Regulating Myofibroblasts in Dupuytren's Disease

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Regulating Myofibroblasts in Dupuytren's Disease

Het reguleren van myofibroblasten in de ziekte van Dupuytren

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Contents

Chapter 1

Introduction

General introduction

Dupuytren's disease is a common fibroproliferative disorder of the palmar fascia. It is named after Baron Guillaume Dupuytren, the surgeon who described the condition in a now famous lecture "La rétraction permanente des doigts" in 1831, despite earlier descriptions by Felix Platter (1680), Henry Cline (1808) and Sir Astley Cooper (1818).

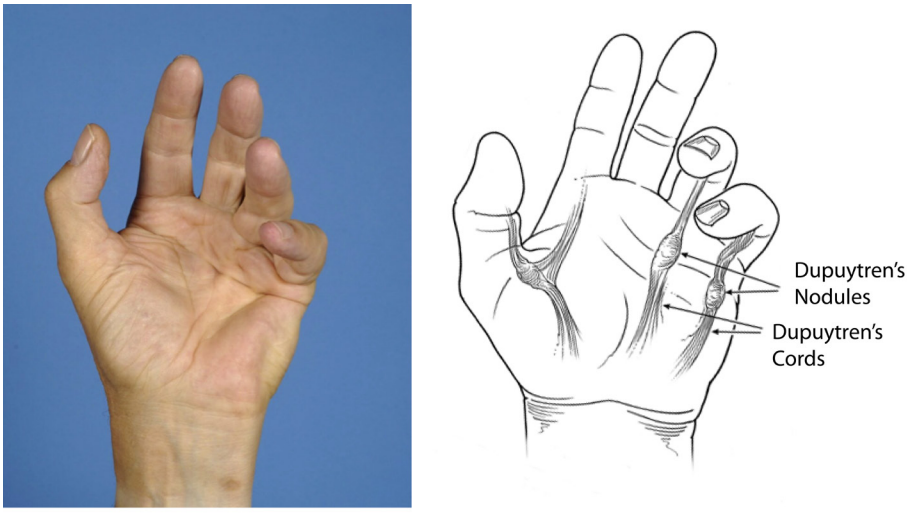

Figure 1. Photograph and schematic showing Dupuytren's contracture affecting the little finger, ring finger, thumb and first webspace. Schematic is adapted from Schnitz, the Indiana hand and shoulder centre, retrieved from indianahandtoschoulder.com.

The classic description of Dupuytren's disease progression is the initial appearance of palmar nodules characterized by dense cellularity and cell proliferation, followed by the development of cords. As the disease progresses this phase is followed by a final fibrotic stage leading to relatively less cellular cords and digital contractures resulting in significant impairment of hand function[1] (Fig 1).

The prevalence of the disease, influenced by the study parameters used, varies according to geographical location and ranges from 0.2% to 56%[2]. Epidemiology studies indicate that Dupuytren's disease affects 4-6% of all Caucasians[3], although it is most common amongst Caucasians of Northern European extraction. In Norway the disease prevalence has been reported reaching 30% in individuals aged over 60 years[4].

Dupuytren's disease has a familial predisposition, suggesting a genetic link. Previous studies have used DNA microarray and linkage studies, allowing differential analysis of expression of multiple genes. Results have varied and included genes involved in extracellular matrix organisation and metalloproteinases, fibronectin, collagens type III, IV and VI and transforming growth factor-β[5-7].

Analysis of subcutaneous fat, fascia and skin overlying Dupuytren's disease revealed differential gene expression in comparison to control tissue[8]. Recently in a large gene wide association study, a role for the Wnt-signaling pathway in the pathogenesis of Dupuytren's disease has been suggested[9].

Dupuytren's has often been associated with various environmental factors. These include smoking, excessive alcohol consumption, diabetes, epilepsy, occupation and trauma. However, their role in the pathogenesis of the disease remains unclear[2].

Clinical presentation

The disease onset is characterized by thickening and pitting of the palmar skin and the presence of nodules in the palm. Clinical nodules result from cell proliferation and extracellular matrix synthesis, whilst skin pits observed are caused by contraction of the saggital fibres[10]. In the later stages of Dupuytren's disease, nodules mature to form collagen rich fibrotic cords, which through gradual contraction lead to digital contractures[1].

Dupuytren's disease is associated with subdermal fat deficiency as pathologic fibrosis displaces the fat. As a result, the thickened palmar skin becomes tethered and loses its normal pliability[11,12].

Dupuytren's disease predominantly affects the longitudinally orientated fascial fibres in the palm. The normal anatomical bands lie along lines of tension in the hand and in response to increased tension thicken to develop into pathological cords[13]. The ring finger and little finger are most commonly affected. Some patients have a greater predisposition for developing the disorder, thereby influencing the disease course and pattern of recurrence. These high-risk patients are said to have a strong "diathesis"[14], and risk factors include a positive family history (1st degree relatives), bilateral disease, ectopic lesions (thickened knuckle pads), male gender and age at onset of younger then 50 years[15].

Treatment

Different treatment options, both surgical and non-surgical, have been tested for the management of Dupuytren's disease. These treatments can be broadly classified as invasive or minimally invasive.

Established flexion deformities of the digits are most commonly treated by limited fasciectomy that involves surgical excision of the diseased fascia[15]. Recurrence rates following fasciectomy have been reported at approximately 12% at 3 years[16] and 20-50% at 5 years[17,18].

Dermofasciectomy is typically considered in cases of recurrence or very aggressive primary disease, and involves surgical excision of the affected tissue combined with replacement of the overlying palmar skin with full thickness non-palmar skin grafts[12]. Recurrence following dermofasciectomy has been reported as ranging between nil[19], or up to 8% between 2-12 years follow-up[20]. The inconsistency in recurrence rates following these different treatment options may also be in part related to the different definitions of recurrence used[21].

Less invasive surgical alternatives include division of the cord by percutaneous needle fasciotomy. More recently enzymatic digestion with collagenase injections has been extensively trailed[22]. These less invasive alternatives promote earlier postoperative recovery, although recurrence rates following these procedures remain high. Recurrence rates after needle fasciotomy are reported to be as high as 85% at 5 years[18] and following collagenase injections 35% at 3 years[23].

In an attempt to overcome high recurrence rates after minimally invasive needle fasciotomy, a novel approach is being tested[24]. This approach disintegrates the fibrous cord through a more extensive percutaneous needle aponeurotomy technique, applying numerous superficial nicks along the cord. Following percutaneous release of the skin from the subcutaneous layer with a needle, autologous lipoaspirates are grafted in the treated area to restore the subdermal fat deficiency[11]. Preliminary results following this new technique show shortened recovery time, restored subcutaneous fat deficiency and scar-free supple skin[24].

Non-surgical management options have also been trailed for the treatment of early Dupuytren's disease. These primarily include local radiotherapy[25] and steroid injection directly into the nodules[26]. Radiotherapy has been used in different hyperproliferative and inflammatory diseases and has demonstrated some effect in the prevention of disease progression in Dupuytren's disease[27], although there remain potential issues with treating a benign disorder with radiotherapy. Steroid injections may result in temporary resolution of nodules, and combined with percutaneous needle aponeurotomy resulted in a greater degree of correction and reduced recurrent deformity at 6 months compared to percutaneous needle aponeurotomy alone[28]. It has been suggested that this limited therapeutic benefit may be due to diminished leukocyte recruitment, as well as increased apoptosis of macrophages and fibroblasts, with reduced proliferation of the latter[29,30].

Despite all these efforts, recurrence of Dupuytren's disease remains a significant problem. Data from studies that have looked more closely at the

pathobiology of Dupuytren's disease suggest that treatment strategies for Dupuytren's disease should be targeted at inhibiting the myofibroblast, the cell responsible for both extracellular matrix deposition and contraction in Dupuytren's disease (Fig. 2).

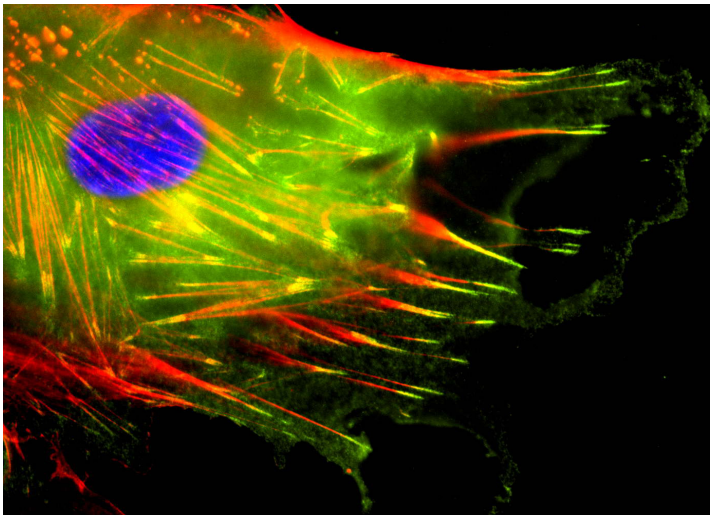

Figure 2. Immunofluoresence staining of cultured Dupuytren's myofibroblast. Cell morphology and actin cytoskeleton were visualized with α-SMA (red) that were connected to the extracellular matrix at the sites of focal adhesions (green, visualized with Vinculin) and the nucleus was stained with DAPI (blue).

Myofibroblasts

Myofibroblasts were first described by Gabbiani in 1971 by electron microscopy in granulation tissue of healing wounds, exhibiting features between fibroblasts and smooth muscle cells[31]. Myofibroblasts differ from fibroblasts by the presence of intracellular contractile microfilaments, supermature focal adhesions and intercellular adherens and gap junctions[32,33] (Fig. 2 and 3).

Myofibroblasts characteristically express α-smooth muscle actin (α-SMA), which is the actin isoform typical of vascular smooth muscle cells[34]. Fibroblast to myofibroblast differentiation is characterized by α-SMA expression, and exposure of the cells to stress leads to the incorporation of α-SMA protein into stress fibers[35], leading to contraction.

Unlike granulation tissue during wound healing, where the expression of α-SMA is transient, α-SMA expression is persistent in Dupuytren's disease[36]. The differentiation of myofibroblasts has been shown to be dependent on a number of different environmental factors, including high extracellular stress from the mechanical properties of the extracellular matrix, cell to cell interactions and exposure to a variety of different growth factors and cytokines[35].

The origin of the myofibroblast in Dupuytren's disease remains unknown. They may arise from the in situ activation of normally quiescent resident fibroblasts in the overlying palmar dermis[14], or within the palmar fascia[13] in response to extracellular changes. Fibroblasts from normal dermal skin and normal palmar fascia both have the potential to develop into myofibroblasts[37]. Alternatively, myofibroblasts may arise from other cell types, including vascular smooth muscle cells, pericytes, fibrocytes, epithelial cells undergoing epithelial-to-mesenchymal transition and possibly astrocytes[38].

Myofibroblasts and the mechanical environment

The extracellular matrix (ECM) is the substrate for cell adhesion, growth and differentiation, and it provides mechanical support to surrounding tissue. In vivo cells adapt to this complex physical environment, whereby mechanical tension and load determine cell morphology, phenotype and function[39]. Myofibroblasts only maintain their phenotype under stress and loss of tension is associated with disassembly of α-SMA stress fibres within minutes[40].

There is no animal model for Dupuytren's disease and in vivo conditions are most reliably examined by studying myofibroblasts in 3D collagen lattices under isometric tension, and isometric contractile forces have been well characterised and measured using a culture force monitor[41].

The ECM is not static but is constantly being remodeled in response to the local mechanical environment. This ECM is dynamic and has a constant turnover of its various components including collagens, elastins, structural organizing proteins, glycosaminoglycans and proteoglycans. Increased tension in the ECM is associated with recruitment of α-SMA stress fibres, a classic hallmark for myofibroblast development[41,42]. These stress fibres terminate at the myofibroblast surface at sites of focal adhesions and provide a mechano-transduction whereby tension in the matrix can be perceived by the myofibroblasts and where force generated by the myofibroblasts can be transmitted to the surrounding matrix. Also, alterations in the mechanical environment can lead to changes in synthesis of matrix metalloproteinases (MMPs).

MMPs function in the degradation of the ECM as a part of controlled matrix remodeling. Under physiological conditions MMPs are usually present at low levels and the activity of these enzymes is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs)[43]. An imbalance of proteolysis may lead to fibrosis and has been reported to be associated with Dupuytren's disease[44,45].

The mechanical environment depends not only on features of individual cells and ECM molecules but also on the complex organizational and reciprocal interactions that occur between aggregates of cells[46].

Intercellular communication

Dupuytren's nodules from patients with active disease are comprised of densely packed myofibroblasts, although these contractile cells also exist in aggregates within more mature cord tissue[13,47].

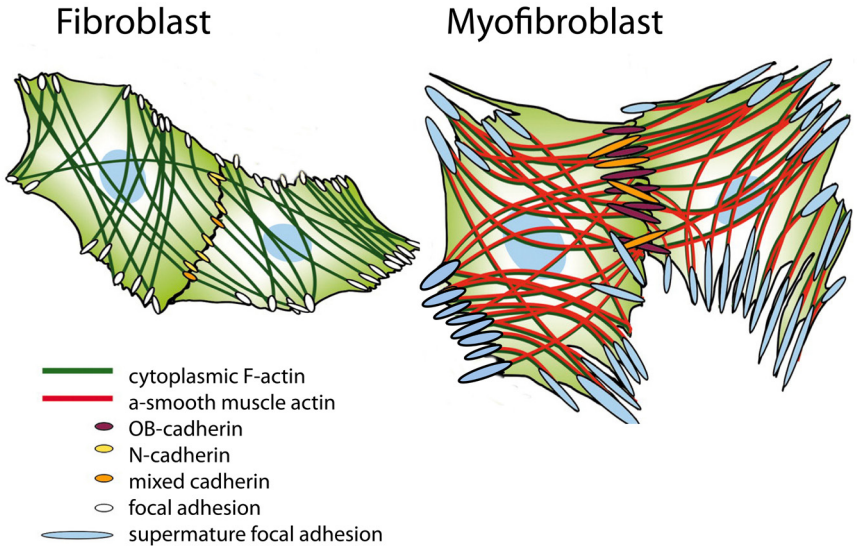

Figure 3. Model of adherence junctions in fibroblasts and myofibroblasts. Fibroblasts exhibit cytoplasmic F-actin filaments, which are connected to the ECM at sites of focal adhesions and between cells at sites of adherence junctions that predominantly express N-cadherin. Myofibroblasts form α-SMA positive stress fibers. The enhanced contractile activity mediated by α-SMA incorporation leads to the development of supermature focal adhesions and predominantly OB-cadherin expression. Adapted from Hinz, Mol Biol Cell, 2004.

Cells primarily communicate via three distinct means: adherens junctions, mechanosensitive junctions and gap junctions. Adherens junctions are composed of cadherins that extend through the plasma membrane and mediate calcium-dependent cell-cell adhesion[48]. Myofibroblast development is associated with upregulation of cadherin expression, and increased α-SMA expression is correlated with markedly higher levels of OB-cadherin[48]. OB-cadherin junctions exhibit higher adhesion strength than N-cadherin, which is the most commonly expressed cadherin in fibroblasts[49,50] (Fig. 3). Mechanosensitive channels open when force is transmitted by tension applied via adherens junctions and lead

to an influx of calcium ions (Fig. 4). Adjacent cells can also communicate via gap junctions to allow passage of molecules of up to 1kD, such as calcium ions, between cells via hydrophilic channels.

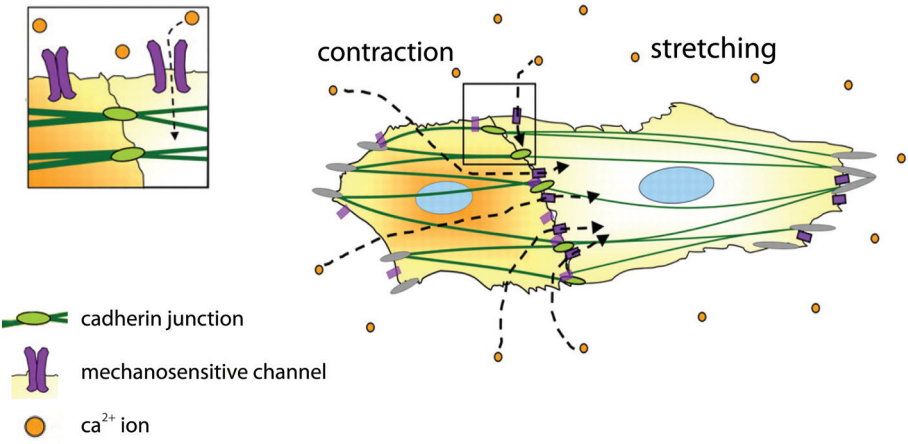

Figure 4. Model of communication between contacting myofibroblasts. The α-SMA stress fibers (green) of contacting myofibroblasts are connected to the ECM at sites of focal adhesions (grey) and intercellularly at sites of cadherin junctions (green). Mechanosensitive channels open when force is transmitted by tension applied via adherens junctions and lead to an influx of Ca2+. The resulting influx of Ca2+ through mechanosensitive channels then triggers a contractile event in an adjacent myofibroblast (right myofibroblast) that perpetuates back (to the left myofibroblast). Adapted from Follonier, J Cell Science, 2008.

These mechanical and electrochemical intercellular communication channels have been implicated in myofibroblast development and contractility[33]. It remains to be investigated how myofibroblasts from patients with Dupuytren's disease act in a coordinated fashion to contract the matrix and hence the digits.

Another potential strategy for the regulation of fibrosis is the use of stem cells.

Stem cells

In recent years, the effect of stem cell-based therapy has increasingly been explored. There is now increasing evidence for the role of mesenchymal stem cells (MSCs) as a potential treatment strategy to alleviate fibrosis. MSCs are multipotent cells that can be easily obtained from a variety of sources, and have been shown to play a role in decreasing fibrosis in models of lung, liver, kidney, heart and also in an animal model of related Peyronie's disease[51-54].

Stem cells from adipose and bone marrow tissue are both attractive sources for cell-based therapy. However, whether these cell populations from different sources are functionally distinct remains unclear. In vivo and in vitro adipose- and bone marrow-derived stem cells display the same ability to differentiate towards adipogenic, osteogenic and chondrogenic lineages[55], although differences at transcriptional and proteomic levels between both cell type according to their tissue origin have been reported[56].

Inflammation is also known to play a crucial role in fibrosis and various proinflammatory cytokines have been implicated in driving the progression of fibrotic diseases[57]. MSCs exhibit potent immunomodulatory and anti-inflammatory effects, either by direct cell contact or indirectly by secretion of various soluble factors[58].

Growth factors and inflammation

Fibrosis is a common pathological end point of many inflammatory disorders. There is considerable evidence demonstrating raised levels of various cytokines and growth factors in Dupuytren's disease. These include transforming growth factor-β1 (TGF-β1), TGF-β2, platelet-derived growth factor (PDGF), tumor necrosis factor (TNF), interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and connective tissue growth factor (CTGF)[59-61].

Of these cytokines and growth factors, TGF-β1 has been studied the most and is considered the primary growth factor responsible for directly promoting myofibroblast differentiation[62], contractile force generation and increased ECM deposition[63]. Dupuytren's myofibroblasts examined in vitro using the culture force monitor exhibit increased isometric contraction of collagen lattices when exposed to 12.5ng/ml of TGF-β1, with higher doses being inhibitory[64].

TNF is another potent inflammatory cytokine that has been implicated in the development of bleomycin-induced pulmonary fibrosis and that has been reported as a key driver of fibrosis in many experimental studies[65,66]. Targeting upstream proinflammatory pathways, by inhibiting TNF, has been initiated in clinical trials to treat pulmonary fibrosis and other fibrotic disorders[67]. However, other studies have suggested an inhibitory role of TNF in the development and contractility of myofibroblasts in dermal fibroblasts[68]. In Dupuytren's disease high levels of TNF mRNA have been reported[69], although no causal link between TNF and Dupupuytren's disease has been demonstrated.

Histological studies have identified the presence of immune cells in Dupuytren's disease and in particular the number of macrophages was shown to correlate with the quantity of myofibroblasts[70]. However, the link between disease progression and inflammation in Dupuytren's tissue remains unclear and molecular mechanisms by which inflammatory cytokines directly drive myofibroblast differentiation remain unknown.

Aim and outline of this thesis

Dupuytren's disease is a very common progressive fibrosis of the palm leading to flexion deformities of the digits that impair hand function. The cell responsible for development of the disease is the myofibroblast. In order to develop new management strategies for treating Dupuytren's disease, it is essential to examine the underlying pathobiology. We set out to examine what factors regulate the myofibroblast phenotype in terms of differentiation, proliferation, ECM production and contractility. Our aim was to examine how myofibroblasts may be regulated through mechanical tension, intercellular interactions, stem cells and inflammatory mediators.

Dupuytren's disease is a fibroproliferative disorder that progresses from active cellular nodules to fibrotic cords in end stage disease. Therefore, in **chapter 2** we will describe the role of mechanical tension and load on Dupuytren's myofibroblast contractility, and matrix metalloproteinases mediated remodeling of the extracellular matrix. We will also describe the difference in contractility between Dupuytren's cells isolated from nodule and cord tissue.

Nodules in Dupuytren's disease are comprised of dense aggregates of myofibroblasts. In **chapter 3**, we explore the hypothesis that in Dupuytren's disease, myofibrobasts act in a coordinated fashion by communicating with each other through adherens, mechanosensitive and gap junctions. Disruption of these intercellular junctions may inhibit extracellular matrix production and contractility.

