Linköping University Medical Dissertations No. 1005

Stimulation of tendon repair by platelet concentrate, CDMP-2 and mechanical loading in animal models

Olena Virchenko

Linköping University
FACULTY OF HEALTH SCIENCES

Division of Orthopaedics, Department of Neuroscience and Locomotion
Faculty of Health Sciences, SE-581 85 Linköping Sweden

Linköping 2007

Drawings by Per Aspenberg

Printed in Sweden by Liu-Tryck, Linköping 2007
ISBN: 978-91-85831-88-3
ISSN: 0345-0082

Contents

Abstract

Growth factor delivery may be useful to accelerate the rate of tendon healing. We studied Platelet Concentrate, which in effect can be regarded as a cocktail of growth factors relevant for tendon healing. In a rat Achilles tendon transection model, one postoperative injection of Platelet Concentrate resulted in increased strength even 3 weeks later.

Mechanical stimulation improves the repair of ruptured tendons. We studied the effects of platelets upon Achilles tendon regenerates in rats 3, 5 and 14 days after transection, either unloaded or mechanically stimulated. At 14 days, physical activity and platelets increased repair independently. Unloading decreased the mechanical properties of the repair tissue to less than half of normal. Moreover, the platelets had no effect without loading.

Thrombin, which we used for platelet activation, improved healing of the rat Achilles tendon by itself. Conversely, continuous inhibition of thrombin by low molecular weight heparin (LMWH) inhibited tendon repair. However, intermittent inhibition, similar to clinical thromboprophylaxis, had no effect on tendon healing.

Cartilage Derived Morphogenetic Protein-2 (CDMP-2) can improve tendon healing in loaded defect models. We now studied unloaded repair in a rabbit patellar tendon model. Two hours postoperative, the rabbits received CDMP-2 injected into the haematoma. The healing tendon became 65 % stronger than controls. We then studied Achilles tendon healing with CDMP-2 injections in sheep, to get a bigger animal model. There was an unexpectedly high variation of repair in these animals, and the study turned out to be underpowered. Spontaneous ruptures in humans have a more variable geometry than in our sheep model, so humans can also be expected to vary a lot in mechanical characteristics of Achilles tendon repair. This accentuates the importance of individualized rehabilitation programs.

In conclusion, both platelet concentrate and CDMP-2 injections might be of interest for clinical use as a complement to surgical or conservative treatment of tendon ruptures. Platelet treatment for tendon ruptures should probably be combined with early physiotherapy.

List of Papers

I. Aspenberg P, Virchenko O. Platelet concentrate injection improves Achilles tendon repair in rats. Acta Orthop Scand. 2004; 75: 93-99

II. Virchenko O, Grenegård M, Aspenberg P. Independent and additive stimulation of tendon repair by thrombin and platelets. Acta Orthop Scand. 2006; 77: 960-6

III. Virchenko O, Aspenberg P. How can one platelet injection after tendon injury lead to a stronger tendon after 4 weeks? Interplay between early regeneration and mechanical stimulation. Acta Orthop Scand. 2006; 77: 806-812

IV. Virchenko O, Lindahl T, Aspenberg P. Low Molecular Weight Heparin impairs tendon repair. Submitted manuscript.

V. Virchenko O, Skoglund B, Fahlgren A, Aspenberg P. CDMP-2 injection improves early tendon healing in a rabbit model for surgical repair. Scand J Med Sci Sports. 2005; 15: 260-264

VI. Virchenko O, Fahlgren A, Rundgren M, Aspenberg P. Early Achilles tendon repair in sheep. Submitted manuscript.

This thesis was supported by the Swedish National Centre for Sports Research, “Materials in Medicine”, Linköping, Sweden, and the Swedish Research Council, project 2031.
During the course of the research underlying this thesis, Olena Virchenko was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

Abbreviations

BMP	Bone Morphogenetic Protein
COX-2	cyclooxygenase-2
CDMP-2	Cartilage Derived Morphogenetic Protein-2
ECGF	Epithelial Cell Growth Factor
EGF	Epidermal Growth Factor
FGF-2	Fibroblast Growth Factor-2
IGF	Insulin-like Growth Factor
LMWH	Low Molecular Weight Heparin
PC	Platelet Concentrate
PPP	Platelet-Poor Plasma
PDGF	platelet-derived growth factor
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor

Introduction

Tendons are soft connective tissues consisting of parallel collagen fibres embedded within an extracellular matrix. Tendons connect muscle to bone and transmit tensile force generated by muscles to move and stabilize joints. They must be capable of resisting high tensile forces with limited elongation (Best et al., 1994; Buckwalter, 2000). There are some difficulties to measure in vivo force of the Achilles tendon. Komi et al. were first to use an implanted buckle force transducer to study the in vivo forces of the Achilles tendon in humans during different activities (Komi, 1990; Komi et al., 1992; Komi et al., 1987). During cycling, a force of less than 1000 N was produced, during slow walking 2600 N and running 9000 N. The force produced while running corresponds to more than 12 times the weight of the human body. When expressed per cross sectional area the value was 11100 N/cm² (11 MPa). It is obvious that during sports, the tendon is exposed to high stress for a long time, which can lead to overuse.

Achilles tendon rupture affects middle-aged adults and is associated with prolonged periods off work and much longer abstinence from sports. Tendon injury is generally considered to be acute or chronic and direct or indirect. Direct acute tendon injuries occur due to laceration by a sharp object. Indirect injuries are the result of acute tensile overload or overuse. Achilles tendon ruptures can be total or partial.

Total Achilles tendon ruptures most often occur in physically active people (average age 40 years). The exact reason why the Achilles tendon ruptures is not known. Two main theories are advocated. According to one, the “degeneration theory”, repeated high loading of the tendon causes microscopic damage between fibrils, hypovascularity and chronic degeneration, which leads to a complete rupture without excessive loads being applied (Ahmed et al., 1998; Carr and Norris, 1989). Kannus and Jozsa reported a histological study of 891 ruptured tendons and 445 healthy controls from individuals killed in accidents. Completely healthy structures were not seen in any of the spontaneously ruptured tendons. Most changes were of a degenerative type (97%). Degenerative changes were also found in 149 control tendons (34 %), which is significantly less. Kannus and Jozsa concluded that degenerative changes may be common in people over the age of 35 years, and that this may predispose to rupture (Kannus and Jozsa, 1991).

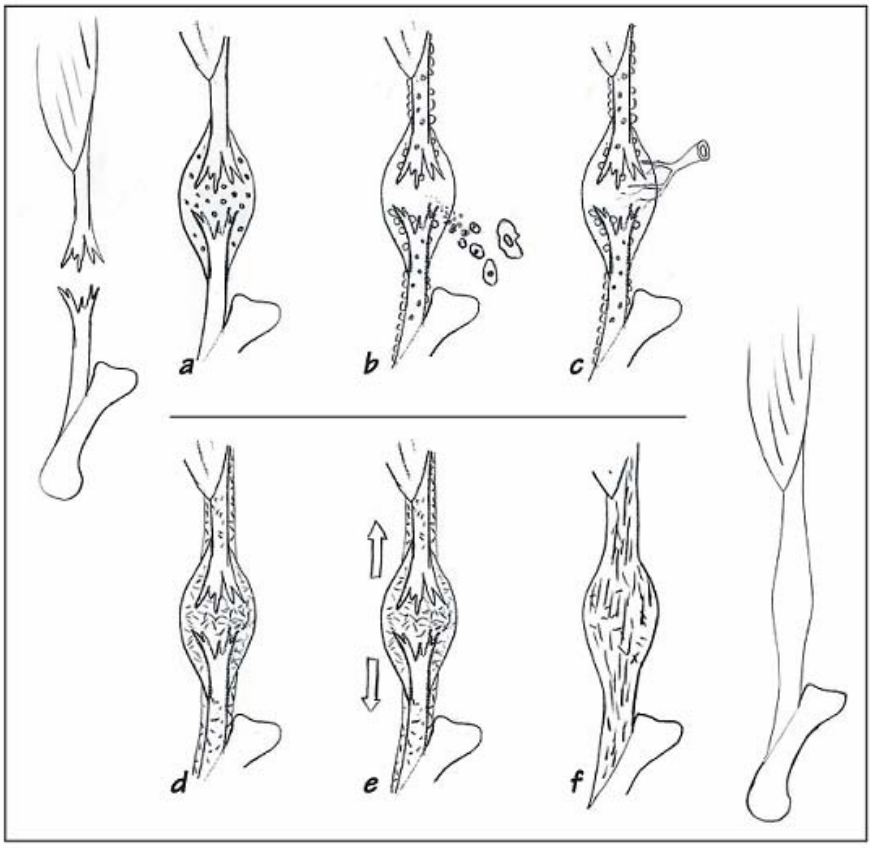

Figure 1. Scheme of tendon repair.
a) Haematoma with platelet activation.
b) Invasion of cells and proliferation of paratenon.
c) Vascular and neuronal ingrowth.
d) Loose collagenous callus formation.
e) Mechanical stimulation.
f) Maturation and remodelling.

According to the "mechanical theory", the Achilles tendon ruptures due to malfunction of the normal inhibitory mechanism of the musculotendinous junction. Barfred suggested that Achilles tendon ruptures can occur in a normal tendon if an excessive load is applied (Barfred, 1973). Another study shows lipid degeneration in the calf muscles of patients who had Achilles tendon rupture. Those patients had been inactive for a period of time before their Achilles tendons ruptured (Hoffmeyer et al., 1990).

The incidence of Achilles tendon rupture is increasing. Jozsa et al. showed that 59 % of ruptures were sustained during sports activities, with a predominance of males (Jarvinen et al., 2005; Jozsa, 1997; Jozsa et al., 1989).

