the most common practice, no matter if the ruptured tendon was surgically or non-surgically treated. Since we found that CDMP-2 is more osteoinductive in an unloaded environment than in a loaded environment, early rehabilitation would minimize a risk for ossification of the tendon after CDMP-2 treatment.

The absence of cartilage or bone in the rabbit study may seem reassuring, but one must keep in mind that the tendons in this model were not immobilized, and therefore further experiments with injections of CDMP in an unloaded model need to be performed. For this reason, we have recently developed a model where the patellar tendon in the rabbit is transsected and unloaded with wires fixated in the patella and tibia.

After having made several studies, mostly on CDMP-2, we finally had the opportunity to compare all 3 CDMPs. Two questions were addressed;

would there be differences in the improvement of tendon regenerate biomechanics, and would the osteogenic properties be the same. In Paper 2 we found trends towards CDMP-2 being more efficient than CDMP-1 (p=0.1 analyses not shown). We therefore chose to concentrate further studies of the effects on CDMP-2. However, there were several reasons to suppose that CDMP-1 and CDMP-3 should also be efficient for stimulating tendon repair. In CDMP-1 deficient brachypodism mice, the Achilles tendon has been shown to be 50% weaker than the controls (Mikic et al. 2001), so CDMP-1 may have a beneficial effect also on tendon healing. CDMP-3 has been shown to be a tendon and ligament inducer (Wolfman et al. 1997) and a tendon and cartilage-like tissue inducer (Lou et al. 1999). CDMP-3 has been speculated to be the primary tendon and ligament-like tissue inducer among the different CDMPs.

Regarding the osteogenetic effect of CDMPs, comparisons have previously been made between CDMP-1 and -2. Evaluated by Alkaline phosphatase activity, CDMP-1 has been found to be more osteogenic than CDMP-2 (Erlacher et al. 1998, Gruber et al. 2000). CDMP-1 has been shown to be localized in cells at ossified sites (Nakase et al. 2001). CDMP-3 has on the contrary only been shown to influence the cell differentiation into a non-osteoblast lineage (Lou et al. 1999). Therefore we were surprised to find that CDMP-3 as well as CDMP-1 tended to be more osteogenic than CDMP-2, as evaluated with histology and Ca analyses. All 3 CDMPs showed responses ranging from no cartilage or bone, to obvious bone ossicles. Even though no significant differences were found, it is obvious that, at least in our model, CDMP-3 can not be said to be less osteogenetic than the others to a clinically relevant extent.

CDMPs may also be used in situations other than Achilles tendon rupture. Recovery after tendinosis may be accelerated with injections of CDMP. Since tendons injected with collagenase (Gehlsen et al. 1999), carrageenan (Kurtz et al. 1999) or PGE1 (Sullo et al. 2001) in rats develop symptoms of tendinosis, it would be interesting to evaluate the effect of CDMP-2 in one of these models.

In contrast to earlier belief, we could show that CDMP-2 did not need a collagen carrier. The locally injected CDMP-2 might bind to the exposed collagen of the tendon ends (Paper 3), which makes it easy to work with in the clinical situation. We believe that conservative treatment of Achilles tendon rupture with injections of CDMP in combination with early rehabilitation might afford a good alternative to surgical treatment.

Conclusions

OP-1 has a detrimental effect upon tendon repair in rats. A lot of bone was produced at the expense of tensile strength.

CDMP-2 improves tendon healing in rats and rabbits mainly by increasing tendon callus mass.

CDMP-2 can be administrated by a local injection without carrier.

CDMP-2 induces more bone and cartilage in a mechanically unloaded environment than in a loaded environment.

In our model, the different CDMPs had similar effects on the biomechanical properties of healing tendons, and no significant differences in cartilage or bone formation could be found.

Thesis at a glance

Study	Species	Tendon model	Treatment	Dose (µg)	Time (days)	Evaluation	Conslusion
1	Rat	Transsection ± denervation	OP-1 collagen, OP-1 injection	10 100	14	Biomechanics Histology	OP-1 induced bone formation at the expence of tensile shength
2	Rat	Transsection + denervation	CDMP-1 collagen, CDMP-2 collagen	1 10	14	Biomechanics	CDMP-1 and -2 improved tendon healing
3	Rat	Defect	CDMP-2 injection	2 10 50	8	Biomechanics	Injected CDMP-2 improved tendon healing. No dose-dependency
4	Rat	Defect ± fore-foot amputation	CDMP-2 collagen, CDMP-2 injection	10	14, 28	Histology	Loading prevents bone formation by CDMP-2
5	Rabbit	Transsection	CDMP-2 injection	10	8, 14, 56	Biomechanics Histology	CDMP-2 improved tendon healing also in rabbit. No bone formation
6	Rat	Defect	CDMP-1 injection, CDMP-2 injection, CDMP-3 injection, OP-1 injection	0.4 2 10	8, 28	Biomechanics Histology	No large difference between the CDMPs

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