The introduction of another cell type in co-culture with Dupuytren's myofibroblasts may also inhibit direct intercellular communication between contractile myofibroblasts. There is increasing evidence for the role of mesenchymal stem cells in the treatment of fibrosis, either by direct cell to cell contact or indirectly through the release of various soluble factors and cytokines. Therefore, in **chapter 4**, we examine the direct and indirect effect of adipose and bone marrow-derived stem cells on Dupuytren's myofibroblast proliferation and contractility.

Inflammation is known to play a key role in fibrosis and various proinflammatory cytokines have been implicated in driving the progression of fibrotic diseases. Therefore, in **chapter 5**, we will test the effect of proinflammatory cytokines on dermal fibroblasts from different anatomical sites, from patients with Dupuytren's disease and individuals unaffected by Dupuytren's disease. We also will examine the molecular mechanisms by which inflammatory cytokines directly drive myofibroblast differentiation.

Finally, in **chapter 6**, we discuss the main findings from this thesis and implications for future research.

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Chapter 2

The mechanical environment in Dupuytren's contracture determines cell contractility and associated MMP mediated matrix remodeling

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Abstract

MMPs are expressed in Dupuytren's contracture and play a role in matrix remodeling. We tested the role of tension on contractility and MMP expression in Dupuytren's nodule and cord cells. Cells were subjected to pre-determined loading patterns of known repeatable magnitudes (static load, unloading and overloading) and tested for MMP gene expression (MMP-1, -2, -9, -13, and TIMP-1, -2) and force generation using a tension-culture force monitor. Matrix remodeling was assessed by addition of cytochalasin D and residual matrix tension was quantified. Nodule compared to cord and control cells demonstrate greater force generation and remodeling ($p<0.05$). Nodule cells subjected to a reduced load and overloading led to threefold increase of MMP-1, -2 and -9 compared to static load, whilst cord and control cells only showed a twofold increase of MMP-9. Nodule cells subjected to overloading showed a 2-fold increase in TIMP-2 expression, whilst cord and control cells showed a twofold increase in TIMP-1 expression. Nodule cells differ from cord cells by increased force generation in response to changes in the mechanical environment and related MMP/TIMP mediated matrix remodeling. In turn this may lead to permanent matrix shortening and digital contracture. Interventional therapies should be aimed at nodule cells to prevent contraction and subsequent permanent matrix remodeling.

Introduction

Dupuytren's contracture is a fibroproliferative condition of the palmar fascia, typically described in terms of contractile cellular nodules that progress to form fibrotic cords and ultimately lead to digital contracture[1,2]. The mainstay for treatment is surgery although there has been recent interest in the role of enzymatic treatment with injecting bacterial collagenase clostridium histolyticum[3], leading to disruption of the contracted Dupuytren's cord. It has been proposed that the mechanism of contracture is a result of two separate but related processes: cell mediated contraction of the matrix whereby cells contract to cause a physical deformation[4] and secondly, continuous matrix remodeling, leading to the permanence of contracture[5].

Tension is known to be a contributing factor in Dupuytren's contracture[6] and alterations in the mechanical environment in which Dupuytren's cells reside can lead to changes in the production of matrix metalloproteinases (MMPs)[7]. MMPs are a major group of zinc-dependent endopeptidases that function in the degradation of the extracellular matrix (ECM) as a part of controlled matrix remodeling[8]. Under physiological conditions MMPs are usually present at low levels and the activity of these enzymes is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs)[8]. An imbalance of these enzymes has been reported to be associated with Dupuytren's contracture[9-11]. Therefore, we tested the effect of externally applied mechanical tension on contractility and MMP expression in Fibroblast Populated Collagen Lattices (FPCLs) seeded with Dupuytren's nodule and cord cells compared to control flexor retinaculum (FR) cells.

Methods

Cell Culture

Following local ethical committee approval and informed written consent, Dupuytren's samples were obtained from patients undergoing primary fasciectomy. Dupuytren's nodule and cord (n=5) tissue were distinguished clinically based on gross morphology and by histological evaluation[12,13]. In line with previous studies, age and sex matched flexor retinaculum tissue (n=5) was used as control and excised from patients with no evidence of coexisting Dupuytren's contracture at routine carpal tunnel decompression. The mean age of the patients was 65 years (range 56-71). Dupuytren's patients were classified into Tubiana stages, the mean Tubiana's grade was 1.8 (range 1-3). An explant method was used to establish cell cultures, which were maintained in culture with normal growth medium (NGM), consisting of Dulbecco's Modified Eagle's medium, supplemented with 2mM Glutamax, penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% foetal calf serum (all Gibco, UK), at 37°C and 5% CO2. All cultures were studied at or below passage five.

Tension Culture Force Monitor

The tension culture force monitor (t-CFM) (Fig.1) is an instrument which measures isometric contractile forces generated by cells within a 3D FPCL and applies load to the FPCL via a microprocessor-controlled stepping motor (Michromech, Braintree, UK), as previously described[14]. It comprises of a rectangular, three-dimensional FPCL, which is cast and floated in medium, tethered to two flotation bars on either of the short edges, and attached to a fixed point at one end and a force transducer at the other. Briefly, 5 x 10^6 cells from either Dupuytren's nodule, Dupuytren's cord or flexor retinaculum cells were dispersed uniformly through 5 ml of neutralized type I collagen gel (2.3mg/ml First link, West Midlands, UK), the gel was set for 30 minutes prior to being floated in 20 ml NGM.

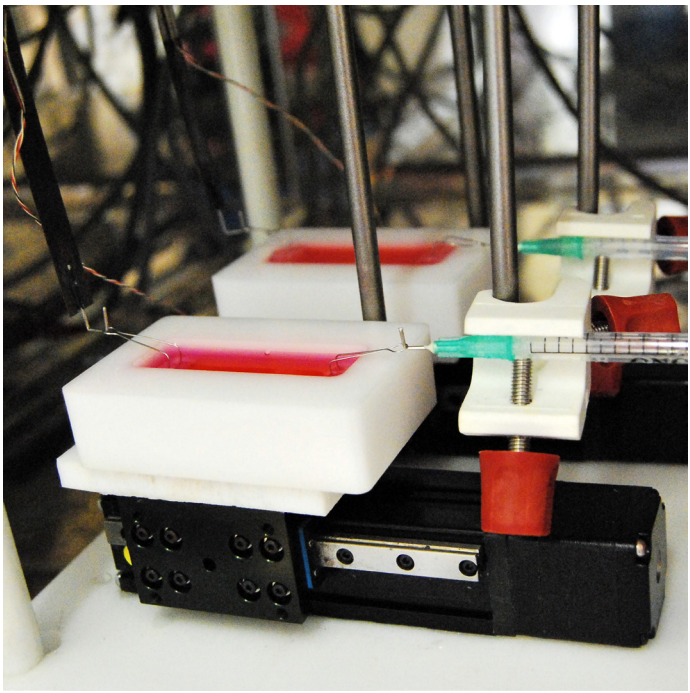

Figure 1. The tension culture force monitor (t-CFM). The collagen gels were cast and floated in medium in a mould positioned on a platform on a microprocessor-controlled stepping motor. The collagen gel was tethered between two floatation bars one is attached to a fixed point, whilst the other is attached to a force transducer.

For the MMP force generation, data was collected every second and a contractility profile was generated over 22hr. After this period, the FPCLs were allowed to contract for a further 2hr (static load), subjected to a series of uniaxial reductions of load forces (unload), or subjected to a series of rapid uniaxial loading forces (overload). A reduction in load and an overload was applied to the FPCLs via a microprocessor-controlled stepping motor. The motion of the culture platform was calibrated against the force transducer. Since the force transducer has a linear response the relationship between force and movement was simple to derive[14]. This ensured us reproducibly and precise loading and unloading rates.

The loading cycle comprised a decrease or an increase in tension of 30 dynes[15] over a 30 sec period, followed by a 30 min resting phase. This process was repeated for 4 cycles.

Quantification of Residual Matrix Tension (RMT)

For the permanent matrix remodeling experiments, cell contractile profiles within the t-CFM were generated for 12, 24 and 48 hr periods. Following these different time periods in culture, cell-mediated force generation was abolished by addition of a saturating dose of 20 µg/ml cytochalasin D as previously described[16,17]. Eliminating cell-generated force revealed force components, force due to active cellular contraction and force due to fixed tension within the matrix due to collagen remodeling by the cells (residual matrix tension - RMT). RMT was measured 2 hr post-delivery of cytochalasin-D as changes in cellular contractility after this period had reached a plateau.

Gene Expression

At the conclusion of the t-CFM experiments, RNA was extracted from FPCLs using an RNeasy MiniKit (Qiagen, UK) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA which was used in PCR reactions with primers specific for MMP-1, -2, -9, -13, TIMP-1, -2 and GAPDH. The sequences of the primers are shown in Table 1. PCR products were run on a 2% agarose gel containing ethidium bromide. The expression levels of the genes of interest were normalized in relation to control GAPDH.

Gene	forward primers	reverse primers
MMP-1	5'-CGA CTC TAG AAA CAC AAG AGC AAG A-3'	5'-AAG GTT AGC TTA CTG TCA CAC GCT T-3'
MMP-2	5'-GTG CTG AAG GAC ACA CTA AAG AAG A-3'	5'-TTG CCA TCC TTC TCA AAG TTG TAG G-3'
MMP-9	5'-CAC TGT CCA CCC CTC AGA GC-3'	5'-GCC ACT TGT CGG CGA TAA GG-3'
MMP-13	5'-TGC TGG CTC ATG CTT TTC CTC-3'	5'-GGT TGG GGT CTT CAT CTC CTG-3'
TIMP-1	5'-ACC ACC TTA TAC CAG CGT TAT GAG-3'	5'-GAG GAG CTG GTC CGT CCA CAA GCA-3'
TIMP-2	5'-CGC TGG ACG TTG GAG GAA AGA AGG-3'	5'-GGG TCC TCG ATG TCG AGA AAC TCC-3'
GAPDH	5'-AAG AAG ATG CGG CTG ACT GTC GAG CCA CAT-3'	5'-TCT CAT GGT TCA CAC CCA TGA CGA ACA TG-3'

Table 1. Sequences of the primers used in assessment of gene expression.

Immunofluorescence staining

Following contraction in the t-CFM, FPCLs were fixed under tension[18] with 4% paraformaldehyde for 30 min prior to being processed for immunofluorescence labeling. To visualize cellular morphology F-actin was stained using Phalloidin-Alexa Fluor 594 and DAPI for nuclear staining (Invitrogen, UK)[19].

Statistical Analysis

Results are presented as means of three independent experiments and standard error of the mean (SEM) was calculated for each experiment analyzed for Dupuytren's nodule, cord and control flexor retinaculum cells. Comparisons between groups were made using a Mann-Whitney nonparametric test to compare intragroup data, or a one-way ANOVA for intergroup data, using Prism software (GraphPad Software). Significance was achieved if $P < 0.05$.

Results

Effect of a single static load on FPCLs contractility and MMP / TIMP expression

Force generation in nodule cells was significantly greater ($P<0.001$), compared to cord and FR cells. Nodule cells continued to contract to a maximum of 137 dynes (mean: 3.3±1.1 dynes/hr) at 24 hr and did not reach tensional homeostasis, for cord cells this was a maximum of 72.8 dynes (mean: 2.0±0.9 dynes/hr) (Fig.2A,D). In contrast, FR cells contracted to a maximum force of 48 dynes at 24 hr and reached tensional homeostasis on average after 15 hr.

Under static load there was no significant difference in expression of MMP-1, -2, -9, -13, TIMP-1 and -2 in all three cell types ($P=0.3$). There was a higher TIMP / MMP mRNA expression ratio amongst all cell types, with significantly more TIMPs expressed compared to MMPs ($P=0.02$) (Fig.3).

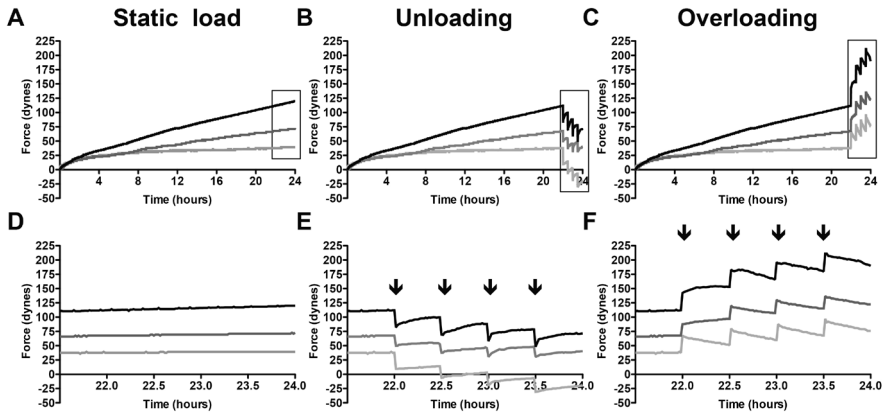

Figure 2. Effect of mechanical loading on FPCL contraction. Isometric contraction of collagen gels seeded with 5 million cells from either FR, cord or nodule (n=5). (A-F) FPCLs were cultured for 24hr in the t-CFM and subjected to a static load (A, magnified in D), to 4 sequential uniaxial reductions in load (B, magnified in E) and overloadings (C, magnified in F) of 30 dynes, each separated by 30 minutes. Real-time isometric force contraction was quantified. Data shown represent the mean of triplicate experiments.

Effect of unloading on FPCL contractility and MMP / TIMP Expression

Next we tested whether mechanically unloading the FPCL led to changes in contractile profiles and gene expression. All three cell types responded to this reduction in load by significantly increasing cellular contraction (Fig. 2B,E). Nodule cells subjected to a reduced load significantly upregulated expression of MMP-1 (threefold), -2 (fivefold) and -9 (threefold) compared to a static load (P=0.01) (Fig.3). In contrast, cord and FR cells subjected to a reduced load only upregulated expression of MMP-9 (twofold) compared to a static load (P=0.02). Nodule cells showed no changes in the expression of TIMPs after a reduction in load although cord cells significantly upregulated TIMP-1 expression (twofold) after a reduction in load (P=0.04).

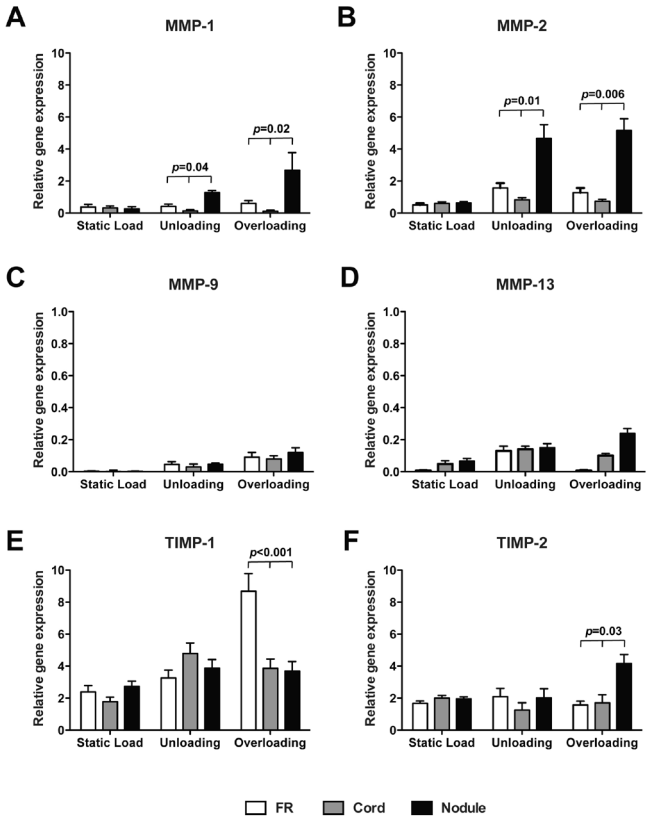

Figure 3. MMP/TIMP expression with mechanical loading. Relative gene expression of MMPs and their TIMPs in collagen gels seeded with 5 million cells from either FR, cord or nodule (n=5). (A-F) FPCLs were cultured for 24hr in the t-CFM and subjected to a static load, unloading and overloading. After 24hr cells from the FPCLs were isolated and levels of MMP-1, -2, -9, -13, TIMP-1 and -2 mRNA were compared to GAPDH by quantitative pcr. Data are shown as the mean of triplicate experiments (±SEM).

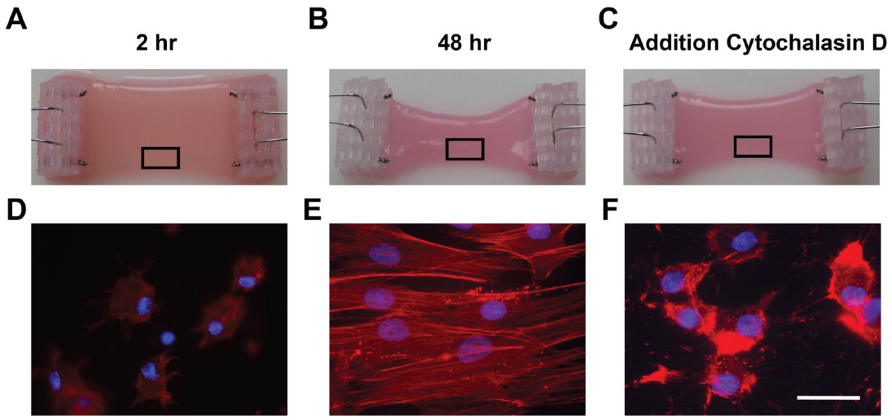

Figure 4. Immunofluorescence labeling and matrix remodeling over 48hr. Fibroblast seeded collagen gels with embedded flotation bars cultured in the t-CFM, tethered between a fixed point and a force transducer. (A-C) Cells contract and remodel the collagen gel in a time-dependent manner. (D-F) To visualize the cellular morphology collagen gels seeded with nodule cells were fixed after 2hr (A,D), 48hr (B,E) and after the addition of cytochalasin D (C,F) to abolish cell-mediated force generation. Representative sections of FPCL highlighted in section (A-C) were processed for immunofluorescence using phalloidin (red) and DAPI (blue). Scale bar: 30 µm.

Effect of overload on contractility and MMP / TIMP expression

Next we assessed whether externally applied overloads led to changes in contractile profiles and gene expression. Nodule subjected to overload responded by significantly increased cellular contraction ($P<0.01$) (Fig. 2C,F), a greater increase compared to cord cells. In subsequent 3 post overload periods the nodule and cord cells responded by decreasing cell generated force to a negative gradient, which corresponded to gradients of FR cells. Nodule cells subjected to an overload upregulated expression of MMP-1 (threefold), -2 (fivefold), -9 (fourfold) and -13 (twofold) compared to a static load ($P=0.02$). Conversely, cord and FR cells only significantly upregulated MMP-9 (threefold) compared to static load ($P=0.03$) (Fig.3). TIMP-2 expression was significantly increased (twofold) in

nodule cells (P=0.03), but not for cord and FR cells (P=0.7). In contrast, TIMP-1 expression was significantly increased in cord (twofold) (P=0.02) and FR cells (fourfold) (P<0.001), but not for nodule cells (P=0.3).

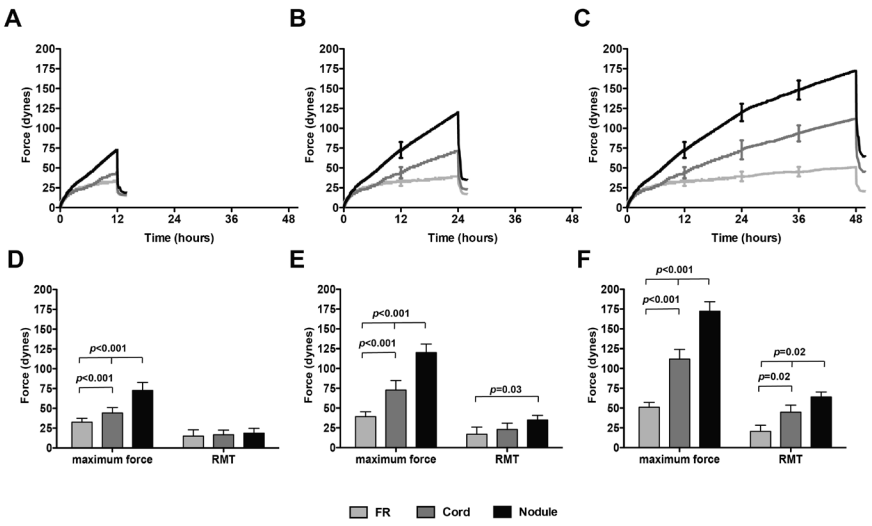

Figure 5. Force generation and matrix remodeling over 48hr in the t-CFM. FPCLs were seeded with 5 million cells from either FR, cord or nodule cells (n=5). (A-C) FPCLs were cultured for 12hr (A), 24hr (B) and 48hr (C) in the t-CFM and real-time isometric force contraction was quantified. Following these time points, cell-mediated force generation was abolished by addition of a saturating dose of cytochalasin D. Data shown represent the mean of triplicate experiments. (D-F) The total force generated by the FPCLs and the residual matrix tension, after cell-mediated force generation was abolished, over 12hr (D), 24hr (E) and 48hr (F) is shown. Data are shown as the mean of triplicate experiments (±SEM).