There is a discussion going on about the choice between either surgical or non-surgical treatment of ruptured tendons. The goal of surgical treatment is the apposition of the tendon ends, which is achieved by a simple end-to-end suture. Surgical treatment brings forth better functional results if compared with non-operative treatment (Amendola, 2002; Bhandari et al., 2002; Moller et al., 2001). The most problematic complications are infections, which may lead to re-ruptures (Amendola, 2002; Khan et al., 2005; Pajala et al., 2002). Khan et al. reviewed twelve trials involving 800 patients with acute Achilles tendon rupture and concluded that "open operative treatment significantly reduces the risk of re-rupture compared with non-operative treatment, but operative treatment is associated with significantly higher risk of other complications". Patients with early mobilization tended to have a lower re-rupture rate (Khan et al., 2005).

The complication rate has been the parameter of major interest in most retrospective studies. Deep venous thrombosis, pulmonary embolism, infection, skin necrosis and re-rupture was mentioned. Lengthening of the Achilles tendon during repair is not uncommon (Cetti et al., 1993; Mortensen et al., 1999). One of the important complications is re-rupturing, and its rate differs dramatically between reports. Möller et al. reported re-ruptures in 21 % of 53 non-surgical patients. In contrast, a consecutive material of 196 cases with non-operative treatment showed only 7 % re-ruptures (Ingvar et al., 2005). This might reflect differences in physiotherapeutic mobilisation regimes (Table 1).

Table 1: Re-rupture rates.

Article	Number of patients	Surgical treatment	Non-surgical treatment
Cetti et al., 1993	4083	1.4 %	13.4 %
Lo et al., 1997	19 trials	2.8 %	11.7 %
Möller 2001	112	1.7 %	20.8 %
Khan et al., 2005	12 trials, 800	3.5 %	12.6 %

Mechanical stimulation

Training increases stiffness and transverse area of tendons (Kannus et al., 1997). Studies on humans show that well trained individuals have a thicker tendon than controls (Maffulli and King, 1992), and long distance runners had an Achilles tendon with a larger cross-sectional area than untrained subjects, as determined by MRI (Magnusson and Kjaer, 2003; Rosager et al., 2002). The stiffness of the human Achilles tendon was obtained in vivo by measuring tendon elongation upon loading-unloading induced by muscle contraction-relaxation in six men. The elongation of the tendon increased parallel with the force acting upon it (Maganaris and Paul, 2002). Reeves et al studied the effect of controlled strength training of the patellar tendon in humans for 14 weeks. Tendon stiffness, as calculated from the gradient of the estimated force-elongation relationship, was increased by training. Mechanical hysteresis calculated as the area between loading-unloading curves, was decreased by training (Reeves et al., 2003). Collagen fibres in a human Achilles tendon subjected to 15 weeks of immobilisation were found to be thinner and disoriented (Jozsa, 1997; Kannus et al., 1997).

Experimental studies have shown a positive effect of mechanical loading on tenocyte function. Repeated loading increased protein synthesis in human tenocytes (Almekinders et al., 1995). Even a quarter minute of cyclic biaxial mechanical stimulation on isolated human tenocytes resulted in proliferation (Zeichen et al., 2000). Banes et al. studied gene expression from either isolated and stimulated or whole flexor digitorum profundus tendons from exercised chickens. The results show that load induced the expression of novel genes as well as some known to be important for tendon healing (Banes et al., 1999). Another study shows that tendon cells are linked via actin-associated adherence junctions along the line of strain. Under load, they appear to attach themselves more strongly together, and the stress fibres can provide an active mechanism for recovery from stretch (Ralphs et al., 2002).

Most studies in this area are performed on animal models, but comparisons between trained and untrained animals can be misleading. Confined to cages, animals probably have a lower quantity of extracellular matrix and a lower force at failure. After physical training they just regain the normal biological parameters of their species (Clark et al., 2000). Postoperative immobilization and mobilization periods in animals cannot be directly compared to a human situation, because at least small animals have faster metabolism and they are four-footed. There are many factors that play an important role in tendon healing, for example genetic

background. Wang et al. compared A/J, B6 and C3H mice found that genetic background plays a critical role in normal tendon growth and development (Wang et al., 2006).

There are not so many studies on the mechanics of tendon repair, because of the difficulties associated with mechanical testing. The tendon has to be fixed in clamps or via the attached bone. There are problems of tendon slippage in the clamp and bone fracture during the mechanical tests. Some groups use glue or sand paper, other a cryo-clamp to fix the soft tissues during the tests. An alternative to mechanical testing is to use histological analysis, but still the mechanical testing has a great importance, as it tests the function of the tendon.

There are many animal models, both loaded and unloaded, which have been used to study the role of mechanics for tendon maintenance or healing. One of them is hindlimb suspension, developed by Morey and used by Almeida-Silveira et al. to study changes in mechanical properties in the unloaded rat Achilles tendon. After 21 days, the stress at failure was 38 % and tangent modulus 41% lower than in tendons of control rats. The maximal strain was similar in both groups (Almeida-Silveira et al., 2000). In another model, a cerclage wire was installed between the patella and the tibial tubercule in rabbits to study the effect of stress shielding. Studies with this model show that complete stress shielding markedly decreased the tensile strength of the patellar tendon (Ishida et al., 1996). Yamamoto et al. used the same model and studied the effects of re-stressing on the mechanical properties and morphology of stress-shielded rabbit patellar tendons. The tendons were unloaded for 1 to 3 weeks and then loaded for the subsequent 3 to 12 weeks. The mechanical properties of the tendons were not completely recovered even after such a prolonged period of re-stressing. The microstructure of the tendon was only partially restored by re-stressing (Yamamoto et al., 1996). After 1 week of stress-shielding, the stress at failure was decreased by 50 percent of control values (Yamamoto et al., 2000), and 3-weeks of stress-shielding decreased the modulus of elasticity and stress at failure to only 9 percent of control values (Yamamoto et al., 1996). These studies only measured material properties and some changes caused by e.g. oedema may not have influenced the force at failure or stiffness of the whole tendon as much as the material properties.

A fiberglass cast is another model for immobilisation for rabbit Achilles tendons. The cast extends from mid-thigh to the toes, spanning the fully flexed knee and ankle held in 45° of plantarflexion so that the calf muscles were in a shortened position. Immobilisation for 4 and

8 weeks caused a decrease in tendon force at failure, stiffness and energy, if compared with controls, but no significant difference was found between 4 and 8 weeks (Matsumoto et al., 2003). Another group used external fixation with Kirschner wires through the femur, tibia, calcaneus, and forefoot connected by triangular frames to study the influence of immobilisation and mobilisation on the functional and mechanical outcome of Achilles tendon healing in rats. After 12 days of fixation, the tendons were twice as deformable and half as stiff as the sham-operated Achilles tendons (Murrell et al., 1994). Palmes et al. studied the long-term effects of postoperative immobilisation as opposed to mobilisation of the Achilles tendon in a mouse model. The transected and sutured tendons were immobilised by fixing the upper ankle joint in equinus position. A cerclage was inserted through the tibiofibular fork and fixed between the calcaneus and the plantar aponeurosis. The were two groups, the immobilisation group had the Achilles tendon unloaded by the cerclage pulled tight so as to put the foot in the equinus position. In the mobilisation group the cerclage was longer, allowing a limited degree of movement. Postoperative mobilisation resulted in significantly more rapid restoration of load to failure in comparison to the immobilisation group after the operation. Mobilised tendons were more mature, with a more parallel fibre bundle arrangement (Palmes et al., 2002).

A partial tenotomy (window) in the extensor digitorum longus in rats is another model to study tendon healing and the role of mechanical loading and unloading. In one study by Iwuagwu et al., specimens were harvested at intervals of 6 hours 1, 3, 5, and 7 days for microscopic cross sections of the window and tendon substance together with recording of cell orientation. In this model one has a possibility to study loaded and unloaded tendons simultaneously. The study demonstrated that the cellular response after injury was affected by tensile loading, with increased cell numbers in both the window and tendon substance of the unloaded tendon (Iwuagwu and McGrouther, 1998). Enwemeka studied the role of functional loading of regenerating rabbit Achilles tendons. First, tendons were surgically tenotomized, repaired and immobilised by plaster/fiberglass casts that the kept ankle fully plantar-flexed and the knee fixed at 90°. Thereafter, loading was initiated by removing the casts after 5 days. At 12 and 18 after surgery, functional loading induced a twofold increase in force at failure and energy uptake compared to controls with prolonged cast immobilization (Enwemeka, 1992). Running exercises improved the strength of repairing Achilles tendons in rats, but not swimming (Murrell et al., 1998; Ng et al., 2004). This suggests that tendon stress may have to reach a rather high level for stimulation to occur, or perhaps that the strain rate is critical.

Table 2: Expression of growth factors in normal tendon.

Factor	Normal tendon		
	Method	Model	Referens
VEGF	ELISA	Human AT	Jackson et al., 1997
	ELISA	Human AT	Pufe et al., 2001; Pufe et al., 2003; Pufe et al., 2005
	ELISA, Western blots, RT-PCR, IHC	Cell culture of rats AT fibroblasts	Petersen et al., 2004
IGF-I	PCR, IHC	Human AT postmortem	Olesen et al., 2006
	IHC	Chicken flexor tendons	Tsuzaki et al., 2000
FGF-2	IHC	Rabbit flexor tendons	Chang et al., 1998
	Heparin-Sepharose Chromatography, Western Immunoblotting	Canine flexor tendons	Duffy et al., 1995
IGF-I, TGF-β-1	IHC, ELISA, PCR, in situ hybridization	Horse flexor tendon	Dahlgren et al., 2005
BMP-12	IHC	Healthy human Patellar tendon	Fu et al., 2003
GDF-5	Gene deletion	Brachypodism mouse with GDF-5-mutation	Mikic et al., 2001
	Gene deletion	Brachypodism mouse3, 5, 7, 9, 11, 14, 28, 42 days	Chhabra et al., 2003

IHC-Immunohistochemistry

Tendon healing and growth factors

Tendon healing involves a large number of complex pathways, where cytokines play a critical role. The process if often described as progressing through three phases, such as inflammation, proliferation and remodelling. This involves a complex pattern of different gene expressions. Discovery of novel genes involved in different phases of healing might lead to new therapies to be applied during these phases.

Which genes are present in a normal tendon and which are expressed directly after rupture? Many groups have tried to answer this question (Table 2). A cascade of secreted growth factors plays an important role in healing. During tendon damage, blood vessels rupture and release blood cells, which, together with the tendon cells, start the inflammatory phase. Upon injury, platelets release growth factors, which are involved in cell growth and differentiation, including PGDF, TGF- β, IGF-I, EGF, ECGF, VEGF, FGF-2. Notably, also BMPs are released from platelets (Sipe et al., 2004). This is of special interest, because proteins of the BMP family, such as the CDMPs, can be used to stimulate tendon repair (see below). Several growth factors have been identified as playing roles in tendon healing, improving soft tissue and bony wound healing, and, when delivered exogenously, stimulate collagen production, improve wound strength, and initiate callus formation (Tables 3 and 4).

CDMP-2

Cartilage-derived morphogenetic proteins (CDMPs) are a subgroup of the bone morphogenetic protein (BMP) family, which are multi-functional growth and differentiation factors. BMPs are important regulators of bone formation during embryogenesis, postnatal growth and remodelling, as well as in regeneration (Chang et al., 1994). It has previously been found that BMPs are expressed during early tendon formation (Macias et al., 1997) and that a single injection of CDMP can improve early tendon callus formation (Forslund and Aspenberg, 2001; Forslund and Aspenberg, 2002). Forslund et al. found no important differences between CDMP-1, -2, and 3 regarding the effects on Achilles tendon healing in rats (Forslund et al., 2003). Mechanical stimulation is of great importance for tissue differentiation and tendon repair. Without this stimulus, CDMP's in rats induce bone or cartilage formation rather than a tendon-like tissue (Forslund and Aspenberg, 2002).

A mutation of the GDF-5 (CDMP-1) gene results in brachypodism in mice (Storm et al., 1994) and chondrodysplasia in humans (Thomas et al., 1996). In brachypodism mouse, both uninjured and repairing tendons show inferior mechanical properties (Mikic, 2004; Mikic et al., 2001), and there is delayed Achilles tendon healing (Chhabra et al., 2003).

Table 3: Expression of growth factors in injured tendon.

Factor	Injured tendon			
	Method	Model	Time	Referens
VEGF	Quantitative Northern blot	Dogs FDP	4, 7, 10, 14 d	Boyer et al., 2001
	IHC, RT-PCR	Sheep ACL	6, 12, 24, 104 w	Petersen et al., 2003
	IHC	Human AT	2 h	Pufe et al., 2001
IGF-I	IHC	Rats AT	1, 2, 3, 5, 7, 10, 14, 28 d	Hansson et al., 1988
	IHC	Rabbit supraspinatus tendon	1, 3, 5, 7, 9, 11, 14, 21, 28 d	Kobayashi et al., 2006
EGF	Heparin-Sepharose Chromatography	Canine flexor tendons	3, 10, 17 d	Duffy et al., 1995
PDGF	IHC	Rabbit ACL and MCL	7, 14 d	Lee et al., 1998
	Heparin-Sepharose Chromatography	Canine flexor tendons	3, 10,17 d	Duffy et al., 1995
	IHC	Rabbit supraspinatus tendon	1, 3, 5, 7, 9, 11, 14, 21, 28 d	Kobayashi et al., 2006
TGF-β	IHC	Rabbit ACL and MCL	7, 14 d	Lee et al., 1998
	IHC, in situ hybridization	Rabbit flexor tendon	1, 3, 7, 14, 28, 56 d	Chang et al., 1997
	IHC	Rabbit supraspinatus tendon	1, 3, 5, 7, 9, 11, 14, 21, 28 d	Kobayashi et al., 2006
FGF-2	IHC	Rabbit ACL and MCL	7, 14 d	Lee et al., 1998
	IHC	Rabbit flexor tendon	1, 3, 7, 14,28, 56 d	Chang et al., 1998
	IHC	Rabbit supraspinatus tendon	1, 3, 5, 7, 9, 11, 14, 21, 28 d	Kobayashi et al., 2006
IGF-I, TGF-β-1	IHC, ELISA, PCR, in situ hybridization	Horse flexor tendon	1, 2, 4, 8, 24 w	Dahlgren et al., 2005

Platelet Concentrate

Another possibility for tendon healing is to use a cocktail of growth factors. For example, IGF-1, PDGF-BB and FGF-2 can be used in combination to maximize tenocyte proliferation (Costa et al., 2006). As mentioned above, such a cocktail and other molecules are released by activated blood platelets. Despite their origin as fragments of bone marrow megacaryocytes, platelets are fully functioning coordinators of coagulation, inflammation and repair. When Platelet Concentrate (PC) is activated by thrombin, the factors are released from the alpha-granules (Anitua et al., 2006; Sanchez et al., 2007; Whitman et al., 1997).

Platelet products have found clinical applications in orthopedic surgery, maxillofacial surgery, dental implant surgery and plastic surgery (Anitua et al., 2004). Human PC improved the bone ingrowth distance into porous hydroxyapatite in nude rats (Siebrecht et al., 2002). In a bone grafting study in goats, Platelet Rich Plasma (PRP) appeared to enhance bone healing considerably (Fennis et al., 2004) and histological analyses of bone defects surrounding titanium implants in dogs showed that PRP increased local bone formation (Kim et al., 2002). However, this literature may be affected by a considerable publication bias, as negative results of experimental studies are seldom published. In skin wounds, PC increased granulation tissue and fibrous tissue formation and epithelial growth (Carter et al., 2003; Henderson et al., 2003; Ksander et al., 1990). Also muscle regeneration was improved by PC in rabbits (Jodczyk et al., 1986).

There are a number of uncontrolled clinical studies reporting a good effect of PC in both bone and soft tissue repair (Tischler, 2002). Marx et al. (1998) showed that combining PRP with autogenous bone in mandibular continuity defects in human patients resulted in faster radiographic maturation and a histomorphometrically denser bone regenerate (Marx et al., 1998). PRP and bovine porous bone mineral combined with guided tissue regeneration was more effective in the treatment of human intrabony periodontal defects than guided tissue regeneration alone (Camargo et al., 2002). There is just one paper to our knowledge describing surgically repaired Achilles tendon ruptures using platelet-rich fibrin matrices in humans. This retrospective study, performed in athletes, showed good functional recovery compared with a matched group with conventional surgery (Sanchez et al., 2007).

Table 4: Papers on applying growth factors to healing tendon or ligament.

Growth factor	Doses	Model	Evaluation	Time	Results	Referens
PDGF	0.5, 1 and 5 µg	Rat MCL	Mech	12 d	Dose-dependent benefical effect	Batten et al., 1996
PDGF-BB	400 ng, 20 µg.	Rabbit MCL	Mech, Hist	6 w	Improved ultimate load, energy and elongation	Hildebrand et al., 1998
PDGF-BB+TGF-β-1	400ng +4ng, 20µg. +200 ng	Rabbit MCL	Mech, Hist	6 w	TGF-beta-1 did not lead to additional improvement	Hildebrand et al., 1998
FGF-2	0, 10, 100 and 1000 ng.	Rat patellar tendon, 1×4 mm window defect	Mech, IHC and pyridinoline crosslink analyses	3, 7, 14 d	Increased cell proliferation and type III collagen expession at 3 d	Chan et al., 2000
VEGF	100 µl (50 µg/ml)	Rat AT repaired with suture	Mech	1, 2, 4 w	Improved early tensile strength. Increased TGF-β expression	Zhang et al., 2003
CDMP-1	CDMP-1-coated suture	Rats AT transected and sutured	Mech, Hist	1, 2, 4, 8 w	Improved repair	Rickert et al., 2001
TGF-β-1	10 ng, 100 ng	Rats AT, transected and sutured	Mech, in situ hibridisation	1, 2, 4 w	Increased expression of procollagen I and III mRNA dose-dependently. The failure load and stiffness increased	Kashiwagi et al., 2004
CDMP-1 CDMP-2	1 µg, 10 µg	Rats AT transection, denervation of the calf muscle	Mech, Hist	2 w	Tensile strength increased dose-dependently	Aspenberg and Forslund, 1999
CDMP-1, 2 and 3	0, 0.4, 2 and 10 µg	Rats AT transection	Mech, Hist	8 d, 4 w	Improved healing dose-dependently. Some bone and cartilage	Forslund et al., 2003
CDMP-2	10 µg	Rabbits AT transection	Mech	8 d	CDMP-2 improved tendon repair. No bone or cartilage	Forslund and Aspenberg, 2001
CDMP-2	0, 2, 10, or 50 µg	Rats AT transection	Mech	8 d	CDMP-2 improved tendon repair	Forslund and Aspenberg, 2003
OP-1	100 µg	Rats AT, transection, with or without denervation of the calf muscle or forefoot amputation	Mech, Hist	2 w	Reduced strength of tendon with or without mechanical unloading. Lots of bone	Forslund and Aspenberg, 1998

Purposes

The general purpose of our studies was to improve tendon healing in animal models.