Permanent matrix remodeling measured by residual matrix tension (RMT)

We quantified permanent matrix remodeling by calculating the RMT following the addition of a saturating dose of cytochalasin D (Fig.4). After 12 hr the RMT was minimal, between 15 and 19 dynes for all three cell types, showing no evidence of permanent remodeling (Fig.5.A,D). RMT at 24 hr was significantly higher for

nodule 35 (±6) dynes (P=0.03) compared to FR 17 (±9) dynes, for cord cells this was 22.9 (±8) dynes. The RMT increased further at 48 hr: nodule was significantly higher, 64 (±6) dynes (P=0.02), compared to cord, 44.8 (±9) dynes, and FR cells, 21 (±8) dynes.

Discussion

Our findings suggest significant differences between Dupuytren's nodule and cord fibroblast in their response to mechanical stimulation, with respect to contractility, MMP / TIMP gene expression and permanent matrix remodeling. Nodule derived cells are more contractile than cord or FR cells. Nodule cells respond to externally applied forces by further increasing contractility and this difference was mirrored by changes in MMP/ TIMP expression, this increase in contractility was greater compared with cord and FR cells. We also found that nodule cells exerted greater RMT compared to cord and FR cells, suggesting that these cells not only have a highly contractile phenotype but also play a crucial role in matrix turnover and remodeling. These findings would be consistent with the in vivo observation, whereby nodules in active disease are comprised mainly of contractile myofibroblasts and fibrous, less cellular cord represents residual or burnt out disease[12].

We measured isometric cell contractility in constrained FPCLs that generate a static load[20]. Nodule cells significantly increased contraction of FPCLs, compared to cord and control cells (P<0.001). Previous studies have shown these differences between Dupuytren's nodule compared to cord and FR cells[2,13,21] or with matched dermal cells[22]. In line with these studies, we found that control cells reached tensional homeostasis, whereas Dupuytren's derived cells continued to contract over a 24h test period.

However, the extracellular matrix is not static but is constantly being remodeled in response to the local, mechanical environment. Therefore, we also tested nodule, cord and FR cells contractility in response to altered mechanical

load. Overloading FPCLs seeded with Dupuytren's derived cells led to increased cellular contraction in the first overload whereas FR cells responded to this externally applied load, by decreasing cell generated force to a negative gradient. All cell types decreased cell generated force in subsequent overloads. We also tested the effect of a reduced load on FPCLs and, interestingly, all cell types responded to this reduction in load by significantly increasing cellular contraction. This suggests that Dupuytren's derived cells have an innate ability to continue to contract in response to externally applied overload, as compared to control cells, whereas all the cell types respond to underloads by increasing contractility equally. An explanation for the latter is tensional homeostasis, whereby cells will establish a level of tension within the collagen matrix, and in the case of a reduced load act to maintain the level of tension against the opposing influence of mechanical unloading[17,23-25]. This mirrors the situation in tendons. In lax tendons, native tendon cells have the ability to contract and re-establish tensional homeostasis. Tendon cells have also been shown to develop a cytoskeletal tensional homeostasis that, in turn, calibrates their mechanoresponsiveness to externally applied loads and, ultimately, their gene expression[24].

Cellular responses to tension include changes in contractility, but also in synthesis of matrix components and regulatory enzymes such as MMPs[26-28]. In Dupuytren's tissue MMP-1, -2, -9 and -13 are significantly increased compared to control palmar fascia[11,29-30]. Therefore, we chose to test these MMP expressions in FPCLs subjected to different loads and looked at expression of their TIMPs which are specific inhibitors that act to control the local activities of MMPs[8]. Under static loads we found very low expression levels of MMPs and TIMPs, implying low matrix turnover. Very low measured basal levels of MMPs and TIMPs are found in non-wounded skin, which is not under altered mechanical tension[31]. However, in Dupuytren's contracture the mechanical environment of the ECM is likely to be more dynamic as it is subjected to frequent loads applied to the hand. Indeed, we found that with both a reduction in load and overloading, nodule cells significantly increased ($P<0.02$) expression of MMP-1, -2, -9, -13 and no significant change in TIMP 1 expression. In contrast, we observed downregulation of

MMP-1 after mechanical stimulation in cord cells and in parallel an upregulation of TIMP-1 in cord and FR cells suggesting differential ECM remodeling in nodule compared to cord and FR cells. The upregulation of MMP-1, -2 and -9 has also been demonstrated in Dupuytren's tissue subjected to defined creep loading in a custom built tensiometer, which simulated the loading regime, found in the continuous elongation technique[32]. However, our findings suggest these changes in MMP expression in Dupuytren's contracture also occur with mechanically unloading. This may be of importance in the clinical setting, when mechanically unloading of the palmar fascia has been observed after fasciotomy[33] and residual Dupuytren's tissue appears to regress only temporarily with high rates of recurrence of 85% within 5 years[34]. These changes in MMP expression and increase in force generation may also explain why when external splints or fixation devices have been used to stretch and straighten contracted digits forcibly, Dupuytren's contractures recurred when the device was removed without surgical excision[35].

The contractile nodule derived cells hold the matrix in a newly shortened position (RMT), while concurrently cells act to permanently remodel the surrounding matrix as suggested by an increase in MMP/TIMP expression. Taken together these findings show that nodule cells have a significantly increased remodeling capacity compared to cord and control cells. Although blocking MMP activity using a broad-spectrum MMP inhibitor, ilomastat, has been shown to decrease RMT and matrix remodeling by Dupuytren's cells using the same model system[36,37], the fundamental problem still appears to be determined by an abnormality of nodule derived cell contraction. Cell contraction over time will lead to subsequent matrix remodeling and in turn digital contracture. Therefore interventional therapies should target nodule cells to prevent permanent matrix remodeling and subsequent contracture.

Acknowledgments

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Chapter 3

Isometric contraction of Dupuytren's myofibroblasts is inhibited by blocking intercellular junctions

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Abstract

Myofibroblasts are responsible for both physiological wound and scar contraction. However, it is not known whether these cells act individually to contract the surrounding matrix or whether they behave in a coordinated manner. Therefore, we studied intercellular junctions of primary human myofibroblasts derived from patients with Dupuytren's disease, a fibrotic disorder of the dermis and subdermal tissues of the palm. The cells were maintained in anchored three-dimensional collagen lattices to closely mimic conditions in vivo. We found that selective blockade of adherens, mechanosensitive or gap junctions effectively inhibited contraction of the collagen matrices and down-regulated the myofibroblast phenotype. Our data indicate that myofibroblasts in part function as a coordinated cellular syncytium and disruption of intercellular communication may provide a novel therapeutic target in diseases characterized by an overabundance of these contractile cells.

Introduction

Dupuytren's disease is a common fibroproliferative disorder with a prevalence of over 7% in the USA[1]. The most frequent manifestation is progressive flexion deformities of the digits of the hand, resulting in significantly compromised function. The cell responsible for the contraction of the palmar fascia and hence the digits in Dupuytren's disease is the myofibroblast. Myofibroblasts characteristically express α-smooth muscle actin (α-SMA), which is the isoform present in vascular smooth muscle cells, in addition to the β- and γ-cytoplasmic actins that are traditionally found in fibroblasts[2]. Myofibroblasts in Dupuytren's tissue exist in aggregates both at the early phase of the disease[3] and also in mature Dupuytren's cords, where they represent the vast majority of the cells in the histological nodules that are found overlying the finger joints[4]. We hypothesized that the myofibroblasts in Dupuytren's nodules coordinate their activity via intercellular junctions.

Cells primarily communicate via three distinct means; adherens junctions, mechanosensitive and gap junctions. Adherens junctions are composed of cadherins that extend through the plasma membrane and mediate calcium-dependent cell-cell adhesion by homophilic association of their ectodomains[5]. The cadherin receptors associate intracellularly with several structural and signaling proteins, most notably the catenins, as well as the actin cytoskeleton[5]. Cadherins comprise a large family, of which most members exhibit a tissue specific distribution. For example, E-cadherins are exclusively expressed in epithelia[6]. Differentiation of primary rat lung or subcutaneous fibroblasts to myofibroblasts in vitro using TGF-β1 is accompanied by an increase of OB-cadherin expression and a decrease of N-cadherin expression, with concomitant upregulation of α-SMA. In vivo, development of myofibroblasts in granulating cutaneous wounds was associated with upregulation of cadherin expression, and increased α-SMA expression correlated with markedly higher levels of OB-cadherin[7]. OB-cadherin junctions exhibit higher adhesion strength than N-cadherin junctions[8]. Inhibition of OB-cadherin, but not of N-cadherin, with

specific peptides reduced the contraction of myofibroblast-populated collagen matrices[7], suggesting that OB-cadherin containing intercellular contacts play an important role in coordinating the contraction of differentiated myofibroblasts.

Mechanosensitive ion channels open when force is transmitted by an adjacent cell via adherens junctions, allowing an influx of cations such as calcium. They have been described in a variety of cells ranging from bacteria[9] to mammalian fibroblasts[10]. In fibroblasts, calcium ion influx through mechanosensitive channels opened by tension applied via N-cadherins at the adherens junctions has been shown to recruit intracellular actin filaments at the intercellular contacts[10].

Adjacent cells can also communicate directly via gap junctions. Gap junctions are composed of six connexin 43 (Cx43) molecules. Hexamers in adjoining cells make direct contact to allow passage of molecules of up to 1kD between cells via hydrophilic channels. Accumulating evidence demonstrates that gap junctions are also important in the generation of myofibroblasts from precursor cells. Infusion of gap junction inhibitors into polyvinyl sponges implanted subcutaneously into rats led to reduced collagen deposition and numbers of α-SMA positive myofibroblasts[11,12]. TGF-β1, a potent inducer of myofibroblast phenotype[7,13], increased Cx43 as well as α-SMA expression in cardiac fibroblasts[14]. Antisense oligodeoxynucleotides to Cx43 applied to excisional skin wounds in mice led to earlier appearance followed by the disappearance of myofibroblasts, and overall accelerated healing. This was accompanied by increased TGF-β1 and collagen-1α expression and reduced numbers of neutrophils and macrophages and mRNA for TNF-α[15].

We explored the hypothesis that myofibrobasts from patients with Dupuytren's disease act in a coordinated fashion by communicating with each other through adherens, mechanosensitive or gap junctions. There is no animal model for Dupuytren's disease. In an effort to emulate the in vivo situation as closely as possible, we employed a validated system whereby we studied cells isolated from nodules excised from patients with Dupuytren's disease and cultured up to a maximum of passage 2 in three dimensional collagen lattices

under isometric conditions in a culture force monitor[16]. Myofibroblasts only maintain their phenotype under stress and loss of tension is associated with disassembly of α-SMA within minutes[17]. To assess the relative contribution of each junction type respectively, we used specific blocking peptides to N- and OB-cadherins[7] as well as inhibitory cadherin antibodies, siRNA blockade of OB- or N-cadherin, carbenoxolone, which is a saponin that inhibits gap junction communication by disassembling Cx43 containing plaques[18] and gadolinium, which is an inhibitor of cationic mechanosensitive channels[10,13,19,20]. Here we show that specific blockade of N- and OB-cadherins, mechosensitive channels or intercellular gap junctions leads to reduced myofibroblast contractility in vitro, suggesting that the cells partly act in concert, effectively as a syncitium.

Methods

Patient samples

Tissue samples were obtained following informed consent (REC 07/H0706/81). Dupuytren's nodular tissue and matched full-thickness skin (harvested from the groin or medial aspect of arm) were obtained from patients with Dupuytren's disease undergoing dermofasciectomy.

Cell culture

Human dermal fibroblasts (HDF) were isolated from non-palmar skin and Dupuytren's myofibroblasts (MF) were isolated from α-SMA-rich nodules[16]. Tissue samples were dissected into small pieces and digested in Dulbecco's modified Eagle's medium (DMEM) (Lonza) with 1% penicillin–streptomycin (PAA) and 5% fetal bovine serum (FBS) (Gibco) with type I collagenase (Worthington Biochemical Corporation) + DNase I (Roche Diagnostics) for up to 2h at 37°C. Cells were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO2. Cells up to passage 2 were used for experiments.

Culture Force Monitor (CFM)

Measurement of the isometric contractile forces generated by cells within 3D collagen matrices was performed as previously described[16]. Briefly, $2x10^6$ cells were seeded in 2.5 ml of type I collagen gel (FirstLink), and the resulting 3D matrices were suspended between two flotation bars and held stationary at one end whilst the other was attached to a force transducer. Fibroblast populated collagen lattice generated tensional forces were continuously measured for 24h and data logged every minute (dynes: $1x10^{-5}$ N). Cell populated matrices were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator for 24h with 5% CO2 and treated with either N- or OB-cadherin peptide blockers (Adherex) and respective control peptide blockers, anti-N-cadherin antibody (Sigma), anti-OB-cadherin antibody (Santa Cruz, Mubio), anti-E-cadherin antibody (Invitrogen), IgG isotype control, gadolinium or carbenoxolone (Sigma). Compounds were added to CFM culture constructs at the start of the experiment except as in supplemental figure 3, where compounds where added either at the start or 12 h later. Cytochalasin D (Sigma) was added at a saturating dose of 20µg/ml cytochalasin D (Sigma) to fibroblast populated collagen matrices[21]. Experiments using each patient sample were performed in triplicate. Data are shown as the mean ± S.E.M from n≥3 patients.

Quantitative RT-PCR

Cells were cultured in monolayer and treated with either N- or OB-cadherin peptide blockers, gadolinium or carbenoxolone for 24h and total RNA was extracted from each sample using the QIAamp RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). For real-time quantitative reverse transcription PCR, Inventoried TaqMan® Gene expression Assays were used for α-SMA (Hs00426835-g1), COL1 (Hs00164004-m1), N-cadherin (Hs00362037-m1), OB-cadherin (Hs00901475-m1) and Cx43 (Hs00748445-s1) (Applied Biosystems) with Reverse Transcriptase qPCR™ Mastermix No ROX (Eurogentec). Samples were run on the ABI 7900HT Fast

Real-Time PCR System (Applied Biosystems). Expression was normalized to GAPDH (Hs02758991-g1) (Applied Biosystems) and compared to the level of gene expression in either baseline respective cell types or to the level of gene expression in HDF, which were assigned the value of 1 using delta CT analysis performed with SDS software (Applied Biosystems). Data are shown as the mean ± S.E.M from n≥3 patients (each assay was performed in triplicate).

Western blots

Cells were cultured in monolayer and treated with either N- or OB-cadherin peptide blockers, gadolinium or carbenoxolone for 24h prior to protein extraction. Cell lysates were prepared in lysis buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, and 1% Nonidet P-40), containing protease inhibitor cocktail (Roche Biochemicals) and then electrophoresed on 10% SDS polyacrylamide gels (Life Technologies), followed by electrotransfer of proteins onto PVDF transfer membranes (Perkin Elmer Life Sciences). Membranes were blocked in 5% BSA/ TBS + 0.05% Tween and incubated overnight at 4°C with primary antibodies against α-SMA primary, anti-N-cadherin (Sigma), anti-OB-cadherin (Mubio), Cx43 or vimentin (Abcam). Horseradish peroxidase-conjugated anti-mouse IgG (Dako) or anti-rabbit IgG (Amersham Biosciences) were used as secondary antibodies. Bound antibody was detected using the enhanced chemiluminescence kit and visualized using Hyperfilm MP (Amersham Biosciences). Protein expression was assessed using vimentin as a loading control. Semi-quantitative analysis of protein expression was performed using densitometry analysis software (Phoretics International, UK). Gels shown are representative of 3 patients.

siRNA

Cells were seeded in 6 well plates at a density of 200,000 cells per well and cultured with DMEM, 10% FBS and 1% penicillin–streptomycin for 16hr. Cells were then washed in PBS and transfected with 90 pmol siRNA and Lipofectamine (both Invitrogen) following the manufacturers protocol. The assay was carried out with siRNA complexes diluted in optiMEM (Invitrogen) with 2% FBS. Inventoried

silencer-select reagents and respective non-targeting negative controls were used for N-cadherin (Hs464829), OB-cadherin (Hs116471) and Cx43 (Hs368353) (Applied Biosystems). siRNA for mechanosensitive junctions is not available. Negative control siRNAs 1 and 2 (Applied Biosystems) were used with sequences that do not target any gene product and provide a baseline to compare siRNA-treated samples. After 24hr of incubation, the transfection medium was replaced by DMEM, 10% FBS and 1% penicillin–streptomycin and the cells were incubated for a further 24hr. PCR and western blot analysis was used to quantify knockdown of gene and protein expression, and these cells were also used for experiments in the CFM. Data are shown as the mean ± S.E.M from n≥3 patients, with each assay was performed in triplicate).

Immunofluoresence

Cells were cultured in monolayer or 3D collagen matrices with DMEM, 10% FBS and 1% penicillin–streptomycin for 24h then fixed for 10 min with 3% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 (Sigma). Cells were stained with either a rabbit monoclonal anti-β catenin or a rabbit polyclonal anti-Cx43 (Abcam) followed by Alexa Fluor 568-conjugated goat anti-rabbit antibody (Invitrogen), Alexa Fluor 488 Phalloidin (Invitrogen) and DNA with DAPI (Sigma). Secondary antibody alone was used as an immunolabelling control. Images were acquired using confocal microscopy oil immersion objectives (60x) and the signal was analyzed by Ultraview confocal microscopy (PerkinElmer). The compact configuration of MF and HDF in 3D collagen lattices meant that we were unable to reliably assess Cx43 labeling under these conditions and therefore staining for Cx43 was performed in monolayer cultures. Cell viability was assessed using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen). Immunofluoresence images shown are representative of cells from 5 patients.

Statistics

The rate of MF or HDF populated collagen lattice contraction (dynes/h) was calculated by measuring the average gradient of the curve between 20 and 24 h. One-way analysis of variance (ANOVA) using Bonferroni's multiple comparison test was used for comparing all conditions. All statistical analyses were performed using software (GraphPad Software version 5.0c). Significance was achieved if $p<0.05$.

Results

Dupuytren's myofibroblasts predominantly express OB-cadherin

We examined adherens and gap junctions in myofibroblasts (MF) and in non-palmar dermal fibroblasts (HDF) from the same patients, and compared the relative expression of OB- and N-cadherins, and Cx43 in each cell type. As expected, MF expressed higher levels of COL1 and α-SMA mRNA and higher levels of α-SMA protein than HDF (Fig 1A). MF also expressed more OB-cadherin at both message and protein levels compared to HDF, but expressed less N-cadherin (Fig 1B) and less Cx43 (Fig 1C) than HDF. Within 3D collagen matrices, adherens junctions, visualized by immunofluoresecent staining for β-catenin, were clearly observed between adjacent MF (Fig 1D). HDF in 3D collagen matrices did not demonstrate the distinctive intracellular organization of α-SMA in stress fibers that is characteristic of MF, and intercellular adherens junctions between HDF (Fig 1E) were less well defined than those between MF. Gap junctions, visualized by immunostaining for Cx43, were more prominent in HDF (Fig 1F). Whilst some of the labeling was localized to the perinuclear cytoplasm in both HDF and MF, distinctive labeling was visualized at sites of intercellular contact, particularly between HDF (white arrows in Fig 1F).

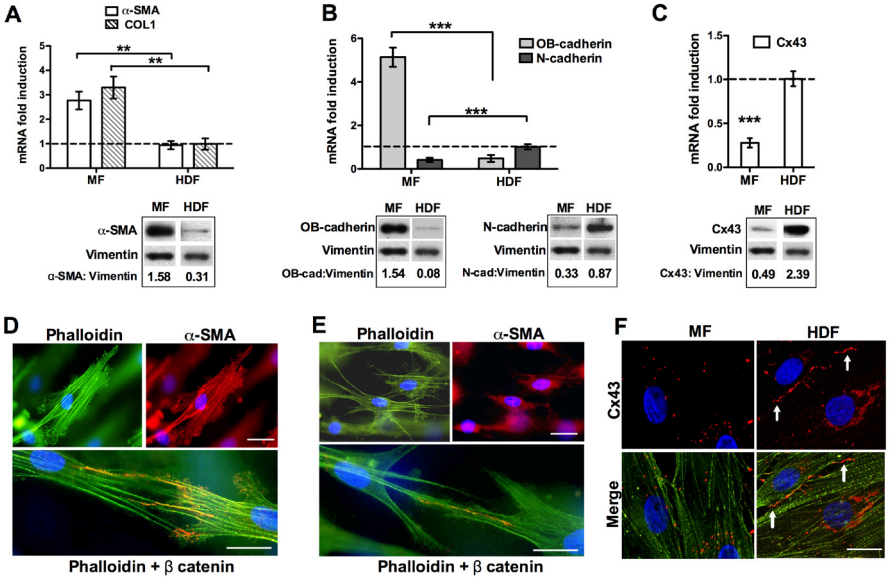

Figure 1. Dupuytren's myofibroblasts predominantly express OB-cadherin. Expression levels of (A) α-SMA, and COL1, (B) OB- and N-cadherin and (C) Cx43 in MF and HDF. mRNA expression levels are compared to baseline HDF. *P < 0.05, ** P < 0.001, *** P < 0.001. N≥3. Immunofluoresence staining of (D) MF and (E) HDF cultured in a 3D collagen matrices for 12h. (Top panels) F-actin stained using phalloidin (green), α-SMA (red) and nuclei stained with DAPI (blue). (Lower panel) F-actin (green), β-catenin (red) and nuclei stained with DAPI (blue). (F) Immunofluoresence staining of MF and HDF cultured in monolayer showing Cx43 (red) and nuclei stained with DAPI (blue). Lower panels show merged images of the same cells also stained for F-actin using phalloidin (green). Scale bar: 10μm.

Blocking OB- or N-cadherins downregulates the myofibroblast phenotype

We next examined the contribution of each type of cell junction to MF and HDF contraction using a number of different approaches. Inhibition of either OB- or N-cadherins with specific blocking peptides resulted in a dose dependent reduction in contraction of MF whilst control peptides had no effect (Fig 2A).

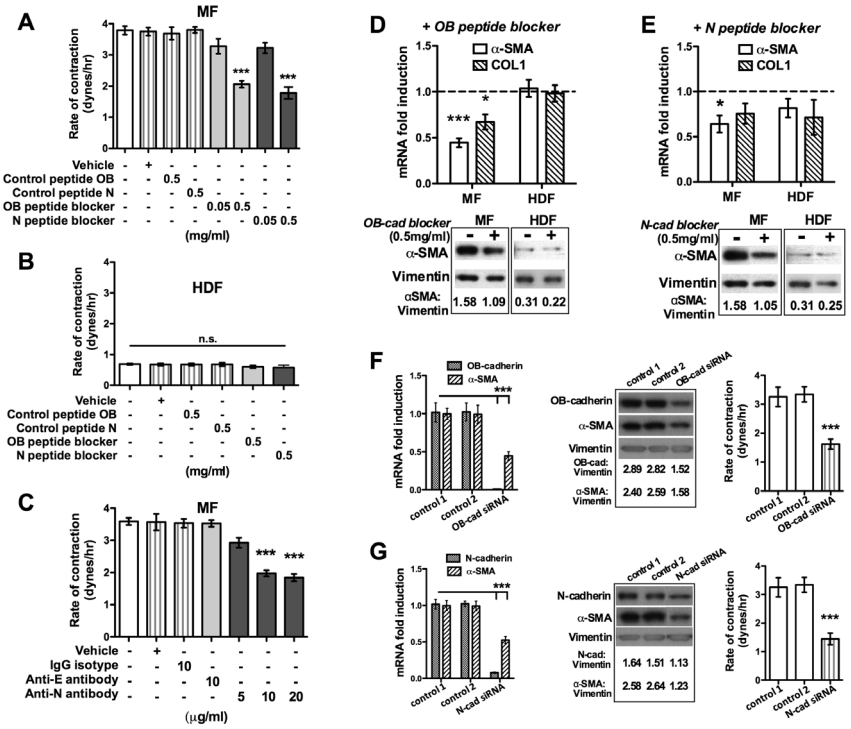

Figure 2. Blocking OB- or N-cadherins downregulates the myofibroblast phenotype. The effect of OB- or N-cadherin peptide blockers (0.05-0.5mg/ml) on MF (A) and HDF (B) contractility. (C) The effect of anti-N cadherin, and anti-E cadherin antibodies (5-20μg/ml) on MF contractility. The effect of OB-cadherin peptide blocker (0.5mg/ml) (D) and N-cadherin peptide blocker (0.5mg/ml) (E) on α-SMA and COL1 mRNA and α-SMA protein in MF and HDF. Effect of silencing of OB-cadherin (F) or N-cadherin (G) on cadherin and α-SMA expression and MF contractility. mRNA expression levels are compared to respective untreated cells. N≥3. *P < 0.05, *** P < 0.001.

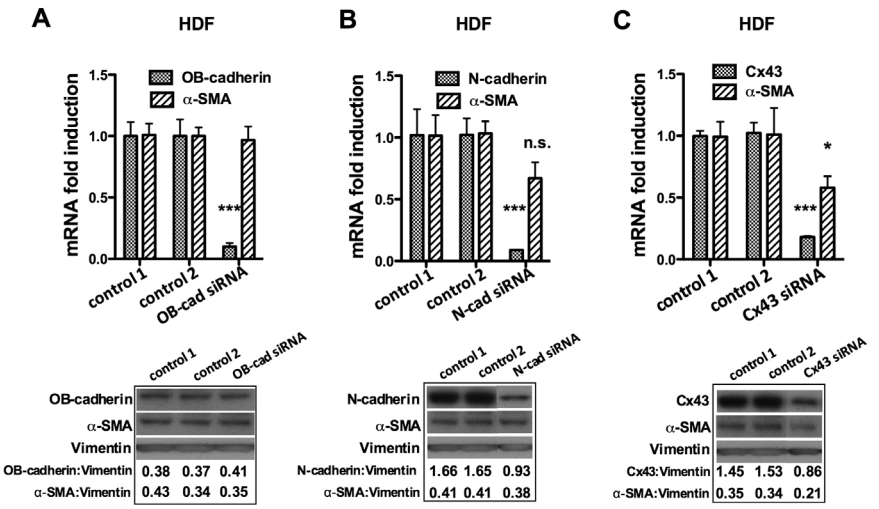

Figure 3. Silencing of OB-, N-cadherin and Cx43 in HDF. (A) OB-cadherin siRNA reduced OB-cadherin gene expression but not α-SMA mRNA and protein levels in HDF. (B) N-cadherin siRNA reduced α-SMA and N-cadherin RNA and protein levels in HDF. (C) Cx43 siRNA reduced α-SMA and Cx43 mRNA and protein levels in HDF. mRNA expression levels are compared to baseline levels in negative control cells. N≥3. **P < 0.05, *** P < 0.001.

Neither cadherin blocking peptide had any effect on the low baseline contraction of HDF (Fig 2B). Neutralizing antibodies for N-cadherin also resulted in reduced MF contractility in a dose dependent manner, compared to isotype controls or antibodies to the epithelial specific E-cadherin (Fig 2C). Blockade of OB-cadherin with peptides resulted in a reduction in α-SMA and COL1 message and α-SMA protein in MF but not HDF (Fig 2D). Peptide blockade of N cadherin also caused a significant reduction in α-SMA message and α-SMA protein in MF but not HDF (Fig 2E). Cell viability was not impaired over 24h following addition of any of the peptides or antibodies. siRNA for OB-cadherin effectively reduced OB-cadherin mRNA and protein levels in MF.

Silencing of this cadherin also caused a reduction in α-SMA expression, and inhibited the isometric contraction of collagen matrices by MF (Fig 2F). Similarly, siRNA for N-cadherin effectively reduced N-cadherin mRNA and protein levels as well as α-SMA expression and MF contractility (Fig 2G). siRNA knockdown of OB-cadherin resulted in reduction in OB-cadherin mRNA in HDF but had no effect on OB-cadherin protein or α-SMA expression (Fig 3A). siRNA knockdown of N-cadherin in HDF resulted in a reduction in N-cadherin mRNA and protein, but had no effect on α-SMA expression (Fig 3B).

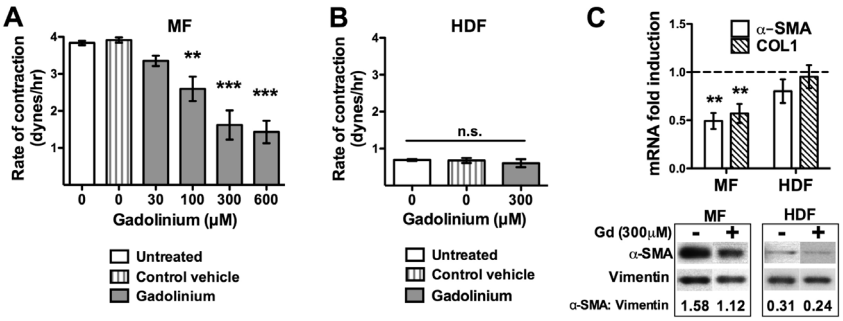

Figure 4. Blocking mechanosensitive junctions downregulates myofibroblast activity. The effect of gadolinium (300μM) on MF (A) and HDF (B) contractility. (C) The effect of gadolinium (300μM) on α-SMA and COL1 mRNA and α-SMA protein expression in MF and HDF. mRNA expression levels are compared to baseline levels in respective untreated cells. N≥3 **P < 0.01, *** P < 0.001.

Blocking mechanosensitive junctions downregulates myofibroblast activity

Cells also communicate via mechanosensitive junctions that open as a result of force transmitted through adherens junctions. Addition of the mechanosensitive channel blocker gadolinium led to dose-dependent reduction in contractility of MF (Fig 4A) but not of HDF (Fig 4B). The reduced contractility of MF was accompanied by a reduction of α-SMA and COL1 message expression as well as α-SMA protein (Fig 4C) in MF but not in HDF. Cell viability was unaffected by gadolinium concentrations up to 600μM, but reduced cell viability was observed at 1200μM.

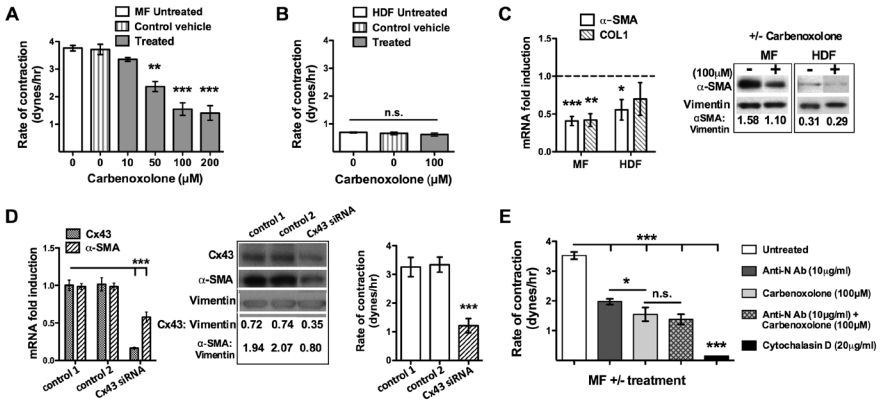

Figure 5. Blocking gap junctions selectively inhibits myofibroblasts. The effect of carbenoxolone (100μM) on (A) MF and (B) HDF contractility. (C) The effect of carbenoxolone (100μM) on α-SMA and COL1 mRNA and α-SMA protein expression in MF and HDF. mRNA expression levels are compared to respective untreated cells (D) The effect of silencing of Cx43 on Cx43 and α-SMA gene, protein expression and MF contractility. (E) The effect of addition of anti-N cadherin antibody (10μg/ml) in combination with carbenoxolone (100μM) on MF contractility compared to carbenoxolone (100μM) alone or to cytochalasin D (20μg/ml). N≥3. *P < 0.05, *** P < 0.001

Blocking gap junctions selectively inhibits myofibroblasts

Finally, we examined the effect of blockade of gap junctions in MF and HDF. Selective blockade of gap junctions by carbenoxolone resulted in a dose dependent decrease in isometric contraction of MF (Fig 5A) but not of HDF (Fig 5B). Carbenoxolone also led to a reduction in expression of COL1 mRNA and α-SMA mRNA and protein in MF; a reduction in α-SMA mRNA only was observed in HDF (Fig 5C). Impaired cell viability was not seen after 24h following addition of carbenoxolone up to a dose of 200μM; higher concentrations (500-1000μM) resulted in cell death. siRNA knockdown of Cx43 in MF reduced mRNA and protein expression of Cx43 and α-SMA, as well as a reduction in contractility of MF in 3D collagen matrices (Fig 5D). Silencing of Cx43 in HDF resulted in reduced expression of Cx43 and α-SMA mRNA and protein (Fig 3C). Combined treatment of MF with neutralizing antibody to N-cadherin and carbenoxolone did not result in reduction of contractility above that seen with carbenoxolone alone. However, contractility was completely abolished by addition of cytochalasin D (Fig 5E).

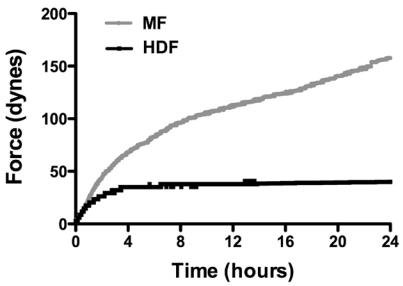

Figure 6. Baseline MF and HDF contractility in the CFM. Representative baseline force contraction (dynes) of collagen lattices populated with either MF (gray) or HDF (black) cells and attached to the CFM. HDF contractility plateaued over the 6-24h period whereas MF continued to contract.

Inhibitors of intercellular junctions are equally effective before or after establishment of junctions in vitro

Both myofibroblasts and fibroblasts contract 3D collagen lattices. However, whilst the latter reach tensional homeostasis at 6h, myofibroblasts continue to contract (Fig 6)[16]. In all the culture force monitor experiments described above the inhibitory compounds were added at the outset of the experiment. It is likely that the freshly disaggregated myofibroblasts need time to re-establish intercellular junctions and cell-matrix adhesion complexes. To discriminate between the possibility that blockade of intercellular communication merely prevents the formation of intercellular contacts, rather than inhibiting established cell connections, inhibitors were also added after cells had been cultured in matrices for 12h and cell-cell junctions established (Fig 1D-F). Comparison of the effect of addition of inhibitors of adherens, mechanosensitive or gap junctions initially or 12h later, showed no difference in the reduction of contractility compared to controls (Fig 7A-C).

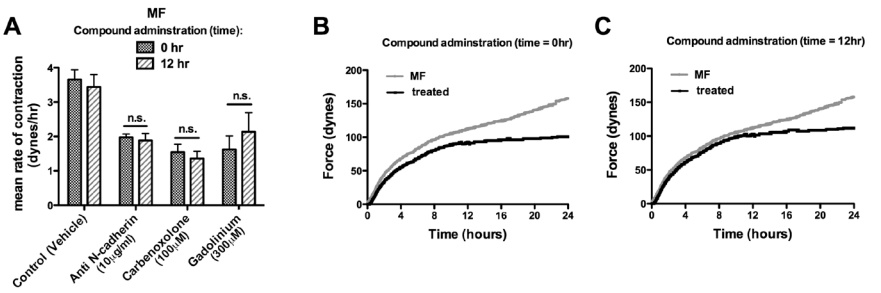

Figure 7. Addition of anti-N cadherin, carbenoxolone and gadolinium at beginning of experiment or after 12 hours had no significant difference on rate of MF contractility. (A) There was no significant difference on the rate of MF contractility when anti-N cadherin (10μg/ml), carbenoxolone (100μM) or gadolinium (300μM) were added initially or after 12 hours. N≥3. (B, C) Representative traces shown after addition of carbenoxolone (100μM) in the CFM either (B) initially or (C) after 12h.

Discussion

We hypothesized that myofibroblasts in vivo may contract in concert, with the activity of groups of cells being coordinated via adherens, mechanosensitive or gap junctions. Consistent with previous reports for myofibroblasts derived following treatment of fibroblasts with TGF-β[8,17], we found that myofibroblasts from Dupuytren's tissue expressed more OB-cadherin at both message and protein levels than HDF, but expressed lower levels of N-cadherin than HDF. However, blocking either N- or OB-cadherin was effective in inhibiting isometric contraction of 3D collagen matrices by primary myofibroblasts from Dupuytren's nodules. This is in contrast to previous publications[7,13] that showed only an OB-cadherin peptide blocker inhibited myofibroblast contraction whilst a N-cadherin peptide blocker only inhibited fibroblast contraction. A possible explanation of the variance of our findings is the source of our cells. We used early passage primary human cells from patients with Dupuytren's disease whereas Follonier et al[13] studied subcutaneous rat fibroblasts up to passage 7 and compared them to myofibroblasts generated by exposure to TGF-β1 over 4 days and Hinz et al[7] studied embryonic rat subcutaneous and lung fibroblasts and myofibroblasts generated by exposure to TGF-β1 over 5 days. An alternative explanation may be that intercellular adherence between myofibroblasts is crucial to their function such that blockade of even the relatively few N-cadherin-containing adherens junctions had a profound effect. We also found that blockade of N-cadherin was equally effective using either a peptide inhibitor or a neutralizing antibody but we are unable to locate an effective commercially available neutralizing antibody to OB-cadherin. These data were confirmed by gene silencing studies. siRNA inhibition of OB-cadherin or N-cadherin resulted in almost complete abolition of their expression at mRNA level and, over 24h, 30-50% reduction in protein expression. This was accompanied by 40-50% reduction in α-SMA protein and 50% reduction in isometric contraction of collagen matrices by MF. In contrast, we found no difference in contractility following OB- or N-cadherin blockade in HDF. Whilst siRNA inhibition of OB-cadherin in HDF resulted in reduced OB-

cadherin expression, no reduction in α-SMA expression was observed. This may be explained by low baseline expression of OB-cadherin in HDF. Similarly, silencing N-cadherin in HDF had no significant effect on α-SMA expression despite relatively more N-cadherin. This may again be due to there being far lower baseline α-SMA expression in HDF compared with MF.

HDF expressed more Cx43 in the resting state and exhibited prominent immunofluorescent labeling compared to myofibroblasts. The only previous study on gap junctions using cells derived from Dupuytren's tissue found that cord-derived cells showed increased intercellular passage of a dye through their gap junctions compared to cells derived from matched nodules[22], which are especially rich in myofibroblasts[4]. We have previously shown using immunohistochemistry that approximately 83 - 92% of cells in Dupuytren's cords are fibroblasts and do not express α-SMA[4]. We found that carbenoxolone, which disrupts gap junctions by dephosphorylation, effectively inhibited isometric contraction of collagen lattices populated by Dupuytren's myofibroblasts. Furthermore, specific knockdown of gap junctions using siRNA directed against Cx43 resulted in a similar reduction in myofibroblast contractility. Follonier et al13 found that the gap junction inhibitors palmitoleic acid or carbenoxolone did not inhibit the spontaneous Ca^{2+} oscillations exhibited by rat myofibroblasts, whereas fibroblast Ca^{2+} oscillations were effectively desynchronized. Together these data demonstrate that gap junctions play a key role in coordinating myofibroblast contraction but not in Ca^{2+} oscillations.

TGF-β1 is a potent inducer of the myofibroblast phenotype in quiescent fibroblasts[7,13] and there is evidence that TGF-β1 and Cx43 are interlinked. However, the precise relationship between the two is unclear and varies according to the system studied. TGF-β1 upregulated Cx43, α-SMA and matrix components in primary human aortic smooth muscle cells[23] and it has been shown that Cx43 positively regulated TGF-β1 activity by competing with Smad 2/3 for binding to microtubules. In neonatal rat cardiac fibroblasts, knockdown of the endogenous Cx43 activity with antisense deoxyneucleotides inhibited α-SMA expression whilst over-expression of Cx43 increased α-SMA expression, and

Cx43 acted cooperatively with TGF-β1 to induce α-SMA expression[14]. The authors speculated that anti-fibrotic therapies based on modification of Cx43 activity may be more appropriate than blocking TGF-β1 signaling by antagonizing TGF-β1 receptors or Smads. Although there was a significant increase in TGF-β1 levels in the tissue post myocardial infarct, with no difference in levels between Cx43-/- and control animals, there was significant down regulation of phosphorylated Smads, which are downstream signaling mediators in the TGF-β1 pathway, in Cx43 deficient mice[24]. Therefore, it is possible that in the heart Cx43 does not directly affect TGF-β1 but instead influences downstream Smad signaling. However, in other systems the relationship between TGF-β1 and Cx43 appears to be reversed. TGF-β1 inhibited Cx43 expression in human detrusor smooth muscle cells found in the bladder[25] and human suburothelial myofibroblasts showed reduced Cx43 on the membrane following exposure to exogenous TGF-β1[26]. It is possible that in our system the carbenoxolone had an effect on the TGF-β1 pathway. Alternatively, carbenoxolone inhibition of myofibroblast contractility may have directly resulted in reduced α-SMA message as a loss of myofibroblast tension has been shown to disassemble α-SMA stress fibers in myofibroblasts within minutes[17]. A direct link between gap junction inhibition and α-SMA protein expression and cell contractility is more likely as we observed similar effects with specific knockdown of Cx43 with siRNA.

It has been shown in rat myofibroblasts that coordination may be controlled through calcium ion influx via mechanosensitive channels, as detected by monitoring periodic Ca^{2+} oscillations between cells[13]. Gadolinium is a specific mechanosensitive channel blocker that acts by altering the packing of membrane phospholipids and applying lateral pressure that squeezes the channels closed[19]. We found that inhibition of contraction of the collagen gel populated by myofibroblasts from Dupuytren's nodules occurred on exposure to gadolinium in a dose-dependent manner and was accompanied by reduced expression of COL1 and α-SMA message as well as α-SMA protein levels. Again, this may reflect the rapid reversal of the myofibroblast phenotype on release of external stress[17].

We have described the effects of inhibition of each individual form of

intercellular junction in vitro and the maximal inhibition in contractility using blocking peptides, neutralizing antibodies or siRNA was approximately 50%. In vivo the various junctions are likely to function concurrently. For example, it has been shown that there is an intimate association between gap junctions and adherens junctions, although the latter are classically considered to transmit mechanical force between cells. In 3T3 cells intracellular co-assembly of Cx43 and N-cadherin is required for gap and adherens junction formation, a process that likely underlies the close association between gap and adherens junction formation[27]. Furthermore, mechanosensitive channels are dependent on force transmitted by adherens junctions. Inhibition of each type of intercellular junction individually failed to abolish tension in the collagen matrices completely. Of all the blockers of intercellular junctions, carbenoxolone had the greatest inhibitory effect on cell contraction in the culture force monitor. Combined inhibition of gap junctions and adherens junctions did not result in any further reduction over that achieved by gap junction blockade alone. However, the addition of cytochalasin D, an actin depolymerizing agent[28], resulted in complete abolition of contractility. These data confirm that MF contraction is wholly dependent on an intact cellular cytoskeleton and suggest that in the absence of effective intercellular communication residual tension exerted by individual cells on the matrix also contributes to MF contraction.