Specific purposes were:

1. To find out if PC can be used to improve tendon healing.
2. To find out if thrombin alone improves tendon healing and, conversely, if thromboprophylaxis can delay tendon healing.
3. To explore the relation between mechanical stimulation and the effect of PC.
4. To find out if CDMP-2 can be used to improve tendon healing in a mechanically unloaded model.
5. To find out if CDMP-2 induces cartilage or bone in loaded or unloaded models in other species than the rat.
6. To transfer previous findings of improved tendon healing in rats and rabbits to a larger animal model.

Materials and methods

All animals, except the sheep, were anesthetized with Isoflurane gas (Forene®, Abbot Scandinavia, Solna, Sweden) in an anaesthetic induction chamber and then operated with Isoflurane via a mask.

Loaded rat and sheep Achilles tendon model

Operation

This model was used in studies № I-IV, and VI, was adopted from the rat Achilles tendon model described by Murrell et al. (1994) and further developed by Forslund (1998). The skin was shaved and the operation was performed under aseptic conditions. A transverse incision was made in the skin lateral to the right Achilles tendon. The surrounding fascia was cut longitudinally and the Achilles tendon complex was exposed. The plantaris tendon was removed. The rat Achilles tendon was cut transversely, proximal to the calcaneal insertion, and a 3 mm long segment was removed to enlarge the defect. The wound was closed.
In the sheep Achilles tendon model no segment was removed. Before the tendon was cut, three longitudinal cuts were made to somewhat simulate the fringing of the human tendon rupture.

Unloaded rat and rabbit tendon models

Operation

This model was used in study V. The right knee was operated on, with the left knee left intact. The skin was shaved and the operation performed under aseptic conditions. An incision was made in the skin medial to the patellar tendon. The superficial surrounding fascia was incised longitudinally and the patellar tendon complex visualized. Transverse Kirschner-wires were inserted through the patella and the tibial tubercle. Specially designed knobs were fastened to the ends of the Kirschner-wires to protect the skin and enable fixation of the steel wires that linked the two transverses Kirschner-wires on either side of the patellar tendon (Figure 2). Thereafter, the deep fascia overlying the tendon was opened and the tendon transected. The resection ends remained in close contact with each other. Finally the skin was closed by intracutaneus sutures. The animals were allowed free movement within their cages.

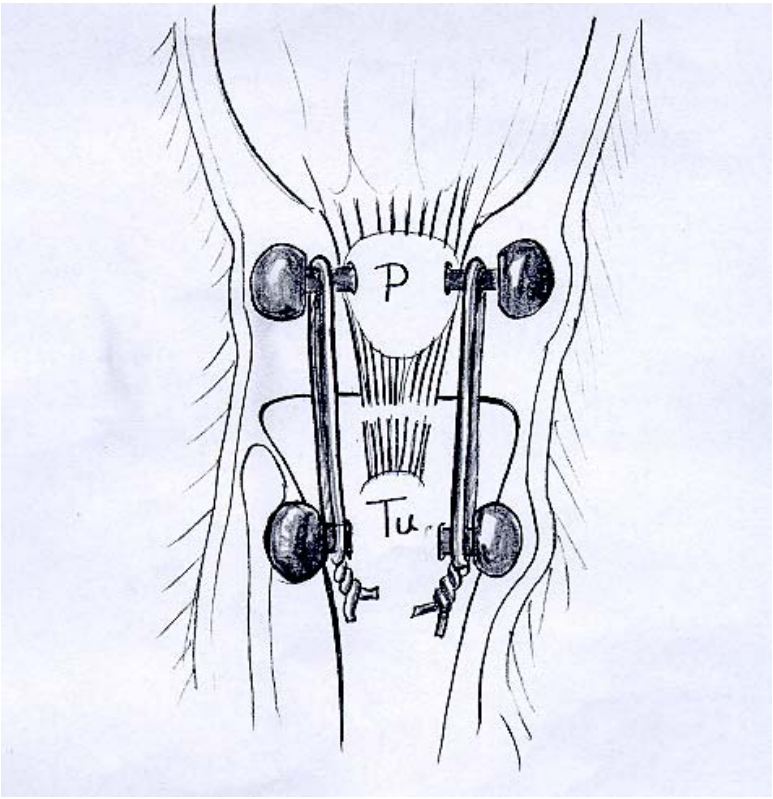

Figure 2: Unloaded rabbit tendon model.

Botox injections

This method was used in study III. The rats were anaesthetized and the skin on the right limb was shaved. The Botulinum toxin A was injected into the gastrocnemius lateralis and medianus and the soleus muscles at a dose of 1 U in each muscle (total dose 3 U). The total injected volume was 0.06 mL.

Platelet concentrate and platelet gel preparation

Whole blood was collected from female Sprague Dawley rats (200 g., M&B, Ry, Denmark). Normally, 3 donor rats were sufficient for an experiment on 10 recipient rats. The rats were anesthetized with Isoflurane®, and 4 to 6 ml of whole blood was collected by cardial puncture using a 10 ml syringe containing 1.5 mL anticoagulant citrate phosphonate dextrose (CPD) buffer (0.15 mg CPD/mL). A 1.2 mm syringe was used. The CPD buffer was taken from a plasma separation bag (Biopack CPDA-1, Firma Biotrans, Dreieich, Germany). 5 mL blood

was mixed with 1.5 mL CPDA-1. After blood collection, the animals were killed by an intra-cardiac injection of an overdose of pentobarbital. The anticoagulated blood was then centrifuged at 220×g for 20 minutes. The supernatant, containing platelet rich plasma, was then centrifuged at 480×g for 20 minutes to form a pellet of rat platelets.

In study I, the platelets were re-suspends in plasma and the cell density was adjusted to 1.5 x 10^{12} platelets/L. The PC was then activated by adding thrombin 20 U per mL PC (USP Thrombostat® Parke-Davis, Morris Plains, USA, 200 units/ml). In order to reduce the risk of a graft versus host reaction, the PC was irradiated at 25 Gy according to international blood banking standards to inactivate the white blood cells. Up until the operation, the platelet concentrate was stored at +4º for a maximum of 24 h. Control solutions consisted of one part CPD buffer as above, added to nine parts saline.

In study II, the platelets the cell density was adjusted to 8.3×10^{12} platelets/L. For the preparation of the platelet gel, 1 mL of the platelet concentrate was dispersed in 20 microwells, 50 µL in each. 20 microwells were activated by adding 0.25 U thrombin from bovine plasma (Sigma Chemical Co., St. Louis, MO, USA, 500 Units) and calcium chloride 10% (Braun Melsungen; 1000 IE/ml $CaCl_2$–2sg) at 37°C 4 mM. The platelet concentrate and platelet gel were stored at +4º for a maximum time of 24 h.

In study III, the platelets the cell density was adjusted to 8.3×10^{12} platelets/L which was used for platelet injections. For the platelet gel preparation the same procedure was used as in study II. The gels in 10 of the microwells also received Hirudin (recombinant from yeast, Fluka Chemie GmbH, 20.75 U/vial) 0.5 U per microwell to neutralize the thrombin.

Administration of growth factors

The administration of growth factors was done in two different ways:

1. A portion of platelet gel was placed in the defect during the operation.
2. PC or CDMP-2 was injected percutaneously into the defect after surgery was completed.

Mechanical testing of tendons

The tendon diameters were measured in the sagittal and frontal planes with a caliper. By blinded repeat measurement of 2-weeks specimens (n=40), the error of measurement (sd (diff) $x2^{-0.5}$) was found to be 7 % of the mean transverse area. The samples were wrapped in saline-soaked gauze until mechanical testing, which was performed within a few hours except for the sheep tendons. Achilles tendons were fixed between two specially designed metal clamps. The angle between the calcaneal bone and the rat Achilles tendon corresponded 30° dorsefixation of the rat foot, to reproduce the physiological position. Sheep Achilles tendons were fixated in 33° dorsiflexion, relative to the direction of traction.

When testing the patellar tendon, the patella was fixed in a metal clamp and one metal pin was inserted in the tibial bone and connected to the machine with a type of fork. All of the tendons were pulled at a constant speed 1 mm/s until failure. Peak force, stiffness and energy uptake until failure was recorded. The sheep tendons also underwent dynamic testing with 20 loading cycles. Transverse area was calculated assuming an elliptic shape, and use to calculate stress at failure.

Histological evaluation

This method has been used in studies № I, V and VI. The tendons for histology were decalcified in EDTA, prepared with routine methods for paraffin sections and stained with Ehrlich Heamatoxylin and Eosin. The specimens were sectioned parallel to the longitudinal direction of the tendon. Only sections from the middle of the tendon callus were made. 3 to 5 glass slides per specimen, comprising the entire length of the rats tendon callus, were prepared, blinded and labeled with a specimen-related code number, so that all slides from one specimen could be evaluated together, without knowledge of treatment and follow-up time. The specimens were analysed in random order, in a microscope using mostly a 12x objective. They were classified according to an arbitrary scoring system from 1 to 5, were 1 represented an immature loose callus and 5 represented a dense organized fibrous tissue with mostly parallel fibres. Rabbit and sheep tendons were analysed following the same preparing and blinding procedures, but we were only looked for signs of cartilage or bone formation.

Radiographic analysis

Radiographs were made to exclude bone formation in study № V.

CT-analyses were used for the same purpose in study № VI.

Discussion

I believe that the use of different growth factors in the care of Achilles tendon rupture patients can lead to significantly faster healing, allowing earlier mobilization, and possibly better end results. However, most research on tendon repair so far has been performed in animal models, and the translation from these models to clinical care is difficult.