Taken together our data suggest that myofibroblasts act in concert to contract 3D collagen matrices and our in vitro findings indicate that this coordinated activity is responsible for approximately 50% of the contractile effect. The remainder is likely to be due to the action of the individual myofibroblasts on the surrounding matrix. It has been suggested that the force generated by cell contraction is transmitted to the extracellular matrix, which is remodelled to a shortened position, a mechanism termed 'lock-step'[13]. Our data show that inhibitors that disrupt adherens, mechanosensitive or gap junctions can each result in a significant decrease in myofibroblast activity, and hence contraction. This may offer a novel therapeutic approach to downregulate myofibroblast activity in cutaneous and musculoskeletal fibrotic disorders.

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Chapter 4

Adipose-derived stem cells inhibit the contractile myofibroblast in Dupuytren's disease

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Abstract

Background: In an attempt to provide minimally invasive treatment for Dupuytren's disease, percutaneous disruption of the affected tissue followed by lipografting is being trialled. Contractile myofibroblasts drive this fibroproliferative disorder whereas stem cells have recently been implicated in preventing fibrosis. Therefore, we tested the role of stem cells in modulating myofibroblast activity in Dupuytren's disease.

Methods: We compared the effect of co-culturing Dupuytren's myofibroblasts with either adipose or bone marrow-derived stem cells on isometric force generation and associated levels of α-SMA mRNA and protein expression. We also tested the effect of these stem cells on Dupuytren's myofibroblast proliferation and assessed whether this was mediated by cell-cell contact or by a paracrine mechanism.

Results: Addition of adipose-derived stem cells to Dupuytren's myofibroblasts reduced the contraction of the latter with corresponding reduction of α-SMA protein expression, probably through a dilution effect. In contrast, bone marrow-derived stem cells increased the myofibroblast contractility. In addition, adipose-derived stem cells inhibit myofibroblast proliferation and mediate these effects by soluble factors, influenced by cell-cell contact dependent signalling.

Conclusion: Adipose-derived stem cells inhibit the contractile myofibroblast in Dupuytren's disease and these findings lend support to the potential benefit of lipografting in conjunction with aponeurotomy as a novel strategy in the treatment of Dupuytren's disease.

Introduction

Dupuytren's disease is a common fibroproliferative disorder, characterised by development of contractile cellular nodules that progress to form fibrotic cords leading to digital contracture[1,2].

Contractures are most commonly treated by surgical excision or using less invasive alternatives such as division of the cord by percutaneous needle fasciotomy, or more recently by enzymatic digestion with collagenase injections[3]. These less invasive treatment alternatives promote earlier postoperative recovery, although recurrence rates following these procedures remain high[4-6].

In an attempt to overcome high recurrence rates after minimally invasive needle fasciotomy, a novel approach is being trialled using a more extensive percutaneous needle aponeurotomy technique. With this technique numerous superficial nicks are performed along the affected tissue to disintegrate the fibrous cord. Subsequent release of the skin from the subcutaneous layer followed by injection with autologous lipoaspirates restores the subcutaneous fat layer[7].

There is now increasing evidence for the role of mesenchymal stem cells (MSCs) as a potential treatment strategy to alleviate fibrosis. MSCs are multipotent cells that can be easily obtained from a variance of sources, and have been shown to play a role in decreasing fibrosis in models of lung, liver, kidney, heart and also in an animal model of related Peyronie's disease[8-11]. MSCs exhibit potent immunomodulatory and anti-inflammatory effects, either by direct cell-cell contact or by indirect secretion of various soluble factors[12]. Their use has also been examined in anti-tumour therapy where they show anti-proliferative effects[13-16].

In Dupuytren's disease the cell responsible for both contraction and extracellular matrix deposition is the myofibroblast[17], and is characterised by the expression of highly contractile α-smooth muscle actin (α-SMA). Research into treatment strategies should be targeted at modulating these myofibroblasts and inhibiting fibroproliferation. Therefore, we set out to examine the effect of lipoaspirates on myofibroblast activity as these lipoaspirates are known to be a

rich source of stem cells with regenerative potential[18-20]. Specifically, we tested the potential therapeutic benefit of ADMSCs to inhibit fibroproliferation in Dupuytren's disease, and whether this effect was specific to ADMSCs.

Methods

Tissue samples and Cells

Tissue samples were obtained following informed consent (MEC-2010-294). Dupuytren's nodular tissue was obtained from patients with Dupuytren's disease undergoing surgery and normal skin was obtained from adult human skin discarded after reconstructive surgery. Human adipose-derived mesenchymal stem cells (ADMSC) (Lonza) were positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, surface markers and negative for CD14, CD31, CD45 surface markers. Bone marrow-derived mesenchymal stem cells (BDMSC) (Lonza) were positive for CD29, CD44, CD105, CD166, surface markers and negative for CD14, CD34, CD45, surface markers. Cells positive for these markers of ADMSC and BDMSC have been reported to differentiate down various lineages including chondrogenic, osteogenic, adipogenic, myogenic, neural, and endothelial.

Cell culture

Dupuytren's cells, myofibroblasts (MF), were isolated from α-SMA-rich nodules[21,22-23] and human dermal fibroblasts (HDF) were isolated from full-thickness skin samples. Briefly, tissue samples were dissected into small pieces and digested in Dulbecco's modified Eagle's medium (DMEM) (Lonza) containing 1% penicillin–streptomycin (PAA) and 5% fetal bovine serum (FBS) (Gibco) with type I collagenase (Worthington Biochemical Corporation) and frequent gentle agitation for up to 2h at 37°C. Cells were then collected following centrifugation and cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37°C and 5% CO2 in a humidified incubator. Cells up to a maximum of passage 3 were used for experiments to prevent passage-dependent phenotypic alterations.

Culture Force Monitor (CFM)

Measurement of the isometric contractile forces generated by cells within 3D collagen matrices was performed as previously described[21,24]. Briefly, 2 x10^6 cells were seeded in 2.5 ml of type I collagen gel (FirstLink), and 3D matrices tethered between two flotation bars, one attached to a fixed point and the other attached to a force transducer (Fig 1). Fibroblast populated collagen matrices generated tensional forces were continuously measured and data logged every minute (dynes: 1x10^{-5} N) and a contraction profile was generated over 24 h. Cell populated matrices were cultured in DMEM with 2% FBS[25,26] and 1% penicillin–streptomycin at 37°C and 5% CO2 in a humidified incubator.

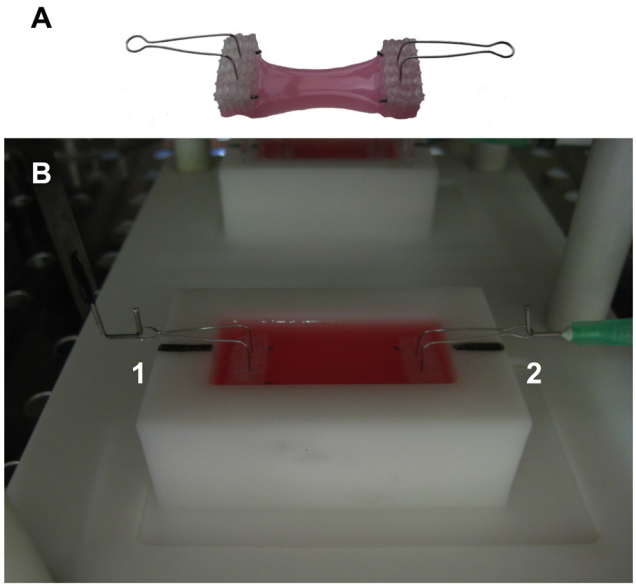

Figure 1. The culture force monitor (CFM). Rectangular seeded collagen gels were caste (A) and floated in medium (B), tethered between two flotation bars one attached to a fixed point (2) and the other attached to a force transducer (1).

Matrices were seeded with co-cultures of different cell proportions (100% MF, 75% MF + 25% ADMSC, BDMSC or HDF, 25% MF + 75% ADMSC, BDMSC or HDF and 100% ADMSC, BDMSC or HDF). The rate of contraction (dynes/h) was calculated by measuring the average gradient of the curve between 8 and 24 h. Experiments shown are from 3 individual patients, each performed in triplicate.

Quantitative RT-PCR

Cells were cultured in monolayer and in either adherent collagen matrices, floating collagen matrices or in tethered collagen matrices in the CFM. Co-cultures were set up with varying cell proportions over 24 h. Total RNA was extracted from each sample using the QIAamp RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). For real-time reverse transcription PCR, Inventoried TaqMan® Gene expression Assays were used for α-SMA (Hs00426835-g1), COL1 (Hs00164004-m1) (Applied Biosystems) with Reverse Transcriptase qPCR™ Mastermix No ROX (Eurogentec). Samples were run on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression was normalized to GAPDH (Hs02758991-g1) (Applied Biosystems) and compared to the level of gene expression in MF, which were assigned the value of 1 using delta delta CT analysis performed with SDS software (Applied Biosystems).

Western blots

Cells were cultured in monolayer, adherent collagen matrices, floating collagen matrices or in tethered collagen matrices cultured in the CFM with co-cultures of different cell proportions for 24 h (as stated above). Cell lysates were prepared using lysis buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, and 1% Nonidet P-40), containing protease inhibitor cocktail (Roche Biochemicals), and then electrophoresed on 10% SDS polyacrylamide gels, followed by electrotransfer of proteins onto PVDF transfer membranes (Perkin Elmer Life Sciences). Membranes were blocked in 5% BSA/TBS + 0.05% Tween and incubated overnight at 4°C with primary antibodies against α-SMA (Sigma) and vimentin (Abcam). Horseradish

peroxidase-conjugated anti-mouse IgG (Amersham Biosciences) was used as secondary antibody. Bound antibody was detected using the enhanced chemiluminescence kit (Amersham Biosciences) and visualized using Hyperfilm MP (Amersham Biosciences).

Cell proliferation

Cell proliferation was measured using an EdU (5-ethynyl-2'-deoxyuridine) microplate assay (Invitrogen), which incorporates into DNA during active DNA synthesis. Co-cultures of different cell proportions were cultured in monolayer or adherent collagen matrices and incubated with Edu (10mM) for 12 h. Edu incorporation was assessed with a fluorescent microplate reader (Gemini XPS) by measuring the excitation/emission at a wavelength of 560/585 nm. To assess DNA content for proliferation studies, co-cultures of different cell proportions were cultured in monolayer or seeded in adherent collagen matrices up to 5 days and stained with Hoechst 33258 dye (Sigma) at day 1, 3 and 5. Briefly, the cells were lysed by freeze thawing in distilled water and stained with the fluorochrome in TNE buffer (10mM Tris, 1mM EDTA, 2M NaCl, pH 7.4). The samples were analysed with a fluorescent microplate reader by measuring the excitation/emission at a wavelength of 350/460 nm. DNA from calf thymus (Sigma) was used as a standard.

Conditioned medium

ADMSC or MF were incubated with 2% DMEM at 37 °C 5% CO2 for 24h after which point supernatants were harvested and used as conditioned medium from non-contact culture experiments. For experiments examining conditioned medium of direct contact cultures, co-cultures of 50% ADMSC + 50% MF were incubated with 2% DMEM at 37 °C 5% CO2 for 24h after which point supernatants were harvested. Transwell chambers (8mm membrane insert) (Sigma) were used to collect conditioned medium from indirect contact cultures: co-cultures of 50% ADMSC (upper chamber) and 50% MF (lower chamber) were incubated with 2% DMEM at 37 °C 5% CO2 for 24 h and supernatants were harvested. Conditioned

medium was filtered through 0.22mm (Sigma) and stored at -80°C for later use in proliferation experiments.

Statistics

A paired t-test was used to compare proliferation of MF in control medium and ADMSC conditioned medium from non-contact cultures, conditioned medium from direct contact cultures or conditioned medium from indirect contact cultures. One way analysis of variance (ANOVA) using Bonferroni's multiple comparison test was used to compare all other conditions. All statistical analyses were performed using software (GraphPad Software version 5.0c). Significance was achieved if $p<0.05$.

Results

Effect of the mechanical environment on the myofibroblast phenotype

We compared the effect of 2D monolayer, 3D adherent matrices and floating collagen matrices on COL1 and α-SMA mRNA and protein expression in Dupuytren's myofibroblasts (MF), ADMSC, BDMSC and human dermal fibroblasts (HDF).

BDMSC expressed significantly higher levels of COL1 mRNA and α-SMA mRNA and protein under all environments investigated ($p<0.05$) when compared to MF, ADMSC and HDF (Fig 2). ADMSC and HDF expressed lower levels of α-SMA mRNA and protein when cultured in floating matrices ($p<0.05$) under minimal tension, compared to MF and BDMSC (Fig 2). The stiffness of both 3D adherent matrices and monolayer cultures exerts effects on cell differentiation on the cell types used, and cells exhibited a similar contractile myofibroblast phenotype under these conditions. Therefore, all our experiments were done under these conditions.

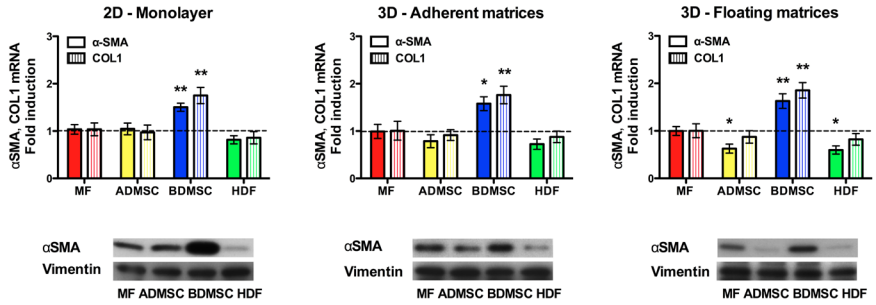

Figure 2. Cells studied in monolayer, adherent 3D collagen matrices and free-floating 3D collagen matrices. (Left panel) In monolayer (2D) levels of α-SMA and COL mRNA, and α-SMA protein expression were significantly higher in BDMSC compared to the other cell types. HDF demonstrated a decrease in α-SMA protein expression although no significant difference in α-SMA and COL mRNA expression. ADMSC and MF demonstrated no differences in α-SMA or COL gene or α-SMA protein expression. (Middle panel) Adherent 3D collagen matrices led to a significant increase of α-SMA and COL1 mRNA and α-SMA protein expression in only BDMSC. There was a decrease in α-SMA protein expression in ADMSC and HDF compared to MF. (Right panel) Free-floating 3D matrices demonstrated significantly increased α-SMA and COL1 mRNA and α-SMA protein expression in BDMSC compared to the other cell types. ADMSC and HDF demonstrated a significant decrease in α-SMA protein expression (although not COL1), and a concomitant decrease in α-SMA protein expression compared to MF. Gene expression was assessed by quantitative RT-PCR and data are presented as fold change compared to GAPDH, and normalized to expression in MF. Protein expression was assessed by western blotting, using vimentin as a loading control. Gels shown are representative of gels from 3 patients. Data are shown as the mean ± S.E.M from n=3 patients (each assay was performed in triplicate)*$p < 0.05$, ** $p < 0.01$.

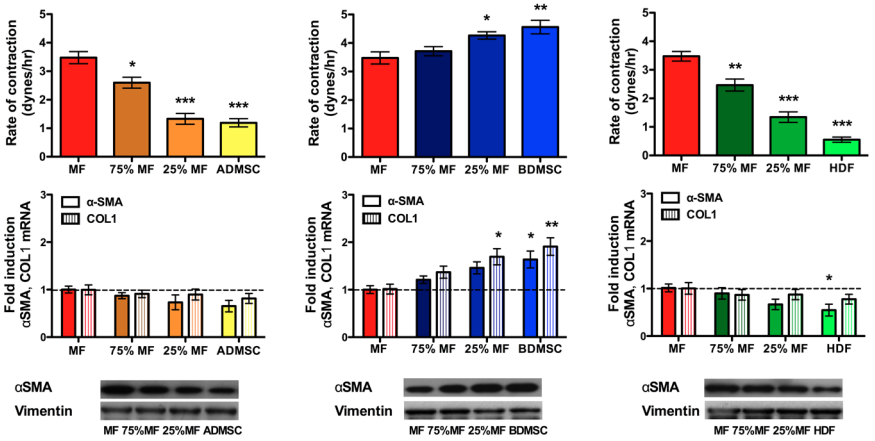

Figure 3. Co-cultures with ADMSC led to inhibition of the contractile myofibroblast in Dupuytren's disease, whilst BDMSC promoted myofibroblast contractility. (Left panels) Co-cultures of Dupuytren's MF and ADMSC led to a dose dependent decrease in contractility when cultured in the CFM. There was a concomitant decrease in α-SMA protein expression, although α-SMA and COL1mRNA expression demonstrated no significant change. (Middle panels) Co-cultures with MF and BDMSC led to a dose dependent increase in contractility in the CFM. A corresponding increase in α-SMA and COL1 mRNA expression and an increase in α-SMA protein expression were observed. (Right panels) Co-cultures with MF and HDF led to a dose dependent decrease in isometric contraction in the CFM. α-SMA and COL1 mRNA expression demonstrated a corresponding decrease, as did α-SMA protein expression. Gene expression was assessed by quantitative RT-PCR and data are presented as fold change compared to GAPDH, and normalized to expression in MF. Protein expression was assessed by western blotting, using vimentin as a loading control. Gels shown are representative of gels from 3 patients. Data are shown as the mean ± S.E.M from n=3 patients (each assay was performed in triplicate). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Adipose derived stem cells reduce the Dupuytren's myofibroblast phenotype, whereas bone marrow derived stem cells increase the myofibroblast phenotype

To test the effect of ADMSC on Dupuytren's MF we co-cultured different percentages of ADMSCs with MF, and examined their effect on the MF phenotype compared to control co-cultures of BDMSC or HDF, in the CFM.

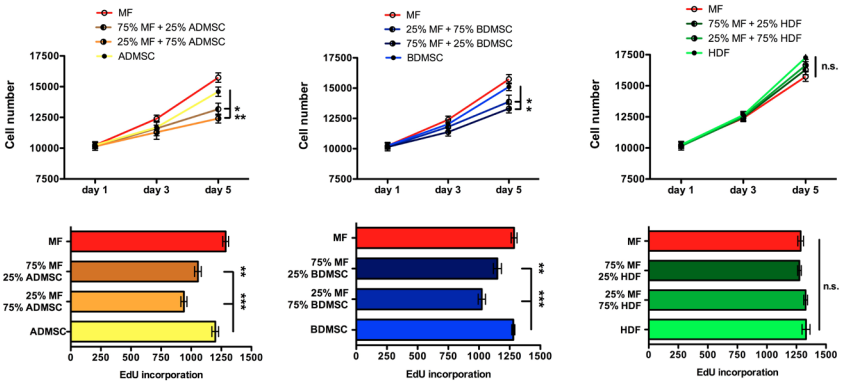

Figure 4. Stem cells inhibit proliferation of Dupuytren's myofibroblasts . (Left panels) Co-cultures of Dupuytren's MF with ADMSC were cultured up to 5 days and stained with Hoechst dye to quantify DNA content. Co-cultures of MF with ADMSC demonstrated a significant decrease in cell number compared to either MF or ADMSC. MF co-cultured with ADMSC demonstrated a corresponding Significant decrease in DNA synthesis compared to respective cell types alone, evaluated by EdU incorporation. (Middle panels) Similarly co-cultures of Dupuytren's MF with BDMSC demonstrated a significant decrease in cell number and DNA synthesis compared to respective cell types alone. (Right panels) Co-cultures of Dupuytren's MF with HDF demonstrated no difference in cell number or DNA synthesis compared to respective cell types alone. Data are shown as the mean ± S.E.M from n=3 patients (each assay was performed in triplicate). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Co-cultures with ADMSC resulted in a dose dependent decrease in contractility of MF ($p<0.05$) associated with a corresponding reduction of α-SMA protein expression. However, there was no effect on α-SMA mRNA expression (Fig 3). Control HDF demonstrated a similar decreasing effect on MF contractility and α-SMA protein expression ($p<0.01$), again without affecting α-SMA mRNA expression (Fig 3). In contrast, co-cultures with BDMSC led to a dose dependent increase in contractility and α-SMA protein expression, without affecting α-SMA mRNA expression (Fig 3).

Stem cells inhibit proliferation of Dupuytren's myofibroblasts

Co-cultures in monolayer of ADMSC with MF led to a reduction in cell numbers at day 5 and a decrease in DNA synthesis after 12h compared to MF or ADMSC in culture alone ($p<0.05$). Similarly co-cultures of BDMSC with MF demonstrated a decrease in cell number ($p<0.05$) and corresponding decrease in DNA synthesis, whilst co-cultures of MF with control HDF demonstrated no difference in cell proliferation. Testing proliferation of co-cultures in adherent collagen matrices yielded similar results as compared to monolayer (Fig 4).

Conditioned medium from adipose-derived stem cells and myofibroblast co-cultures leads to reduced Dupuytren's myofibroblast proliferation

First we tested the effect of conditioned medium of ADMSC or MF to examine the effect of key soluble factors secreted by these cells when cultured alone, having no contact with each other. Conditioned medium taken from ADMSC led to decreased MF cell number at day 5 (83%, $p<0.05$), whilst conditioned medium taken from MF cultures had no effect (104%, $p=0.93$) (Fig 5). Next we tested whether MF influenced the production of soluble inhibitory factors by ADMSC in an indirect fashion, in a situation when there would be no direct cell-cell contact between these cells. We cultured ADMSC and MF in a transwell chamber, separating these cells by a 0.8 μm membrane, only allowing communication via culture media. Conditioned medium taken from indirect contact co-cultures also led to decreased MF cell number at day 5 (73%, $p<0.001$) (Fig 5). Subsequently

we tested whether MF influenced the production of soluble inhibitory factors by ADMSC in a direct fashion, in a situation when ADMSC would be in a direct contact with MF. The production of soluble inhibitory factors by direct cell-cell cultures led to decreased MF cell number at day 5 (69%, $p<0.001$) and a significant decrease compared to conditioned medium from ADMSC of non-contact cultures ($p<0.05$) (Fig 6).

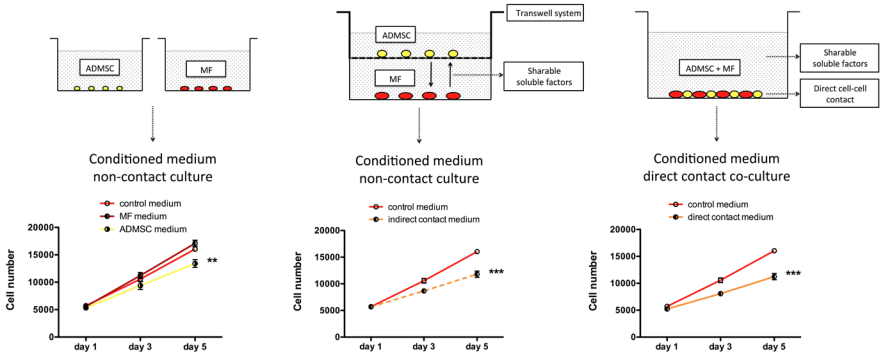

Figure 5. Conditioned medium of adipose-derived mesenchymal stem cells and adipose-derived mesenchymal stem cells and myofibroblast co-cultures inhibited Dupuytren's myofibroblast proliferation.

(Left) Myofibroblasts were cultured in monolayer up to 5 days treated with conditioned medium from noncontact cultures of either adipose-derived mesenchymal stem cells or myofibroblasts, and stained with Hoechst dye. Conditioned medium from adipose-derived mesenchymal stem cells led to a decreased myofibroblasts cell number, whereas conditioned medium from myofibroblasts had no effect on cell number. (Center) Conditioned medium from indirect contact co-cultures in a Transwell chamber with 50 percent adipose-derived mesenchymal stem cells and 50 percent myofibroblasts led to significantly decreased myofibroblast cell number. (Right) Similarly, conditioned medium from direct contact co-cultures of 50 percent adipose-derived mesenchymal stem cells and 50 percent myofibroblasts resulted in a significantly decreased myofibroblasts cell number. Data are shown as the mean ± SEM from three patients (each assay was performed in triplicate)($^{**}p < 0.01$, $^{***}p < 0.001$).

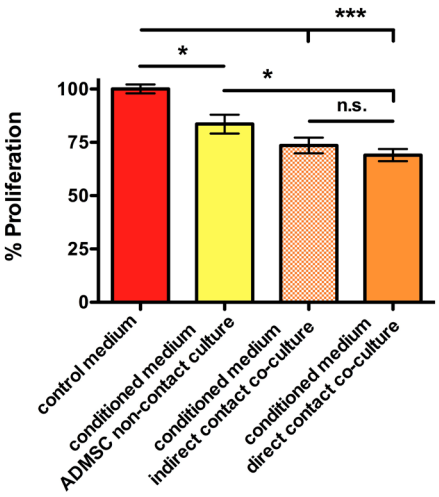

Figure 6. Adipose-derived stem cells inhibit myofibroblast pro- liferation by soluble factors, influenced by cell-cell contact- dependent signaling.

Conditioned medium from direct contact co-cultures in a Transwell chamber with 50 percent adipose-derived mesenchymal stem cells and 50 percent myofibroblasts led to a significantly decreased myofibroblast cell number com- pared with conditioned medium from noncontact cultures of adipose-derived mesenchymal stem cells. Data are shown as the mean ± SEM from three patients (each assay was performed in triplicate) (*$p < 0.05$, ***$p < 0.001$).

Discussion

This study shows that adipose-derived stem cells (ADMSCs) release soluble factors through cell-cell contact dependent signalling to inhibit Dupuytren's myofibroblast proliferation. Furthermore using the culture force monitor (CFM) as a functional outcome, we showed that ADMSCs reduce myofibroblast contractility with a corresponding reduction of α-SMA protein expression, probably through a dilution effect. Myofibroblasts are the primary cells that contribute to the

pathological processes leading to fibrosis and digital contractures in Dupuytren's disease[27]. Therefore, our findings represent a potential translational strategy using ADMSCs in the treatment of Dupuytren's disease. We also demonstrate that MSCs isolated from different tissues have similar characteristics, but they can exhibit differential effects on myofibroblast contractility, and hence the source of MSCs should be considered when planning their use in the clinical setting[28,29].

There is no animal model for Dupuytren's disease and in vivo conditions are most reliably examined by studying myofibroblasts in 3D collagen lattices under isometric tension[24]. Myofibroblasts only maintain their phenotype under stress and loss of tension is associated with disassembly of α-SMA stress fibres[30]. The substrate stiffness of both 3D adherent matrices and monolayer cultures exerts effects on cell differentiation on the cell types used, and in our system cells exhibited a similar contractile myofibroblast phenotype under these conditions. Therefore, all our experiments were performed under these conditions to ensure the culture conditions would similarly influence cell-cell and cell-matrix adhesion formation, and also cell differentiation[31,32].

The CFM permits real-time force quantification in 3D collagen lattices under isometric tension. Using this measure of contractility and associated α-SMA gene and protein expression, we found that myofibroblasts co-cultured with ADMSCs and human dermal fibroblasts (HDF) led to a dose dependent decrease in the contractile myofibroblast phenotype. This dose dependent decrease is most likely to be due to serial dilution of myofibroblasts, and in turn reduced overall force generation[33]. In direct contrast to our findings with ADMSCs, we found a dose dependent increase in the myofibroblast phenotype when BDMSCs were co-cultured with myofibroblasts. Sarraf et al.[34] showed a comparable contractility of BDMSC although these data were from mouse derived cells examined in the CFM. Other authors have also shown lower contractility of BDMSCs[35], although these studies used cells up to passage 10, which is recognised to have a negative influence on cell contractility[33]. It is worth noting that our co-culture model experiments were only performed over 24h in the CFM, and therefore, not truly representative of the processes involved in vivo as progression of Dupuytren's

disease may evolve over several months or years.

To further characterize the interaction between MSCs and myofibroblasts, we tested whether stem cells inhibited myofibroblast proliferation. MSCs have been shown to inhibit proliferation in various tumour and non-tumour cells, including hematopoietic cancers and in cardiac and renal fibrosis[11,13,36]. In line with this we found a decrease in proliferation of Dupuytren's myofibroblasts with both ADMSCs and BDMSCs when the cells were cultured in direct contact.

Several lines of evidence suggest that MSCs may in part mediate their inhibitory effects on proliferation by paracrine mechanisms. We examined whether proliferation of myofibroblasts was inhibited in response to soluble factors produced by ADMSCs by culturing conditioned medium from ADMSCs and from myofibroblasts separated by either a transwell insert to prevent cell-cell contact, or by allowing direct ADMSC and myofibroblast cell-cell contact. We found that under all conditions Dupuytren's myofibroblast proliferation was inhibited, and using conditioned medium from ADMSCs similar inhibitory results were seen as have been reported in the literature using conditioned medium from BDMSCs and cultured with cardiac fibroblasts[11]. However, greatest inhibition of proliferation was seen when direct cell-cell contact was permitted with co-cultures of ADMSCs and Dupuytren's myofibroblasts. This greatest inhibition of proliferation with direct cell-cell contact is also reported in literature using BDMSCs cultured with T cell leukemia, colon adenocarcinoma and small-cell lung cancer cell lines[37].

In Dupuytren's disease, localised inflammation has been shown to contribute to development and progression of the disease and cells derived from Dupuytren's tissue produce an appreciable amount of these pro-inflammatory cytokines[38-40]. In a related condition to Dupuytren's disease injections of ADMSCs in an animal model of Peyronie's disease, led to the prevention of fibrosis at the early inflammatory stage with downregulation of collagen III protein expression[8]. Similarly, MSCs cells have been shown to downregulate inflammatory cytokines and reduce αSMA protein expression in an animal model of renal fibrosis[36].

Therefore, the beneficial anti-inflammatory and anti-fibrotic effects suggest a potential role for adipose-derived stem cells in the prevention of progression of fibrosis in Dupuytren's disease.

It is likely that following extensive percutaneous needle aponeurotomy disruption of the affected tissue will lead to stress-relaxation and subsequent loss of tension. Loss of tension is associated with disassembly of α-SMA stress fibres and in turn may result in apoptosis[30]. Whilst aggregates of myofibroblasts remain, the injection of lipoaspirates containing ADMSCs results in a reduction of density of contractile myofibroblasts. Furthermore, the ADMSCs reduce the recruitment of additional myofibroblasts by inhibiting their proliferation.

These findings are consistent with, and are the first to support the hypothesis that adipose-derived stem cells represent a potential translational strategy in the treatment of Dupuytren's disease, whereby adipose-derived stem cells downregulate the contractile myofibroblast. This lends support to the potential benefit of lipografting in conjunction with percutaneous needle aponeurotomy in the treatment of Dupuytren's disease.

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Chapter 5

Unravelling the signaling pathways promoting fibrosis in Dupuytren's disease reveals TNF as a novel therapeutic target

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Abstract

Dupuytren's disease is a very common progressive fibrosis of the palm leading to flexion deformities of the digits that impair hand function. The cell responsible for development of the disease is the myofibroblast. There is currently no treatment for early disease or for preventing recurrence following surgical excision of affected tissue in advanced disease. Therefore, we sought to unravel the signaling pathways leading to the development of myofibroblasts in Dupuytren's disease. We characterized the cells present in Dupuytren's tissue and found significant numbers of immune cells, including classically activated macrophages. High levels of proinflammatory cytokines were also detected in tissue from Dupuytren's patients.

We compared the effects of these cytokines on contraction and profibrotic signaling pathways in fibroblasts from the palmar and nonpalmar dermis of Dupuytren's patients and palmar fibroblasts from non-Dupuytren's patients. Exogenous addition of TNF, but not other cytokines, including IL-6 and IL-1β, promoted differentiation into specifically of palmar dermal fibroblasts from Dupuytren's patients in to myofibroblasts. We also demonstrated that TNF acts via the Wnt signaling pathway to drive contraction and profibrotic signaling in these cells. Finally, we examined the effects of targeted cytokine inhibition. Neutralizing antibodies to TNF inhibited the contractile activity of myofibroblasts derived from Dupuytren's patients, reduced their expression of α-smooth muscle actin, and mediated disassembly of the contractile apparatus. Therefore, we showed that localized inflammation in Dupuytren's disease contributes to the development and progression of this fibroproliferative disorder and identified TNF as a therapeutic target to down-regulate myofibroblast differentiation and activity.

Introduction

Dupuytren's disease is a common fibroproliferative disorder with a prevalence of over 7% in the USA[1]. The classic description of disease progression is the initial appearance of palmar 'nodules' characterised by high cellularity and cell proliferation, followed by the development of 'cords'. This is followed by a final fibrotic stage that is associated with maturation of the cords and digital contractures resulting in significant impairment of hand function[2]. Established flexion deformities of the digits are most commonly treated by surgical excision (fasciectomy) of the cord. The long recovery time following surgery has led to description of alternative techniques of disrupting the cord with a needle (percutaneous fasciotomy)[3] or enzymatic digestion using collagenase injections[4]. However, even following surgery, patients often have significant residual dysfunction due to irreversible fixed flexion deformities of the joints. Currently there is no specific treatment for early disease or prevention of recurrence following fasciectomy or fasciotomy of Dupuytren's cords. Therefore, we sought to unravel the cellular mechanisms leading to the development of this disease to reveal new potential therapeutic targets.

The cell responsible for matrix deposition and contraction in Dupuytren's disease is the myofibroblast[5]. Myofibroblasts characteristically express α-smooth muscle actin (α-SMA), which is the actin isoform typical of vascular smooth muscle cells[6]. Fibroblast to myofibroblast differentiation is characterised by α-SMA expression and exposure of the cells to stress leads to the incorporation of α-SMA protein into stress fibres[7]. Unlike in granulation tissue, where the expression of α-SMA is transient[8], α-SMA expression is persistent in Dupuytren's disease. The development of myofibroblasts has been shown to be dependent on a number of different environmental cues, including tension in the matrix and exposure to a variety of different soluble mediators[7]. The best studied of these is TGF-β1. The expression of TGF-β1, and associated signaling molecules is elevated in Dupuytren's disease[9] and Dupuytren's myofibroblasts or dermal fibroblasts from the same patients proliferated in response to 1 or 5ng/ml TGF-β1[10]. Normal

human dermal fibroblasts cultured in stressed collagen lattices also showed increased α-SMA expression when treated with 1ng/ml TGF-β1[11]. Fibroblasts derived from the transverse carpal ligament of patients unaffected by Dupuytren's disease exposed to 2ng/ml TGF-β1 showed no increase in α-SMA staining[12]. However, these fibroblasts, as well as Dupuytren's myofibroblasts, did exhibit increased isometric contraction of collagen lattices when exposed to 12.5ng/ml of TGF-β1, with higher doses being inhibitory[13]. Therefore, TGF-β1 can promote cell proliferation and increase α-SMA expression and matrix contraction in a manner dependent on both the dose and the tissue of origin of the cells.

Inflammation is also known to play a crucial role in fibrosis and various pro-inflammatory cytokines have been implicated in driving the progression of fibrotic diseases[14]. Histological studies have identified the presence of immune cells in Dupuytren's disease; in particular, the number of macrophages was shown to correlate with the quantity of myofibroblasts[15]. Intralesional steroid injections led to temporary softening and flattening of Dupuytren's nodules[16] and reduced recurrence following percutaneous needle fasciotomy[17]. It has been suggested that this therapeutic benefit of steroids in early Dupuytren's disease may be due to diminished leukocyte recruitment[18] as well as increased apoptosis of macrophages and fibroblasts, with reduced proliferation of the latter[19]. However, the link between disease progression and localized inflammation in Dupuytren's tissue remains unclear and molecular mechanisms by which inflammatory cytokines directly drive myofibroblast differentiation remain unknown.

To explore these issues we systematically studied the cytokines produced by freshly disaggregated cells from Dupuytren's nodules. TNF was identified as a key regulator of the myofibroblast phenotype and TNF blockade in vitro led to down regulation of the myofibroblast phenotype. Our data indicate that progression of early palmar nodules of Dupuytren's disease to deposition of extracellular matrix in cords and subsequent digital contractures in vivo may be prevented by local administration of a TNF inhibitor. This approach may also be effective in preventing recurrence following surgery, percutaneous needle fasciotomy or collagenase treatment.

Methods

Patient samples

Tissue samples were obtained following informed consent (REC 07/H0706/81). Dupuytren's nodular tissue, palmar skin (uninvolved skin overlying Dupuytren's tissue) and non-palmar skin (full-thickness skin harvested from the groin or medial aspect of arm) were obtained from patients with Dupuytren's disease undergoing dermofasciectomy. Tissue was also obtained from palmar skin of patients unaffected by Dupuytren's disease.

Cell culture

Fibroblasts from the palmar (PF-D) and non-palmar dermis (NPF-D) of patients with Dupuytren's disease and palmar fibroblasts (PF-N) from individuals unaffected by Dupuytren's disease were isolated from full-thickness skin samples. Dupuytren's myofibroblasts (MF-D) were isolated from α-SMA-rich nodules[20]. Tissue samples were dissected into small pieces and digested in Dulbecco's modified Eagle's medium (DMEM) (Lonza) with 1% penicillin–streptomycin (PAA) and 5% fetal bovine serum (FBS) (Gibco) and type I collagenase (Worthington Biochemical Corporation) + DNase I (Roche Diagnostics) for up to 2h at 37°C. Cells were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO2. Cells up to passage 2 were used for experiments.

Culture Force Monitor (CFM)

Measurement of the isometric contractile forces generated by cells within 3D collagen matrices was performed as previously described[21]. Briefly, 1.5 x10^6 cells were seeded in 2.5 ml of type I collagen gel (FirstLink), and 3D matrices tethered between two flotation bars and held stationary at one end whilst the other attached to a force transducer. Fibroblast populated collagen matrix generated tensional forces were continuously measured and data logged every minute (dynes: 1x10^{-5} N). Cell populated matrices were cultured in DMEM with 10%

FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator for 24hr with 5% CO2 and treated with either TGF-β1, TNF (Peprotech), rhIL-1β, rhIL-6, rhIL-10, anti-IL1β, anti-IL6, anti-TNF, anti-TNF (R&D Systems), LiCl (Invitrogen), TNF in combination with LiCl, SD208 (TOCRIS bioscience), Dkk-1 (R&D Systems), Adalimumab (Abbott), Certolizumab (UCB), Etanercept (Pfizer) and Golimumab (Merck). Experiments using each patient sample were performed in triplicate.

Quantitative RT-PCR

Cells were cultured in monolayer and treated with either TGFβ1, SD208, TNF, anti-TNF, LiCl, SB216763 or TNF in combination with LiCl for 24hr and total RNA was extracted from each sample using the QIAamp RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). For real-time reverse transcription PCR, Inventoried TaqMan® Gene expression Assays were used for α-SMA (Hs00426835-g1), COL1a1 (Hs00164004-m1), GSK (Hs01047719-m1), TNFR1 (Hs01042313-m1) and TNFR2 (Hs00961749-m1) (Applied Biosystems) with Reverse Transcriptase qPCR™ Mastermix No ROX (Eurogentec). Samples were run on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression was normalized to GAPDH (Hs02758991-g1) (Applied Biosystems) and compared to the level of gene expression in either baseline respective cell types or to the level of gene expression in NPF-D, which were assigned the value of 1 using delta delta CT analysis performed with SDS software (Applied Biosystems).

Western blots

Cells were cultured in monolayer and treated with either TGF-β1, SD208, TNF, anti-TNF, LiCl, SB216763 or TNF in combination with LiCl for 30 minutes or 24hr prior to protein extraction. Cell lysates were prepared in lysis buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, and 1% Nonidet P-40), containing protease inhibitor cocktail (Roche Biochemicals) and phosphatase inhibitor cocktail (Invitrogen), and then electrophoresed on 10% SDS polyacrylamide gels, followed by electrotransfer of

proteins onto PVDF transfer membranes (Perkin Elmer Life Sciences). Membranes were blocked in 5% BSA/TBS + 0.05% Tween and incubated overnight at 4°C with primary antibodies against α-SMA primary antibody (Sigma), TNFR1, TNFR2, phosphorylated GSK-3β, total GSK-3β, β-actin (all from Cell Signaling), and vimentin (Abcam). Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences) were used as secondary antibodies. Bound antibody was detected using the enhanced chemiluminescence kit (Amersham Biosciences) and visualized using Hyperfilm MP (Amersham Biosciences).

Flow cytometry

For detection of α-SMA, cells were permeabilized overnight at 4 °C with PBS containing 1% FCS, 0.01% sodium azide and 0.05% saponin, and then stained with anti-α-SMA (abcam) and subsequently with phycoerythrin-conjugated goat anti-mouse IgG (Southern Biotechnology) for 10 min at 4 °C. For detection of macrophages, cells were stained for 30 min at 4 °C with Pacific blue–conjugated anti-CD1a (e-Bioscience) and phycoerythrin-conjugated anti-CD163 (R&D Systems), fixed in Cytofix (BD Bioscience), permeabilized overnight and stained with fluorescein isothiocyanate–conjugated anti-CD68 (DAKO). For intracellular cytokine staining, cells were stimulated with brefeldin A (Sigma-Aldrich), permeabilized and stained with allophycocyanin-conjugated anti-TNF (e-Bioscience). Samples were analyzed on a FACSCanto II (BD Bioscience) and data analyzed using FlowJo software (TreeStar).

Immunohistochemistry

All Dupuytren's tissue samples were fixed in formalin, longitudinally bisected, embedded in paraffin wax, and 7-µm sections from the cut surface were processed for immunohistochemistry[20]. Sequential sections were stained with mouse monoclonal anti–α-SMA antibody (Sigma), anti-CD68 antibody (DAKO) and mouse monoclonal anti-desmin (DAKO) antibody. Antibodies were detected using a 2-staged polymer enhancer system (Sigma). Mouse serum at the same protein concentration as the monoclonal antibody solution was used as a control.

Mesoscale ELISA

Freshly disaggregated cells from whole tissue samples were isolated as described previously[22]. Dupuytren's nodular tissue samples were dissected into small pieces and digested in DMEM with 5% FBS, 1% penicillin–streptomycin and type I collagenase + DNase I for up to 2hr at 37°C. 5×10^5 freshly disaggregated cells were immediately plated in a 6 well plate in 4ml DMEM (5% FBS, 1% penicillin–streptomycin). Cells were incubated at 37 °C 5% CO2 for 24 h and then supernatants were harvested (passage 0). The remaining freshly disaggregated cells were cultured and 5×10^5 cells at passage 1 or passage 2 from the same donor plated in a 6 well plate with 4ml DMEM (5% FBS, 1% penicillin–streptomycin) and supernatants harvested after 24 h. Supernatants were analysed for IL-1β, IL-6, IL-8, IL-10, GM-CSF, IFγ and TNF cytokines using a Human Pro-Inflammatory 9-Plex Ultra-sensitive kit (N05007A-1 Meso Scale Discovery), and for TGFβ1 using a MSD Human TGFβ1 kit (L451UA-1 Meso Scale Discovery).