There are some differences between transected tendons in animals and ruptured tendons in humans. First, the tendons in our models are cut sharply, and their ends are not frayed. The ruptured human tendon has frayed ends. Possibly, the large area of exposed collagen in the frayed ends can provide sites for binding some of the exogenously applied growth factors, and serve as a kind of slow-release surface. On other hand, the degenerated tendon ends in humans can have areas of previous necrosis and also a poor blood supply as a result of acute vascular damage. Human tendon ruptures often have a history of degenerative pathological changes (Ahmed et al., 1998; Carr and Norris, 1989; Kannus and Jozsa, 1991), while our experiments are performed in healthy tendons. The lack of degenerative changes in the animal models is probably not so important for the healing process, because with or without previous degeneration there will be necrosis as a result of the trauma and the ensuing inflammation. In our larger model, in sheep, we made three longitudinal cuts in the tendon tissue to increase the vascular damage and the exposed collagen surface, thereby somewhat mimicking the human situation.

According to general allometric scaling laws, the metabolic activity in rats should be approximately 4 times faster than in humans, i.e. for some aspects, our 8 days experiments in rats will correspond to 4 weeks in humans (Long et al., 2006; Suarez et al., 2004; West and Brown, 2005). On the other hand, the time course of e.g. an inflammatory response may be more similar to the human situation. Size is an important issue: not only the number of cell generations needed to repopulate the defect will be larger with increasing size, also diffusion distances for chemotactic factors as well as nutrition will differ dramatically, and thereby e.g. the relative importance of revascularization. The differing time course between various events during healing in species of different size will affect the response to an injected growth factor. It is possible that injections in a larger species will have to be given at a later time point, or perhaps repeated. Our experiment in sheep must therefore be regarded as a first attempt only.

Tendons must be capable of resisting high tensile forces with limited elongation. They are designed to transmit loads with minimal energy loss and no permanent deformation. Because of the tendons' mechanical functions, our experiments mainly focus on mechanical parameters as outcome variables. Comparison of force at failure between transected treated and control groups in our experiments was generally chosen to test our hypotheses, but force at failure values of intact tendons also have a great value. These, however, are not easy to obtain. In the sheep, the calcaneal bone was imbedded in a methyl methacrylate resin and fixed in a specially designed metal clamp. The gastrocnemius muscles were scratched away from the aponeurosis, so that thin tendon fibres could bee fixed between metal clamps, using a fibre polish cloth between the clamp and the tissue. Still, during testing it could be seen with the naked eye that tendon fibres ruptured one after another, starting close to the clamp.

In the rats, however, it was possible to create a rupture in the mid -tendon substance in most cases. The reason is unknown, but the method involves meticulous fanning of the proximal fibres to be clamped, to get an even stress distribution. Also, the dorsiflexed position of the calcaneus was developed to achieves a physiologic loading angle and avoid tearing off of the tendon from the insertion. In vivo, tendon rotation plays an important role in human Achilles tendon pathology. The collagen fibres are twisted along the tendon's longitudinal axis, which can produce high stress concentration and hydrostatic pressure in the tendon (Curwin et al., 1988; Stanish et al., 1985). In the Achilles tendon, this appears to happen mainly in the area 2-5 cm above the calcaneal insertion (Barfred, 1973), which is the area where most complete ruptures occurs. This mechanical situation is hard to mimic during mechanical testing. Tractional forces are not distributed evenly between all fibres, but concentrate at the edges of the clamps which hold the tendon. The alternative to our testing device could be the cryo-jaw. The basic principle is that the clamps are chilled with fluid nitrogen so that the adjacent tissue is snap frozen together with the clamp (Wieloch et al., 2004).

Another possibility is to remove parts of the midtendon substance, so that a "waist" is produced, where the tendon is predetermined to rupture. One can then calculate the total force at failure from the original and waist transverse areas (Bruns et al., 2000). However, there is a risk that the tendon structure is variable, making the waist portion not representative. The waist segment also needs to be parallel with the collagen fibres, which is difficult to achieve. These problems are likely to result in too low values.

We used qualitative histology to determinate the maturing of the tendon callus and identify or exclude bone or cartilage formation. It is possible to quantify tendon callus maturity by use of special staining in combination with polarized light. The better longitudinal organization of the collagen, the more polarized transmitted light will be (Ehrlich et al., 2005). We have not utilized this method, because mechanics (especially stress at failure) is our opinion a more direct measure of callus maturity.

Loading plays a critical role in the differentiation of tendon tissue. This is likely true during organ development, and definitively during the later phases of healing. Running and swimming are the most used methods for tendon loading in animals (Murrell et al., 1998; Ng et al., 2004). We tried activity cages as a new method for Achilles tendon loading in rats. The cages consist of two floors connected by a narrow pass. The rats had nest boxes on the ground floor from which they could jump up to the first floor, which was constructed as a maze. In two corners of the maze, food pellets were placed (Carlsson, 2005). In a study on behaviour, nesting, grooming, feeding and sleeping of untreated rats in this environment was observed during two periods with one week in between. The rats spent 75% of the time in the maze, and used less time for sleeping and grooming. In other tests, rats from the activity cages showed less anxiety related behaviours. However, more research is needed to quantitate the activity differences between activity and control cages (Carlsson, 2005).

Interestingly we noted that not only did the increased physical activity lead to a stronger callus, it also led to significant callus shortening (unpublished data). In a blinded fashion, the tendons were held up against a lamp, and the original tendon stumps could be seen as shadows in the translucent callus. The distance between the stumps was decreased from 9.2 to 8.6 mm.

There is a variety of animal models for unloading of tendons, like hindlimb suspension, partial tenotomy (window), internal fixation with cerclage wires, external fixation with fibreglass cast or with Kirschner wires connected by external frames (Almeida-Silveira et al., 2000; Iwuagwu and McGrouther, 1998; Matsumoto et al., 2003; Murrell et al., 1994; Palmes et al., 2002; Yamamoto et al., 1996). Our group has previously used denervation and forefoot amputation (Forslund and Aspenberg, 1998; Forslund and Aspenberg, 2002).

As a new and practical model for unloading of the healing tendon, we used Botox injection into the gastrocnemius muscles. Botox is a highly specific inhibitor of acetylcholine release in the neuromuscular endplate, and should have no effects on the tendon other than from the absence of muscle contraction. As long as the muscle is relaxed and pliable, traction forces in the tendon callus should be low. Earlier models have used immobilization, and in animals it is unclear to what extent immobilization really leads to unloading. Human patients probably load their injured Achilles tendons while in plaster (Benum et al., 1984), and similarly, most animals probably strain their tendons by muscle contraction in external or fixation. Therefore, the dramatic inhibition of repair by unloading in the Botox model appears to give us new opportunities to study the influence of loading in tendon repair.

The theory of mechanical control of differentiation postulates that an undifferentiated mesenchymal tissue develops into fibrous tissue, cartilage or bone depending on its mechanical loading history. In previous work of our group, a decrease in load led to an increase in bone formation in response to exogenously applied CDMP-2. Thus, the bone formative response to CDMP-2 is influenced by the local mechanical environment, and not only by the origin of the responding cells. This extends the theory of mechanical control of differentiation to include also the response to signalling proteins.

CDMP-2 did not induce bone formation in the unloaded rabbit Patellar tendons. This was either because of species differences in the response to CDMPs, or because unloading was incomplete. The normal position of the sitting rabbit's knee is flexion, in which the patellar tendon is slightly curved around the femoral condyles. We observed that the unloading wires between the patella and the tibial tubercle were also slightly bent around the condyles, and straightened out in extension. A mismatch in the curvature of the tendon and the wires could probably provide a small amount of motion in the patellar tendon defect, which might have been enough for mechanical stimulation. We observed a change in the size of the gap between the tendon ends of 1 to 2 mm during flexion and extension.

Platelets had no effect on repair at 2 weeks without mechanical loading. Moreover, with loading, a further increase in physical activity improved repair but not the response to platelets. Our interpretation of these findings is that the healing process can be mechanically stimulated first when the early healing response has produced a matrix capable of transferring some load. We showed that platelets stimulated the early phases of regeneration. This

suggests that clinical use of platelets might be successful only if combined with some kind of mechanical stimulation. It is possible that patients with an Achilles tendon rupture while immobilized in a short leg cast, as estimate from EMG activity (Benum et al., 1984). Platelets not only play a role in tendon healing, but also have an antibacterial effect (Dankert et al., 2001), which might be beneficial if it is to be used clinically.

There are some aspects on platelet concentrate preparation. In our experiments, the platelet concentrate was activated by calcium chloride and thrombin. A gelatinous structure was formed, that could be grasped by a forceps and implanted into the tendon defect. We used thrombin to be sure that the platelets would become activated and growth factors released from the alpha-granules. There are some variations in how platelet gels are prepared in different studies. For example, addition of calcium chloride alone can promote the formation of native thrombin, mimicking the physiological clotting process and enabling a more sustained release of growth factors, so that adding of thrombin is not needed to activate platelets (Tsay et al., 2005). This procedure eliminates the risk of disease transmission and immunological reactions associated with the use of exogenous bovine thrombin (Landesberg et al., 1998). Another risk with platelet gel preparations is that they may contain neutrophiles, which are viewed as the “smart bomb” of inflammation. They express matrix metalloproteinases (MMP-8 and MMP-9), which are matrix-degrading enzymes, and can release reactive oxygen species, which are non-specifically toxic and destroy all surrounding cells, no matter if they are injured or healthy (Scott et al., 2004). In order to reduce the risk of a graft versus host reaction, the platelet concentrate was irradiated at 25 Gy at the beginning of our work with them. In later years, this has become regarded as an unnecessary. Interestingly, neutrophil depletion resulted in faster repair in a mouse wound model, which no adverse effect on strength (Dovi et al., 2003). This finding may open for a discussion about the role of neutrophils in a surgically sterile wound and the possibilities of local neutrophil-inhibition.