Immunofluoresence

Cells were cultured in 3D collagen matrices with DMEM, 10% FBS and 1% penicillin–streptomycin, treated with or without anti-TNF (10µg/ml) for 24hr then fixed for 10 min with 3% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 (Sigma) for 30 min. Cells were stained with a mouse monoclonal α-SMA (Sigma) followed by Alexa Fluor 568-conjugated rabbit anti-mouse antibody (Invitrogen) and DNA with DAPI (Sigma). Secondary antibody alone was used as an immunolabelling control. Matrices were compressed to enhance visualization of cell morphology and intracellular α-SMA filaments. Images were acquired using confocal microscopy oil immersion objectives (60x) and the signal was analyzed by Ultraview confocal microscopy (PerkinElmer). Cell viability was assessed using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen).

Topflash

Cells were transfected using the Amaxa Human Dermal Fibroblast Nucleofector Kit (Lonza) with the Wnt reporter plasmid TOPflash (Addgene Plasmid 12456) or the Wnt mutant reporter plasmid FOPflash (Addgene Plasmid 12457) both kindly donated by Dr. Randall Moon (Howard Hughes Medical Institute, University of Washington)[23]. Each Wnt vector was co-transfected with PRL-CMV Renilla Luciferase expression plasmid (Promega). After 24 h transfection in Opti-MEM reduced serum media (Life Technologies), cells were stimulated for a further 16 h with either TNF (Peprotech), LiCl (Sigma), TNF with LiCl, or SB-2167613 (Sigma). The cells were then lysed and Firefly and Renilla luciferase activities were analyzed with Dual-Luciferase Assay System (Promega) using a Micro-Beta Jet Luminescence Counter (Perkin Elmer). Values of the Firefly luciferase reporter gene activity were normalized to Renilla luciferase activity.

Statistics

The rate of FPCL contraction (dynes/hr) was calculated by measuring the average gradient of the curve between 6 and 24 h. A paired t-test was used for comparison of TNFR1/2 expression in MF-D, and to compare TOP and FOP TCF/Lef promoter gene activity in NPF-D, PF-N and PF-D. Analysis of single variance was used for comparisons all other conditions. All statistical analyses were performed using software (GraphPad Software version 5.0c). Significance was achieved if $p<0.05$.

Results

Cells in Dupuytren's nodules and the cytokines they produce

To quantify the presence of immune cells in Dupuytren's tissue, flow cytometric analysis was performed on cells directly isolated from Dupuytren's nodules. The majority (87±6%) of cells were myofibroblasts, together with a significant number of macrophages (6.5±2.3%), with the classically activated (CD68+/CD163-) M1 phenotype predominating (4.8±2.2%) (Fig 1a,b). Immunohistochemistry showed

that the macrophages were distributed throughout Dupuytren's nodules (Fig 1c) and clustered around the blood vessels. Serial histological sections revealed no neutrophil elastase positive cells (not shown). Soluble cytokines produced by freshly disaggregated Dupuytren's nodule tissue included variable amounts of TGF-β1 (mean±SD 236±248pg/ml, range 4-852pg/ml), as well as TNF (mean±SD 78±26pg/ml), IL-6 (mean±SD 5591±3215pg/ml) and, consistent with the presence of substantial numbers of classically activated M1 macrophages, GM-CSF (mean±SD 64±24pg/ml). Low levels of IL-1β(mean±SD 17±10pg/ml), IL-10 (mean±SD11±9pg/ml) and IFNγ (mean±SD 7±6pg/ml) were observed (Fig 1d).

Cells from the same patient samples were also maintained in culture and examined for constituent cells and cytokine profiles at passage 2. Macrophages were absent and the passaged cells produced greater amounts of TGF-β1 (mean±SD: 654pg/ml ±158 pg/ml), but less TNF (mean±SD: 4 pg/ml ±4 pg/ml) and IL-6 (mean±SD: 208 pg/ml ±312 pg/ml) than freshly isolated cells.

Dermal fibroblasts treated with TGF-β1 acquire the myofibroblast phenotype

Recent data suggest that the tissues overlying the Dupuytren's nodules, including the dermis, are likely to constitute a source for myofibroblast precursors during disease progression[24]. Therefore we studied the effect of TGF-β1 on dermal fibroblasts from palmar and non-palmar skin from patients with Dupuytren's disease, as well as palmar dermal fibroblasts from normal individuals unaffected by Dupuytren's disease. Cells were stimulated with 0.1, 1.0 and 10 ng/ml of TGF-β1. All cell types showed increased contractility in 3D collagen matrices when treated with 1.0 and 10 ng/ml TGF-β1 (Fig 2a). These doses of TGF-β1 also resulted in increased levels of α-SMA and COL1 mRNA (Fig 2b), as well as augmented α-SMA protein (Fig 2c). Treatment of myofibroblasts from Dupuytren's nodules with SD208, a small molecule activin receptor-like kinase (ALK)5 inhibitor of TGF-β1R1/Smad2/3 interaction[25], resulted in a dose dependent decrease in contractility (Fig 2d), with concomitant decrease in α-SMA and COL1 gene

expression and α-SMA protein level (Fig 2e). These data show that low doses of TGF-β1 (0.1ng/ml) have no effect on dermal fibroblasts and that higher concentrations (1-10 ng/ml) stimulate myofibroblast differentiation of dermal fibroblasts, regardless of the source of origin. Furthermore, inhibition of TGF-β1 signaling in Dupuytren's myofibroblasts can reduce contraction and fibrotic gene expression.

The effect of rhTNF on dermal fibroblasts of different origins and the effect of inhibiting TNF on myofibroblasts

We examined the ability of other cytokines detected in Dupuytren's tissue to promote myofibroblast differentiation. Addition of rhTNF to palmar dermal fibroblasts from patients with Dupuytren's disease in 3D collagen lattices led to increased contraction. This effect peaked at 0.1ng/ml TNF, a physiological concentration, decreasing thereafter, to become inhibitory at 10ng/ml, a very high concentration (Fig 3a, Fig 4a). In contrast, contraction of non-palmar dermal fibroblasts from patients with Dupuytren's disease and palmar cells from normal individuals unaffected by Dupuytren's disease was dose dependently inhibited by rhTNF (Fig 3a). Interestingly, the freshly disaggregated cells from Dupuytren's nodules secreted TNF at levels near optimal (mean±SD 78±26pg/ml) (Fig 1d) for differentiation of palmar dermal fibroblasts into myofibroblasts. Expression of TNF receptors at message and protein level was higher in palmar dermal fibroblasts from Dupuytren's patients and also in Dupuytren's myofibroblasts, where TNFR2 was especially raised (Fig 3b). Addition of 0.1ng/ml rhTNF led to enhanced expression of COL1 and α-SMA mRNA and also α-SMA protein only in palmar dermal fibroblasts from patients with Dupuytren's disease (Fig 3c). Addition of rhIL-6 (Fig 5a) or rhIL-1β (Fig 5b) had no effect on contractility of palmar dermal fibroblasts from patients with Dupuytren's disease.

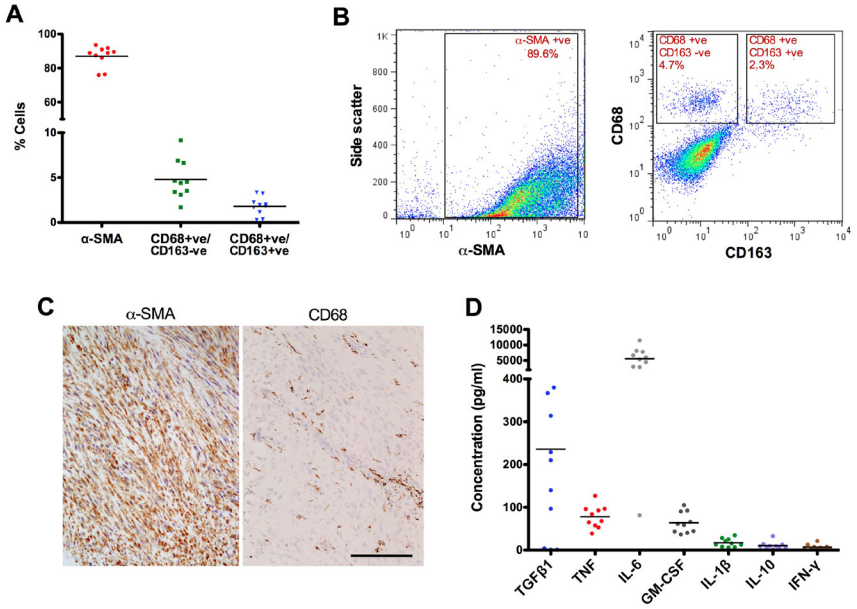

Figure 1. Inflammatory cells are present in Dupuytren's myofibroblast-rich nodules and resident cells in the nodules produce pro-inflammatory cytokines. (a) Flow cytometric analysis of cells isolated from freshly disaggregated Dupuytren's nodular tissue. Intracellular α-SMA positive (myofibroblasts) (mean ±SD: 87±6.1%), cell surface CD68 positive CD163 negative (classically activated M1 macrophages) (mean ±SD: 4.8±2.2%) and CD68 positive CD163 positive (alternatively activated M2 macrophages) (mean ±SD: 1.8±1.0%) cells were quantified. (b) Representative FACS scatter plots showing the proportion of α-SMA positive cells and gating strategy for CD68+ and CD163+ cells in disaggregated Dupuytren's nodular tissue. (c) Serial histological sections of Dupuytren's nodular tissue stained for α-SMA+ and CD68+ cells. CD68+ cells localized around blood vessels. Scale bar: 100μm. (d) Cytokines secreted by freshly isolated nodular cells in monolayer culture using electrochemiluminescence. All data shown are from >10 patient samples.

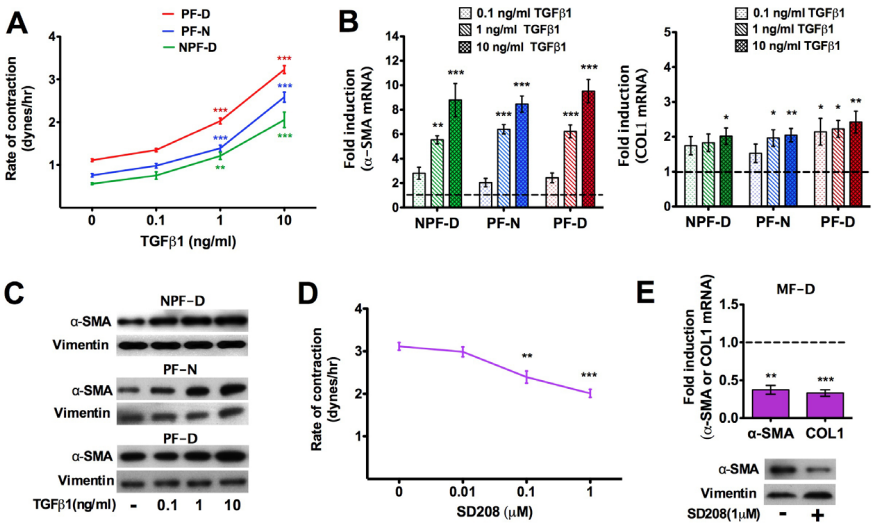

Figure 2. TGF-β1 induces the myofibroblast phenotype indiscriminately in all types of dermal fibroblasts, an effect reversed with the TGF-β1R1/Smad2/3 inhibitor, SD208. (a) TGF-β1 led to increased contractility of palmar (PF-D) and non-palmar (NPF-D) dermal fibroblasts from patients with Dupuytren's disease as well as in palmar dermal fibroblasts from normal individuals unaffected by Dupuytren's disease (PF-N) (b) All three cell types showed an increase in expression of α-SMA and COL1 assessed by quantitative RT-PCR (markers of myofibroblast contractility and matrix deposition respectively) on exposure to TGF-β1 in a dose-dependent manner. Data are shown as relative expression compared to each cell type untreated. (c) Representative western blots showing α-SMA protein expression increased in all dermal fibroblasts cultured with TGF-β1. Vimentin is shown as loading control and remained constant. (d) Dose dependent reduction in contractility of Dupuytren's myofibroblasts on addition of SD208. (e) SD208 (1μM) resulted in decreased α-SMA and COL1 expression in myofibroblasts from Dupuytren's nodules (MF-D) relative to untreated myofibroblasts. Representative western blots showing α-SMA protein expression was reduced in Dupuytren's myofibroblasts treated with SD208 (1μM). Vimentin is shown as loading control. All data are shown as the mean ± S.E.M from n≥3 patients (each assay was performed in triplicate). *P < 0.05, ** P < 0.01, *** P < 0.001.

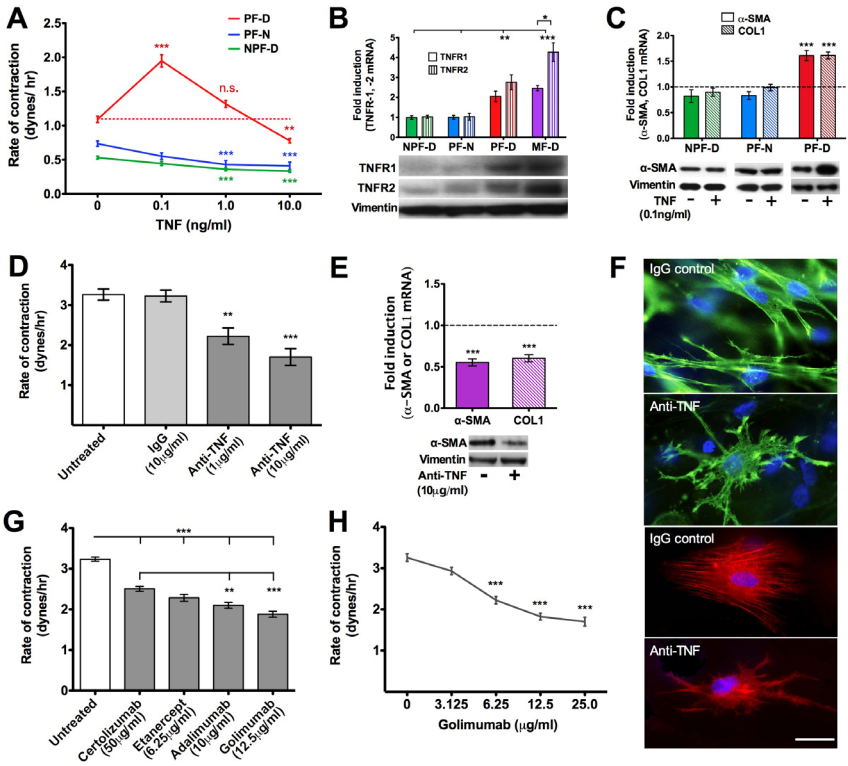

Figure 3. TNF selectively induces the myofibroblast phenotype in Dupuytren's palmar fibroblasts; anti-TNF reverses Dupuytren's myofibroblast phenotype. (a) Contraction of palmar fibroblasts from patients with Dupuytren's disease (PF-D) peaked on addition of 0.1ng/ml TNF. In contrast, TNF treatment of PF-N and NPF-D led to a dose dependent decrease in contractility. (b) Baseline gene expression of TNFR1 and TNFR2 was significantly higher in myofibroblasts and PF-D compared to both PF-N and NPF-D. In Dypuytren's myofibroblasts (MF-D), TNFR2 expression was significantly greater than TNFR1. Fold change was normalized to the baseline expression of NPF-D. (c) α-SMA and COL1 mRNA expression only increased in Dupuytren's palmar fibroblasts and not in other dermal fibroblasts (PF-N and NPF-D) when cultured with TNF (0.1ng/ml) and compared to respective untreated fibroblasts. There was a corresponding increase in α-SMA protein expression in PF-D treated with TNF (0.1ng/ml) but no difference in other dermal fibroblasts demonstrated by western blotting. Vimentin is shown as loading control and remained constant. (d) Dose dependent

reduction in contractility of Dupuytren's myofibroblasts in response to neutralizing antibody to TNF. IgG isotype (10μg/ml) antibody was used as a control. (e) Anti-TNF (10μg/ml) led to a corresponding reduction in mRNA expression of α-SMA and COL1 in Dupuytren's myofibroblasts compared to the respective untreated cells. A concomitant reduction in α-SMA protein expression in Dupuytren's myofibroblasts was seen when treated with anti-TNF (10μg/ml) demonstrated by western blotting. Isotype IgG (0.1ng/ml) control was used for anti-TNF and vimentin is shown as loading control and remained constant. (f) Immunofluoresence staining of Dupuytren's myofibroblasts seeded in 3D collagen matrices. Cell morphology and actin cytoskeleton was visualized with phalloidin (green) or α-SMA (red) and nuclei stained with DAPI (blue). Neutralizing antibody to TNF (10μg/ml) led to disassembly of the cytoskeleton. Isotype IgG control also used at 10μg/ml. Scale bar: 30μM. (g) Comparison of current anti-TNF preparations approved by FDA for subcutaneous administration on the contractility of Dupuytren's myofibroblasts. Doses calculated based on 25% of recommended dose in rheumatoid arthritis (certolizumab 200mg in 1ml every 2 weeks, etanercept 50mg in 1ml every week, adalimumab 40mg in 0.8ml every 2 weeks, golimumab 50mg in 0.5ml every 4 weeks). (h) Dose response of Dupuytren's myofibroblasts to golimumab. All data shown are from n≥3 patients (each in triplicate). Data expressed as mean ± S.E.M. *P < 0.05, ** P < 0.01, *** P < 0.001, n.s. (not significant).

To determine if inhibiting TNF activity would reduce contraction and fibrotic gene expression in Dupuytren's myofibroblasts, we used a number of means of TNF blockade. Addition of neutralizing antibody to TNF reduced isometric contraction of Dupuytren's myofibroblasts in a dose-dependent fashion (Fig 3d), sustained for 72h (Fig 4B) the maximum time the cells could be maintained in culture in the CFM. This was accompanied by reduced expression of COL1 and α-SMA at the message level as well as α-SMA protein (Fig 3e). Conversely, addition of neutralizing antibodies to IL-6 (Fig 5c) or IL-1β(Fig 5d) had no effect on myofibroblast contractility and neither did rhIL-10 (Fig 5e). Inhibiting TNF did not affect myofibroblast viability (Fig 4c). However, there was disassembly of the intracellular α-SMA contractile apparatus (Fig 3f). There are four FDA approved anti-TNF agents suitable for subcutaneous administration. Whilst all were effective, adalimumab and golimumab were somewhat more efficacious in

inhibiting isometric myofibroblast contraction (Fig 3g) and the latter was effective in a dose-dependent manner (Fig 3h). Together these data show that TNF blockade is equally effective in down regulating the Dupuytren's myofibroblast phenotype as TGF-β1 inhibition.

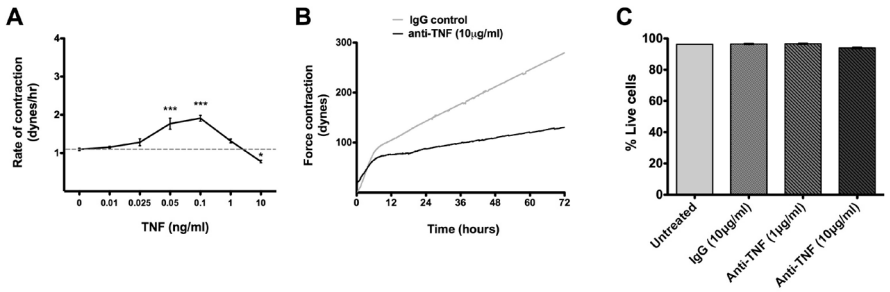

Figure 4. Anti-TNF continues to inhibit myofibroblast contractility over 72 h with no effect on cell viability. (A) The extended dose range for TNF activity was examined by isometric contraction of 3D collagen matrices seeded with palmar fibroblasts from patients with Dupuytren's disease maintained for 24 h in the culture force monitor (CFM). (B) Real-time isometric force contraction (dynes) in 3D collagen matrices seeded with Dupuytren's myofibroblasts treated with neutralizing antibody to TNF (10 μg/mL) continued to decrease over 72 h compared with untreated myofibroblasts in the CFM. (C) No effect on myofibroblast viability was seen over 24 h when assessed with Live/Dead stain. Data are shown for n ≥ 3 patients and expressed as mean ± SEM. *P < 0.05, ***P < 0.0001.

TNF Acts via the Wnt Signaling Pathway in Dupuytren's Disease.