We used thrombin to activate the platelets. Thrombin is a proteolytic enzyme, which activates several proteins and converts fibrinogen to fibrin. It can also act as a growth factor. We found a significant improvement of tendon healing by thrombin alone. We therefore hypothesised that inhibition of thrombin by LMWH (widely used in orthopaedics) could delay tendon healing. We found that continuously given thromboprophylaxis impaired tendon healing, but if given intermittently twice a day, it had no effect. How these results relate to the clinical

situation is unknown, but worrisome. Especially modern thromboprophylactic drugs with a long half-life, and a continuous effect on thrombin, might have deleterious effects.

Conclusions

1. PC can be used to improve tendon healing.
2. Thrombin can be used to improve tendon healing and thrombosprophylaxis might delay tendon healing.
3. Mechanical stimulation improves tendon healing with and without PC, and is a prerequisite for long-time effects of PC.
4. CDMP-2 can improve tendon healing in a mechanically unloaded model.
5. The tendency of CDMPs to induce cartilage or bone formation in a rat tendon was not observed in rabbits and sheep.
6. Sheep showed a large variation in repair, which precluded conclusions regarding CDMP-2 effects.

The future

The next step of our research would be clinical applications of either platelets or CDMP-2 to injured Achilles tendons in combination with early loading. This requires methods for evaluation of the mechanical characteristics of healing tendons in humans. One such method, developed in Linköping, uses implanted marker beads in the tendon in combination with 3D radiography (Röntgen stereometric analysis; RSA) and loading. The strain at different loads allows calculation of tendon stiffness. It is also possible to estimate the load on the Achilles tendon by use of EMG (Benum et al., 1984).

We believe that surgical treatment of Achilles tendons with intraoperative addition of a platelet gel might lead to faster recovery. Another possibility is conservative treatment with percutaneous injection of platelets or CDMP-2, which might become an attractive alternative to surgical treatment. Both treatments should probably be combined with early rehabilitation.

Acknowledgement

Per Aspenberg, my supervisor, for introducing me into orthopaedic research, for his boundless enthusiasm, lot of energy and ideas, great visions and for getting me started with this prospective thesis.

Anna Fahlgren for all discussions about orthopaedics and life in general and for all support and the great time in the lab.

Björn Skoglund for inspiring talks and for the revision of the English texts in the beginning of my work.

Björn Pasternak, as kind-hearted as competent, for discussions in Russian.

Thomas Lindahl, Magnus Grenegård, Anders Kalén, Mats Rundgren for their knowledge, inspiration and support.

Bo Skoog for mechanical tests of sheep tendons and his knowledge in mechanics.

Ali Sodeifi and ***Bibbi Mårdh*** for technical assistance and good times in the lab during all operations. All the others at the Experimental Orthopaedics lab in Linköping for their great support and being such a nice group.

Jan Szymanowski, Dan Linghammar and all staff at the Animal Department for great animal knowledge and taking care of my animals.

My family back home in Ukraine: Mother Mushfika, Father Mykola and brother Ruslan and my family in Sweden: Aleksander, Julia and Albert Szymanowski for their love, support and belief in me.

All my friends for their understanding and support.

Jan Szymanowski for being my family, for his absolutely great support and patience, for showing a world and sharing dreams with me.

Stryker Biotech for CDMP-2 supply and funding.

During the course of the research underlying this thesis, Olena Virchenko was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden. This thesis was supported by the Swedish National Centre for Sports Research, the Strategic Research Project “Materials in Medicine”, Linköping, Sweden; and the Swedish Research Council, project 2031.

References

Ahmed, I. M., Lagopoulos, M., McConnell, P., Soames, R. W., and Sefton, G. K. (1998): Blood supply of the Achilles tendon. *J Orthop Res* **16**, 591-6.

Almeida-Silveira, M. I., Lambertz, D., Perot, C., and Goubel, F. (2000): Changes in stiffness induced by hindlimb suspension in rat Achilles tendon. *Eur J Appl Physiol* **81**, 252-7.

Almekinders, L. C., Baynes, A. J., and Bracey, L. W. (1995): An in vitro investigation into the effects of repetitive motion and nonsteroidal antiinflammatory medication on human tendon fibroblasts. *Am J Sports Med* **23**, 119-23.

Amendola, N. (2002): Surgical treatment of acute rupture of the tendo Achillis led to fewer reruptures and better patient-generated ratings than did nonsurgical treatment. *J Bone Joint Surg Am* **84-A**, 324.

Anitua, E., Andia, I., Ardanza, B., Nurden, P., and Nurden, A. T. (2004): Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost* **91**, 4-15.

Anitua, E., Sanchez, M., Nurden, A. T., Nurden, P., Orive, G., and Andia, I. (2006): New insights into and novel applications for platelet-rich fibrin therapies. *Trends Biotechnol* **24**, 227-34.

Aspenberg, P., and Forslund, C. (1999): Enhanced tendon healing with GDF 5 and 6. *Acta Orthop Scand* **70**, 51-4.

Banes, A. J., Horesovsky, G., Larson, C., Tsuzaki, M., Judex, S., Archambault, J., Zernicke, R., Herzog, W., Kelley, S., and Miller, L. (1999): Mechanical load stimulates expression of novel genes in vivo and in vitro in avian flexor tendon cells. *Osteoarthritis Cartilage* **7**, 141-53.

Barfred, T. (1973): Achilles tendon rupture. Aetiology and pathogenesis of subcutaneous rupture assessed on the basis of the literature and rupture experiments on rats. *Acta Orthop Scand Suppl*, 3-126.

Batten, M. L., Hansen, J. C., and Dahners, L. E. (1996): Influence of dosage and timing of application of platelet-derived growth factor on early healing of the rat medial collateral ligament. *J Orthop Res* **14**, 736-41.

Benum, P., Berg, V., and Fretheim, O. J. (1984): The strain on sutured Achilles tendons in walking cast. An EMG analysis. *Eur Surg Res* **16 Suppl 2**, 14-21.

Best, T. M., McElhaney, J., Garrett, W. E., Jr., and Myers, B. S. (1994): Characterization of the passive responses of live skeletal muscle using the quasi-linear theory of viscoelasticity. *J Biomech* **27**, 413-9.

Bhandari, M., Guyatt, G. H., Siddiqui, F., Morrow, F., Busse, J., Leighton, R. K., Sprague, S., and Schemitsch, E. H. (2002): Treatment of acute Achilles tendon ruptures: a systematic overview and metaanalysis. *Clin Orthop Relat Res*, 190-200.

Boyer, M. I., Watson, J. T., Lou, J., Manske, P. R., Gelberman, R. H., and Cai, S. R. (2001): Quantitative variation in vascular endothelial growth factor mRNA expression during early flexor tendon healing: an investigation in a canine model. *J Orthop Res* **19**, 869-72.

Bruns, J., Kampen, J., Kahrs, J., and Plitz, W. (2000): Achilles tendon rupture: experimental results on spontaneous repair in a sheep-model. *Knee Surg Sports Traumatol Arthrosc* **8**, 364-9.

Buckwalter, J. A., Einhorn, T.A., Simon, S.R. (2000): *Orthopaedic Basic Science: Biology and Biomechanics of the Musculoskeletal System*.

Camargo, P. M., Lekovic, V., Weinlaender, M., Vasilic, N., Madzarevic, M., and Kenney, E. B. (2002): Platelet-rich plasma and bovine porous bone mineral combined with guided tissue regeneration in the treatment of intrabony defects in humans. *J Periodontal Res* **37**, 300-6.

Carlsson, L. (2005): Behavioural effects of different types of environmental enrichment on laboratory mice and rats, IFM, Linköping.

Carr, A. J., and Norris, S. H. (1989): The blood supply of the calcaneal tendon. *J Bone Joint Surg Br* **71**, 100-1.

Carter, C. A., Jolly, D. G., Worden, C. E., Sr., Hendren, D. G., and Kane, C. J. (2003): Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing. *Exp Mol Pathol* **74**, 244-55.

Cetti, R., Christensen, S. E., Ejsted, R., Jensen, N. M., and Jorgensen, U. (1993): Operative versus nonoperative treatment of Achilles tendon rupture. A prospective randomized study and review of the literature. *Am J Sports Med* **21**, 791-9.

Chan, B. P., Fu, S., Qin, L., Lee, K., Rolf, C. G., and Chan, K. (2000): Effects of basic fibroblast growth factor (bFGF) on early stages of tendon healing: a rat patellar tendon model. *Acta Orthop Scand* **71**, 513-8.

Chang, J., Most, D., Stelnicki, E., Siebert, J. W., Longaker, M. T., Hui, K., and Lineaweaver, W. C. (1997): Gene expression of transforming growth factor beta-1 in rabbit zone II flexor tendon wound healing: evidence for dual mechanisms of repair. *Plast Reconstr Surg* **100**, 937-44.

Chang, J., Most, D., Thunder, R., Mehrara, B., Longaker, M. T., and Lineaweaver, W. C. (1998): Molecular studies in flexor tendon wound healing: the role of basic fibroblast growth factor gene expression. *J Hand Surg [Am]* **23**, 1052-8.

Chang, S. C., Hoang, B., Thomas, J. T., Vukicevic, S., Luyten, F. P., Ryba, N. J., Kozak, C. A., Reddi, A. H., and Moos, M., Jr. (1994): Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. *J Biol Chem* **269**, 28227-34.

Chhabra, A., Tsou, D., Clark, R. T., Gaschen, V., Hunziker, E. B., and Mikic, B. (2003): GDF-5 deficiency in mice delays Achilles tendon healing. *J Orthop Res* **21**, 826-35.

Clark, M. G., Clerk, L. H., Newman, J. M., and Rattigan, S. (2000): Interaction between metabolism and flow in tendon and muscle. *Scand J Med Sci Sports* **10**, 338-45.

Costa, M. A., Wu, C., Pham, B. V., Chong, A. K., Pham, H. M., and Chang, J. (2006): Tissue engineering of flexor tendons: optimization of tenocyte proliferation using growth factor supplementation. *Tissue Eng* **12**, 1937-43.

Curwin, S. L., Vailas, A. C., and Wood, J. (1988): Immature tendon adaptation to strenuous exercise. *J Appl Physiol* **65**, 2297-301.

Dahlgren, L. A., Mohammed, H. O., and Nixon, A. J. (2005): Temporal expression of growth factors and matrix molecules in healing tendon lesions. *J Orthop Res* **23**, 84-92.

Dankert, J., Krijgsveld, J., van Der Werff, J., Joldersma, W., and Zaat, S. A. (2001): Platelet microbicidal activity is an important defense factor against viridans streptococcal endocarditis. *J Infect Dis* **184**, 597-605.

Dovi, J. V., He, L. K., and DiPietro, L. A. (2003): Accelerated wound closure in neutrophil-depleted mice. *J Leukoc Biol* **73**, 448-55.

Duffy, F. J., Jr., Seiler, J. G., Gelberman, R. H., and Hergrueter, C. A. (1995): Growth factors and canine flexor tendon healing: initial studies in uninjured and repair models. *J Hand Surg [Am]* **20**, 645-9.

Ehrlich, H. P., Lambert, P. A., Saggers, G. C., Myers, R. L., and Hauck, R. M. (2005): Dynamic changes appearing in collagen fibers during intrinsic tendon repair. *Ann Plast Surg* **54**, 201-6.

Enwemeka, C. S. (1992): Functional loading augments the initial tensile strength and energy absorption capacity of regenerating rabbit Achilles tendons. *Am J Phys Med Rehabil* **71**, 31-8.

Fennis, J. P., Stoelinga, P. J., and Jansen, J. A. (2004): Mandibular reconstruction: a histological and histomorphometric study on the use of autogenous scaffolds, particulate cortico-cancellous bone grafts and platelet rich plasma in goats. *Int J Oral Maxillofac Surg* **33**, 48-55.

Forslund, C., and Aspenberg, P. (1998): OP-1 has more effect than mechanical signals in the control of tissue differentiation in healing rat tendons. *Acta Orthop Scand* **69**, 622-6.

Forslund, C., and Aspenberg, P. (2001): Tendon healing stimulated by injected CDMP-2. *Med Sci Sports Exerc* **33**, 685-7.

Forslund, C., and Aspenberg, P. (2002): CDMP-2 induces bone or tendon-like tissue depending on mechanical stimulation. *J Orthop Res* **20**, 1170-4.

Forslund, C., and Aspenberg, P. (2003): Improved healing of transected rabbit Achilles tendon after a single injection of cartilage-derived morphogenetic protein-2. *Am J Sports Med* **31**, 555-9.

Forslund, C., Rueger, D., and Aspenberg, P. (2003): A comparative dose-response study of cartilage-derived morphogenetic protein (CDMP)-1, -2 and -3 for tendon healing in rats. *J Orthop Res* **21**, 617-21.

Fu, S. C., Wong, Y. P., Chan, B. P., Pau, H. M., Cheuk, Y. C., Lee, K. M., and Chan, K. M. (2003): The roles of bone morphogenetic protein (BMP) 12 in stimulating the proliferation and matrix production of human patellar tendon fibroblasts. *Life Sci* **72**, 2965-74.

Hansson, H. A., Dahlin, L. B., Lundborg, G., Lowenadler, B., Paleus, S., and Skottner, A. (1988): Transiently increased insulin-like growth factor I immunoreactivity in tendons after vibration trauma. An immunohistochemical study on rats. *Scand J Plast Reconstr Surg Hand Surg* **22**, 1-6.

Henderson, J. L., Cupp, C. L., Ross, E. V., Shick, P. C., Keefe, M. A., Wester, D. C., Hannon, T., and McConnell, D. (2003): The effects of autologous platelet gel on wound healing. *Ear Nose Throat J* **82**, 598-602.

Hildebrand, K. A., Woo, S. L., Smith, D. W., Allen, C. R., Deie, M., Taylor, B. J., and Schmidt, C. C. (1998): The effects of platelet-derived growth factor-BB on healing of the rabbit medial collateral ligament. An in vivo study. *Am J Sports Med* **26**, 549-54.

Hoffmeyer, P., Freuler, C., and Cox, J. N. (1990): Pathological changes in the triceps surae muscle after rupture of the Achilles tendon. *Int Orthop* **14**, 183-8.

Ingvar, J., Tagil, M., and Eneroth, M. (2005): Nonoperative treatment of Achilles tendon rupture: 196 consecutive patients with a 7% re-rupture rate. *Acta Orthop* **76**, 597-601.

Ishida, H., Yasuda, K., Hayashi, K., Yamamoto, N., and Kaneda, K. (1996): Effects of resumption of loading on stress-shielded autografts after augmentation procedures. An experimental study. *Am J Sports Med* **24**, 510-7.

Iwuagwu, F. C., and McGrouther, D. A. (1998): Early cellular response in tendon injury: the effect of loading. *Plast Reconstr Surg* **102**, 2064-71.

Jackson, J. R., Minton, J. A., Ho, M. L., Wei, N., and Winkler, J. D. (1997): Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1beta. *J Rheumatol* **24**, 1253-9.

Jarvinen, T. A., Kannus, P., Maffulli, N., and Khan, K. M. (2005): Achilles tendon disorders: etiology and epidemiology. *Foot Ankle Clin* **10**, 255-66.

Jodczyk, K. J., Bankowski, E., and Borys, A. (1986): Stimulatory effect of platelet-breakdown products on muscle regeneration. *Zentralbl Allg Pathol* **131**, 357-61.

Jozsa, L., Kannus, P. (1997): *Human Tendons. Anatomy, Physiology, and Pathology*.

Jozsa, L., Kvist, M., Balint, B. J., Reffy, A., Jarvinen, M., Lehto, M., and Barzo, M. (1989): The role of recreational sport activity in Achilles tendon rupture. A clinical, pathoanatomical, and sociological study of 292 cases. *Am J Sports Med* **17**, 338-43.

Kannus, P., and Jozsa, L. (1991): Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. *J Bone Joint Surg Am* **73**, 1507-25.

Kannus, P., Jozsa, L., Natri, A., and Jarvinen, M. (1997): Effects of training, immobilization and remobilization on tendons. *Scand J Med Sci Sports* **7**, 67-71.

Kashiwagi, K., Mochizuki, Y., Yasunaga, Y., Ishida, O., Deie, M., and Ochi, M. (2004): Effects of transforming growth factor-beta 1 on the early stages of healing of the Achilles tendon in a rat model. *Scand J Plast Reconstr Surg Hand Surg* **38**, 193-7.

Khan, R. J., Fick, D., Keogh, A., Crawford, J., Brammar, T., and Parker, M. (2005): Treatment of acute achilles tendon ruptures. A meta-analysis of randomized, controlled trials. *J Bone Joint Surg Am* **87**, 2202-10.

Kim, S. G., Kim, W. K., Park, J. C., and Kim, H. J. (2002): A comparative study of osseointegration of Avana implants in a demineralized freeze-dried bone alone or with platelet-rich plasma. *J Oral Maxillofac Surg* **60**, 1018-25.

Kobayashi, M., Itoi, E., Minagawa, H., Miyakoshi, N., Takahashi, S., Tuoheti, Y., Okada, K., and Shimada, Y. (2006): Expression of growth factors in the early phase of supraspinatus tendon healing in rabbits. *J Shoulder Elbow Surg* **15**, 371-7.

Komi, P. V. (1990): Relevance of in vivo force measurements to human biomechanics. *J Biomech* **23 Suppl 1**, 23-34.

Komi, P. V., Fukashiro, S., and Jarvinen, M. (1992): Biomechanical loading of Achilles tendon during normal locomotion. *Clin Sports Med* **11**, 521-31.

Komi, P. V., Salonen, M., Jarvinen, M., and Kokko, O. (1987): In vivo registration of Achilles tendon forces in man. I. Methodological development. *Int J Sports Med* **8 Suppl 1**, 3-8.

Ksander, G. A., Sawamura, S. J., Ogawa, Y., Sundsmo, J., and McPherson, J. M. (1990): The effect of platelet releasate on wound healing in animal models. *J Am Acad Dermatol* **22**, 781-91.

Landesberg, R., Moses, M., and Karpatkin, M. (1998): Risks of using platelet rich plasma gel. *J Oral Maxillofac Surg* **56**, 1116-7.

Lee, J., Harwood, F. L., Akeson, W. H., and Amiel, D. (1998): Growth factor expression in healing rabbit medial collateral and anterior cruciate ligaments. *Iowa Orthop J* **18**, 19-25.

Lo, I. K., Kirkley, A., Nonweiler, B., and Kumbhare, D. A. (1997): Operative versus nonoperative treatment of acute Achilles tendon ruptures: a quantitative review. *Clin J Sport Med* **7**, 207-11.

Long, F., Chen, Y. Q., Cheverud, J. M., and Wu, R. (2006): Genetic mapping of allometric scaling laws. *Genet Res* **87**, 207-16.

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A., and Hurle, J. M. (1997): Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* **124**, 1109-17.

Maffulli, N., and King, J. B. (1992): Effects of physical activity on some components of the skeletal system. *Sports Med* **13**, 393-407.

Maganaris, C. N., and Paul, J. P. (2002): Tensile properties of the in vivo human gastrocnemius tendon. *J Biomech* **35**, 1639-46.

Magnusson, S. P., and Kjaer, M. (2003): Region-specific differences in Achilles tendon cross-sectional area in runners and non-runners. *Eur J Appl Physiol* **90**, 549-53.

Marx, R. E., Carlson, E. R., Eichstaedt, R. M., Schimmele, S. R., Strauss, J. E., and Georgeff, K. R. (1998): Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **85**, 638-46.

Matsumoto, F., Trudel, G., Uhthoff, H. K., and Backman, D. S. (2003): Mechanical effects of immobilization on the Achilles' tendon. *Arch Phys Med Rehabil* **84**, 662-7.

Mikic, B. (2004): Multiple effects of GDF-5 deficiency on skeletal tissues: implications for therapeutic bioengineering. *Ann Biomed Eng* **32**, 466-76.

Mikic, B., Schalet, B. J., Clark, R. T., Gaschen, V., and Hunziker, E. B. (2001): GDF-5 deficiency in mice alters the ultrastructure, mechanical properties and composition of the Achilles tendon. *J Orthop Res* **19**, 365-71.

Moller, M., Movin, T., Granhed, H., Lind, K., Faxen, E., and Karlsson, J. (2001): Acute rupture of tendon Achillis. A prospective randomised study of comparison between surgical and non-surgical treatment. *J Bone Joint Surg Br* **83**, 843-8.

Mortensen, H. M., Skov, O., and Jensen, P. E. (1999): Early motion of the ankle after operative treatment of a rupture of the Achilles tendon. A prospective, randomized clinical and radiographic study. *J Bone Joint Surg Am* **81**, 983-90.

Murrell, G. A., Jang, D., Deng, X. H., Hannafin, J. A., and Warren, R. F. (1998): Effects of exercise on Achilles tendon healing in a rat model. *Foot Ankle Int* **19**, 598-603.

Murrell, G. A., Lilly, E. G., 3rd, Goldner, R. D., Seaber, A. V., and Best, T. M. (1994): Effects of immobilization on Achilles tendon healing in a rat model. *J Orthop Res* **12**, 582-91.

Ng, G. Y., Ng, C. O., and See, E. K. (2004): Comparison of therapeutic ultrasound and exercises for augmenting tendon healing in rats. *Ultrasound Med Biol* **30**, 1539-43.

Olesen, J. L., Heinemeier, K. M., Langberg, H., Magnusson, S. P., Kjaer, M., and Flyvbjerg, A. (2006): Expression, content, and localization of insulin-like growth factor I in human achilles tendon. *Connect Tissue Res* **47**, 200-6.

Pajala, A., Kangas, J., Ohtonen, P., and Leppilahti, J. (2002): Rerupture and deep infection following treatment of total Achilles tendon rupture. *J Bone Joint Surg Am* **84-A**, 2016-21.

Palmes, D., Spiegel, H. U., Schneider, T. O., Langer, M., Stratmann, U., Budny, T., and Probst, A. (2002): Achilles tendon healing: long-term biomechanical effects of postoperative mobilization and immobilization in a new mouse model. *J Orthop Res* **20**, 939-46.

Petersen, W., Unterhauser, F., Pufe, T., Zantop, T., Sudkamp, N. P., and Weiler, A. (2003): The angiogenic peptide vascular endothelial growth factor (VEGF) is expressed during the remodeling of free tendon grafts in sheep. *Arch Orthop Trauma Surg* **123**, 168-74.

Petersen, W., Varoga, D., Zantop, T., Hassenpflug, J., Mentlein, R., and Pufe, T. (2004): Cyclic strain influences the expression of the vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-1alpha) in tendon fibroblasts. *J Orthop Res* **22**, 847-53.

Pufe, T., Petersen, W., Kurz, B., Tsokos, M., Tillmann, B., and Mentlein, R. (2003): Mechanical factors influence the expression of endostatin--an inhibitor of angiogenesis--in tendons. *J Orthop Res* **21**, 610-6.

Pufe, T., Petersen, W., Tillmann, B., and Mentlein, R. (2001): The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. *Virchows Arch* **439**, 579-85.

Pufe, T., Petersen, W. J., Mentlein, R., and Tillmann, B. N. (2005): The role of vasculature and angiogenesis for the pathogenesis of degenerative tendons disease. *Scand J Med Sci Sports* **15**, 211-22.

Ralphs, J. R., Waggett, A. D., and Benjamin, M. (2002): Actin stress fibres and cell-cell adhesion molecules in tendons: organisation in vivo and response to mechanical loading of tendon cells in vitro. *Matrix Biol* **21**, 67-74.

Reeves, N. D., Narici, M. V., and Maganaris, C. N. (2003): Strength training alters the viscoelastic properties of tendons in elderly humans. *Muscle Nerve* **28**, 74-81.

Rickert, M., Jung, M., Adiyaman, M., Richter, W., and Simank, H. G. (2001): A growth and differentiation factor-5 (GDF-5)-coated suture stimulates tendon healing in an Achilles tendon model in rats. *Growth Factors* **19**, 115-26.

Rosager, S., Aagaard, P., Dyhre-Poulsen, P., Neergaard, K., Kjaer, M., and Magnusson, S. P. (2002): Load-displacement properties of the human triceps surae aponeurosis and tendon in runners and non-runners. *Scand J Med Sci Sports* **12**, 90-8.

Sanchez, M., Anitua, E., Azofra, J., Andia, I., Padilla, S., and Mujika, I. (2007): Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices. *Am J Sports Med* **35**, 245-51.

Scott, A., Khan, K. M., Roberts, C. R., Cook, J. L., and Duronio, V. (2004): What do we mean by the term "inflammation"? A contemporary basic science update for sports medicine. *Br J Sports Med* **38**, 372-80.

Siebrecht, M. A., De Rooij, P. P., Arm, D. M., Olsson, M. L., and Aspenberg, P. (2002): Platelet concentrate increases bone ingrowth into porous hydroxyapatite. *Orthopedics* **25**, 169-72.

Sipe, J. B., Zhang, J., Waits, C., Skikne, B., Garimella, R., and Anderson, H. C. (2004): Localization of bone morphogenetic proteins (BMPs)-2, -4, and -6 within megakaryocytes and platelets. *Bone* **35**, 1316-22.

Stanish, W. D., Curwin, S., and Rubinovich, M. (1985): Tendinitis: the analysis and treatment for running. *Clin Sports Med* **4**, 593-609.

Storm, E. E., Huynh, T. V., Copeland, N. G., Jenkins, N. A., Kingsley, D. M., and Lee, S. J. (1994): Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. *Nature* **368**, 639-43.

Suarez, R. K., Darveau, C. A., and Childress, J. J. (2004): Metabolic scaling: a many-splendoured thing. *Comp Biochem Physiol B Biochem Mol Biol* **139**, 531-41.

Thomas, J. T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J., and Luyten, F. P. (1996): A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. *Nat Genet* **12**, 315-7.

Tischler, M. (2002): Platelet rich plasma. The use of autologous growth factors to enhance bone and soft tissue grafts. *N Y State Dent J* **68**, 22-4.

Tsay, R. C., Vo, J., Burke, A., Eisig, S. B., Lu, H. H., and Landesberg, R. (2005): Differential growth factor retention by platelet-rich plasma composites. *J Oral Maxillofac Surg* **63**, 521-8.

Tsuzaki, M., Brigman, B. E., Yamamoto, J., Lawrence, W. T., Simmons, J. G., Mohapatra, N. K., Lund, P. K., Van Wyk, J., Hannafin, J. A., Bhargava, M. M., and Banes, A. J. (2000): Insulin-like growth factor-I is expressed by avian flexor tendon cells. *J Orthop Res* **18**, 546-56.

Wang, V. M., Banack, T. M., Tsai, C. W., Flatow, E. L., and Jepsen, K. J. (2006): Variability in tendon and knee joint biomechanics among inbred mouse strains. *J Orthop Res* **24**, 1200-7.

West, G. B., and Brown, J. H. (2005): The origin of allometric scaling laws in biology from genomes to ecosystems: towards a quantitative unifying theory of biological structure and organization. *J Exp Biol* **208**, 1575-92.

Whitman, D. H., Berry, R. L., and Green, D. M. (1997): Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. *J Oral Maxillofac Surg* **55**, 1294-9.

Wieloch, P., Buchmann, G., Roth, W., and Rickert, M. (2004): A cryo-jaw designed for in vitro tensile testing of the healing Achilles tendons in rats. *J Biomech* **37**, 1719-22.

Yamamoto, E., Tokura, S., Yamamoto, N., and Hayashi, K. (2000): Mechanical properties of collagen fascicles from in situ frozen and stress-shielded rabbit patellar tendons. *Clin Biomech (Bristol, Avon)* **15**, 284-91.

Yamamoto, N., Hayashi, K., Kuriyama, H., Ohno, K., Yasuda, K., and Kaneda, K. (1996): Effects of restressing on the mechanical properties of stress-shielded patellar tendons in rabbits. *J Biomech Eng* **118**, 216-20.

Zeichen, J., van Griensven, M., and Bosch, U. (2000): The proliferative response of isolated human tendon fibroblasts to cyclic biaxial mechanical strain. *Am J Sports Med* **28**, 888-92.

Zhang, F., Liu, H., Stile, F., Lei, M. P., Pang, Y., Oswald, T. M., Beck, J., Dorsett-Martin, W., and Lineaweaver, W. C. (2003): Effect of vascular endothelial growth factor on rat Achilles tendon healing. *Plast Reconstr Surg* **112**, 1613-9.