TNF derived from activated macrophages can promoteWnt/β-catenin activity in gastric cancer cells[26]. Moreover, TNF activation of Wnt/β-catenin signaling pathways has been shown to control adipocyte differentiation[27]. Ligation by Wnt of the receptor complex comprising Frizzled and LRP5/6 leads to accumulation of cytoplasmic β-catenin, which translocates to the nucleus, binding the transcription factors TCF/Lef, and promotes transcription of genes typically

associated with myofibroblasts, including COL1 and α-SMA. Because a recent genomewide association study demonstrated that Wnt signaling may be involved in Dupuytren's disease[5], we explored the hypothesis that TNF may directly control myofibroblast differentiation via Wnt signaling. Addition of exogenous Dkk-1, an inhibititor of Wnt ligand binding to the Frizzled receptor complex, did not affect myofibroblast contractility (Fig. 5f), indicating that the myofibroblast phenotype is not dependent on binding of Wnt ligands to their receptor LRP5/6-Frizzled. Using the TOPflash TCF/Lef luciferase reporter assay, we found that stimulation of palmar dermal fibroblasts from Dupuytren's patients with TNF led to increased Wnt signaling. In contrast, this did not occur on TNF treatment of nonpalmar dermal fibroblasts from patients with Dupuytren's disease nor of palmar cells from normal individuals unaffected by Dupuytren's disease (Fig. 6a). These data are consistent with the cell type-specific effect of TNF on cell contractility and gene expression that we observed in Fig. 3a.

Following ligation of Frizzled and LRP5/6 by Wnt, glycogen synthase kinase (GSK-3β) is inactivated by phosphorylation, which prevents the degradation of β-catenin, preserving it within the cell. SB-216736 is a potent selective ATP-competitive GSK-3β inhibitor[28] and induces Wnt signaling pathways in the absence of Wnt ligands. Thus, SB-216736 drove TOPflash TCF/Lef luciferase indiscriminately in all three cells types, indicating that TCF/Lef can be activated in these cells and confirming the specific effect of TNF only in palmar fibroblasts from patients with Dupuytren's disease (Fig. 3A). Interestingly SB-216736 had no effect on α-SMA, COL1 mRNA or α-SMA protein expression in any cell type, whereas TNF led to increased α-SMA and COL1 mRNA and α-SMA protein only in palmar dermal fibroblasts from patients with Dupuytren's disease (Fig. 6B). These data indicate that inactivation of GSK-3β alone is insufficient to drive gene transcription downstream of TCF/Lef activation in contrast to cell stimulation with TNF, which promotes the expression of profibrotic genes. These data indicate that TNF induces additional signaling events required for effective gene expression.

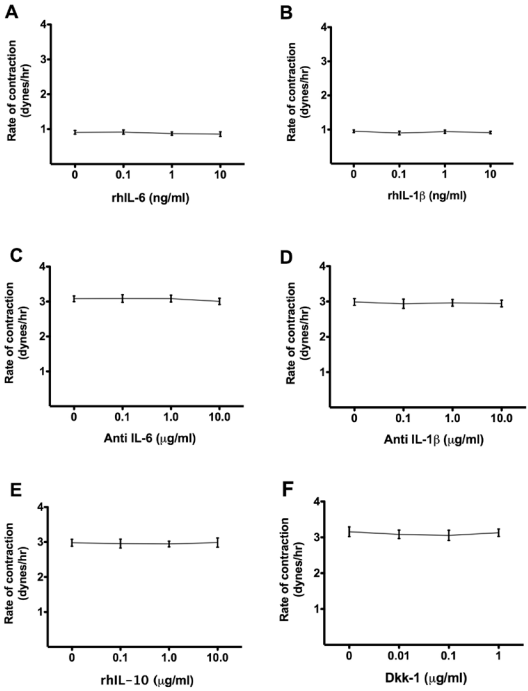

Figure 5. IL-6 and IL-1β have no effect on contractility of palmar dermal fibroblasts from patients with Dupuytren's disease, and myofibroblast contractility is not inhibited with anti–IL-6, anti–IL-1β, recombinant human IL-10 (rh-IL-10), or Dkk-1. Isometric force contraction of 3D collagen matrices seeded with palmar dermal fibroblasts from patients with Dupuytren's disease and cultured for 24 h in the CFM, either alone or with (A) rhIL-6 and (B) rhIL-1β demonstrated no effect on contractility. 3D collagen matrices seeded with Dupuytren's myofibroblasts and cultured for 24 h in the culture force monitor either alone or with (C) anti–IL-6, (D) anti–IL-1β, (E) rhIL-10, and (F) Dkk-1 (a negative regulator of Wnt ligand/receptor complex binding) demonstrated no effect on myofibroblast contractility. Data are shown for n ≥ 3 patients and expressed as mean ± SEM.

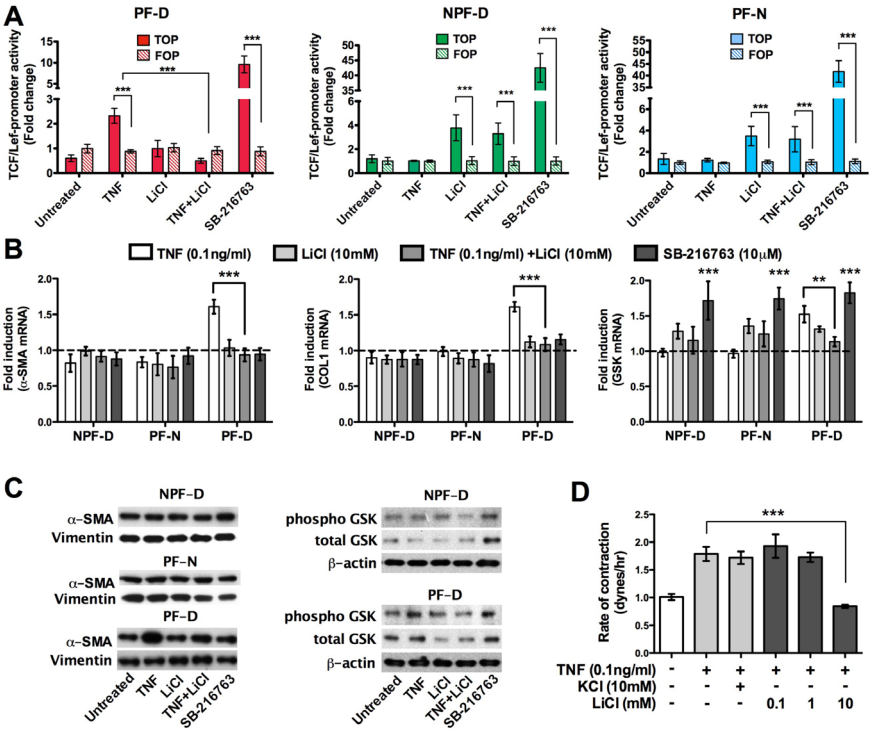

Figure 6. TNF acts via Wnt signaling pathway in Dupuytren's disease. (A) TNF (0.1 ng/mL) stimulated the activity of a TCF/Lef reporter assay, a downstream indicator of activation of the Wnt signaling pathway, in palmar fibroblasts from Dupuytren's patients (PF-D). This effect was reversed on addition of Li ions (10mM), whereas LiCl alone had no effect on PF-D. In contrast, palmar fibroblasts from individuals unaffected by Dupuytren's disease (PF-N) or nonpalmar fibroblasts from Dupuytren's patients (NPF-D) treated with TNF (0.1 ng/mL) did not promote the activity of TCF/Lef reporter construct. LiCl led to increased TCF/Lef reported activity in nonpalmar dermal fibroblasts from Dupuytren's patients (NPF-D) and palmar fibroblasts from individuals unaffected by Dupuytren's disease (PF-N);. SB-216763 (10 μM), a selective inhibitor of GSK-3β24, was used as a positive control and activated TCF/Lef in all cell types. (B) Only PF-D cultured with TNF (0.1 ng/mL) demonstrated an increase in α-SMA, COL1, and GSK-3β mRNA expression compared with respective untreated cells. The increase in GSK-3β expression by PF-D on exposure to TNF was reversed by LiCl (10 mM). SB-216763

(10 μM) was used as a positive control. (C) Palmar fibroblasts from patients with Dupuytren's disease (PF-D) treated with TNF (0.1 ng/mL) showed increased α-SMA protein expression and increased phosphorylated GSK-3β that was reversed by LiCl (10 mM), demonstrated by Western blotting. β-actin is shown as a loading control and remained constant. (D) Isometric contraction of PF-D was increased with TNF treatment (0.1 ng/mL) and reversed in a dose-dependent manner by LiCL (0.1–10 mM). KCl (10 mM) was used as a control for LiCl. All data shown for $n \geq 3$ patients (each performed in triplicate). Data expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

We evaluated the effect of TNF on Wnt signaling events upstream of TOPflash TCF/Lef luciferase activation. Stimulation of palmar dermal fibroblasts from patients with Dupuytren's disease with TNF resulted in increased phosphorylation of GSK-3β (Fig. 6C), demonstrating that TNF drives inactivation of this key molecule in the Wnt signaling pathway. Again, treatment of cells with SB-216736 as a positive control induced phosphorylation of GSK-3β indiscriminately in both palmar and nonpalmar dermal fibroblasts from patients with Dupuytren's disease (Fig. 6C).

Lithium ions are well-established but nonselective modulators of GSK-3β activity; in the majority of cell types, LiCl inhibits GSK-3β and hence enhances Wnt signaling. However, in adipocytes, lithium ions stimulate the activity of GSK-3β by inhibiting phosphorylation of the enzyme[29]. We found that in nonpalmar dermal fibroblasts from Dupuytren's patients and palmar fibro- blasts from individuals unaffected by Dupuytren's disease, LiCl indeed stimulated TCF/Lef activation (Fig. 6A). In contrast, in palmar dermal fibroblasts from patients with Dupuytren's disease, LiCl had no effect on TCF/Lef activation (Fig. 6A). Moreover, in the latter cell type, we found that TNF-mediated TCF/Lef activation was reversed by LiCI. LiCl also inhibited TNF-mediated α-SMA and COL1 gene expression, α-SMA protein expression, and phosphorylation of GSK-3β (Fig. 6 B and C), and these effects translated to the functional outcome of collagen lattice contraction (Fig. 6D). Together, these data indicate that in palmar dermal fibroblasts from Dupuytren's patients LiCl activates GSK-3β activity and are consistent with the findings that, as previously demonstrated in adipocytes, TNF acts via the Wnt signaling pathway[27].

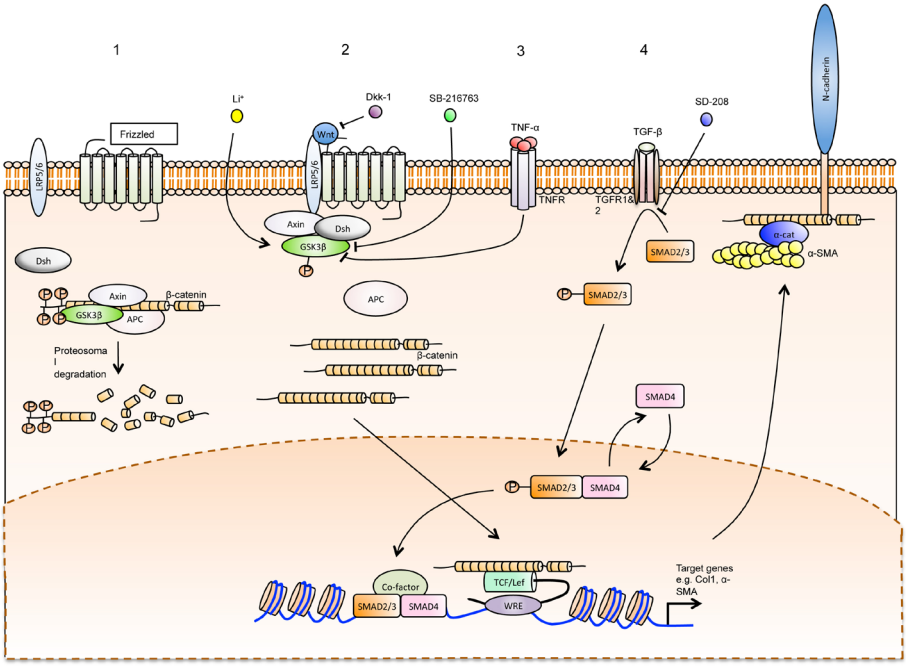

Figure 7. Schematic illustrating how TNF acts via theWnt pathway leading to the development of the myofibroblast phenotype. [1] In the resting state, cytoplasmic β-catenin is phosphorylated by GSK-3β. This modification targets β-catenin for ubiquitination and proteosomal degradation. [2] Ligation by Wnt of the receptor complex comprising Frizzled and LRP5/6 leads to phosphorylation and inhibition of GSK-3β. Thus, β-catenin escapes modification and subsequent degradation, leading to accumulation of cytoplasmic β-catenin, which participates in cell–cell adherens junctions and also translocates to the nucleus. Here it binds to the transcription factors TCF/Lef and promotes the expression of genes typically associatedwithmyofibroblasts: COL1 and α-SMA. Subsequent cytoskeletal assembly of α-SMA protein produces the contractile apparatus of the cell, with attachments to neighboring cells via cadherins and the matrix via integrins. Wnt ligand–receptor binding is competitively inhibited by Dkk-1, leading to resumption of β-catenin degradation. SB-216736 is a small molecule that increases the phosphorylation and thus specifically inhibits the activity of GSK-3β. [3] In palmar dermal fibroblasts from patients with Dupuytren's disease, TNF

binding to TNFR leads to GSK-3β phosphorylation and inhibition, thereby releasing β-catenin from degradation and enabling the transcription of COL1 and α-SMA genes and assembly of an α-SMA–rich cytoskeleton. Li ions restore GSK-3β activity in these fibroblasts, thus enhancing β-catenin degradation and reversing the effect of TNF activation. [4] Binding of TGF-β1 to TGF-β1R1/2 leads to Smad2/3 phosphorylation and activation. The latter recruits Smad4 and, on entering the nucleus, leads to transcription of the same genes as the β-catenin TCF/Lef complex, namely COL1 and α-SMA. The interaction of TGF-β1R1/2 with Smad2/3 is selectively inhibited by SD-208.

Discussion

Currently the management of established Dupuytren's disease once digital contractures have developed is predominantly surgical excision of the affected tissue or, less often, disruption of the cord with a needle or collagenase. Lack of understanding of the signaling pathways driving disease pathogenesis has meant that there is no specific therapeutic for treating early disease or for preventing recurrence following excision or division of the cord. The absence of valid targets has led to empirical treatment with modalities such as local steroid injection[16,17] or radiotherapy[30,31].

To identify the signaling mechanisms responsible for the development and persistence of the myofibroblasts in vivo, we studied freshly isolated cells, obtained from surgically excised Dupuytren's tissue. Using flow cytometry and immunohistology, we found that, although the majority of the cells in these specimens were myofibroblasts, ~7% were macrophages, and the classical M1 proinflammatory phenotype predominated. Classical macrophages have previously been shown to be associated with fibrosis, whereas the role of alternatively activated M2 macrophages is more controversial[32]. Inflammation is known to play a crucial role in fibrosis, and a variety of proinflammatory cytokines have been implicated, including TNF, IL-1β, and IL-6[32]. We found that cells freshly disaggregated from Dupuytren's tissue released appreciable amounts of TNF, IL-6, GM-CSF, and variable amounts of TGF-β1 (Fig. 1).

We documented the effect of these proinflammatory cytokines on dermal fibroblasts from different tissue sources. Our studies were restricted to using cells up to passage 2. Several factors informed our choice of experimental approach. Mature Dupuytren's disease is restricted to certain fibers of the palmar fascia. Many previous studies have compared cells from Dupuytren's nodules or cords with cells from the fascia in the region of the carpal tunnel or the transverse carpal ligament from affected or normal individuals or uninvolved transverse palmar fibers from patients with Dupuytren's disease. However, this approach has limitations. The palmar fascia over the carpal tunnel is rarely affected by Dupuytren's disease in susceptible individuals, and the transverse carpal ligament is always unaffected; hence, it is possible that the constituent cells are inherently different[33]. Furthermore, with the exception of nodules in Dupuytren's disease, fascia is sparsely populated by cells and hence to obtain adequate numbers, most authors use cells to passage 5[12]. However, we and others have shown that at passage 5 the phenotypes of myofibroblasts and normal human dermal fibroblasts tend to merge[21,34]. In addition, whereas the cell of origin for Dupuytren's myofibroblasts remains controversial, there is accumulating evidence that the adjacent tissues, including the overlying dermis, make a significant contribution and that these cells are more akin to Dupuytren's myofibroblasts than fibroblasts from carpal tunnel fascia[24]. Moreover, Dupuytren's disease is restricted to the palm of the hands in patients with a genetic predisposition. Therefore, it is important to avoid variations due to genetic and environmental factors. For these reasons, we compared dermal fibroblasts from palmar and nonpalmar sites from the same group of patients, using palmar dermal fibroblasts from individuals without Dupuytren's disease as controls.

It is well established that TGF-β1 induces the myofibroblast phenotype[7]. Human dermal fibroblasts demonstrate enhanced α-SMA expression at low (1 ng/mL) concentrations of TGF-β1[11,35], whereas fibroblasts from the transverse carpal ligament only responded to higher doses[12,13]. We found that TGF-β1 increased the contractility in a dose-dependent manner of dermal fibroblasts from all three sources at concentrations of 1–10 ng/mL, which is in excess of the range we found

released by freshly disaggregated cells from Dupuytren's nodular tissue. We also found that SD208, a small molecule inhibitor of TGF-β1R1/Smad2/3 interactions, led to a dose-dependent reduction in the myofibroblast phenotype. However, global inhibition of TGF-β1 is undesirable due to the important role of TGF-β1 in a wide range of physiological processes[25] and the increased inflammation, tumor promotion, and cardiac toxicity seen in animal studies[36]. Although no adverse events specifically relating to inhibition of the TGF-β1 pathway have been reported in the limited clinical trials for fibrotic disorders to date, no late-phase studies to date have demonstrated efficacy[25,37].

Fibrosis is a common pathological end point of many inflammatory disorders affecting critical visceral organs. In a murine model, TNF production was found to be essential for the development of bleomycin-induced pulmonary fibrosis, in part through up-regulation of TGF-β1 expression[38], and systemic administration of soluble TNF receptor led to reduction in fibrosis[39]. The utility of these models may, however, be limited because not all strains of mice develop pulmonary fibrosis in response to bleomycin[38], emphasizing the importance of studying primary human tissues. There is no animal model for Dupuytren's disease, and in vivo conditions are most closely emulated in vitro by populating 3D collagen lattices with fibroblasts and maintaining the construct under tension under isometric conditions using a CFM[21,40]. Myofibroblasts only maintain their phenotype under stress, and loss of tension is associated with disassembly of α-SMA stress fibers within minutes[41]. Using the CFM, we found that rhTNF led to a dose-dependent increase in contraction of palmar fibroblasts from patients with Dupuytren's disease. This effect peaked at 100 pg/mL, a concentration similar to that observed in Dupuytren's patient tissue. This effect was comparable to 1 ng/mL of TGF-β1, whereas rhIL-6 and rhIL-1β had no discernible effect. This increased contractility mediated by rhTNF was associated with increased expression of α-SMA and COL1 message and α-SMA protein. In contrast, contraction of palmar fibroblasts from individuals unaffected by Dupuytren's disease was inhibited by rhTNF, as were nonpalmar fibroblasts from patients with Dupuytren's disease, consistent with a previous report[11]. The specific action of TNF is in contrast to

the nonselective effect of TG-Fβ1, which induced the myofibroblast phenotype indiscriminately in all types of dermal fibroblasts. The response to TNF of dermal fibroblasts from different anatomical sites reflects the localization of Dupuytren's disease to the palm in susceptible patients, whereas TGF-β1 increases fibroblast contractility irrespective of the origin of the fibroblasts. It is possible that the dermal fibroblasts derived from palmar skin obtained from patient's with Dupuytren's contracture undergoing dermofasciectomy may have included an appreciable number of myofibroblasts, and this might explain the differing response to TNF. However, this is unlikely because we have previously shown that palmar dermal fibroblasts behave like nonpalmar cells from the same patients in that they reach tensional homeostasis in the CFM, whereas nodule-derived myofibroblasts continue to contract[21].

Fibrosis induced byTGF-β1 has been shown to involve canonical Wnt signaling[42], and there is evidence of cross-talk between Wnt/β-catenin and TGF-β1 signaling pathways, including combinatorial transcriptional regulation of α-SMA for myofibroblast differentiation[43]. Although it is possible that the induction of the myofibroblast phenotype in Dupuytren's palmar fibroblasts was due to downstream activation by TNF of TGF-β1[44], the pathway would be specific for this cell type because TNF reduced the contractility of nonpalmar dermal fibroblasts from Dupuytren's patients and palmar dermal fibroblasts from normal individuals. This is in contrast to the similar effects of TGF-β1 on all three cell types. It is difficult to draw overall conclusions from the literature about interactions between signaling pathways because the source of cells varies widely between studies. The activation of TGF-β1 pathways was shown in 3T3 and murine pulmonary fibroblasts[44], whereas in human dermal fibroblasts from neonatal foreskins, TNF inhibited TGF-β/Smad signaling, again via activator protein1 (AP-1) activation[45].

Wnt/β-catenin signaling has been shown to be intimately involved with fibrosis[46], and a recent genomewide association study showed that this pathway is likely involved in Dupuytren's disease[5]. However, we found that exogenous addition of excess Dkk-1, a negative regulator of Wnt signaling, had no effect

on myofibroblast contractility, suggesting that in the CFM model, contraction is not dependent on a Wnt ligand acting through the canonical pathway[47]. We hypothesized that TNF may be acting via the Wnt/β-catenin signaling pathway, as previously demonstrated in adipocytes[27,48,49]. In the unstimulated cell, β-catenin is phosphorylated by GSK-3β and undergoes ubiquitination and proteosomal degradation. Ligation by Wnt of the receptor complex comprising Frizzled and LRP5/6 leads to phosphorylation and inhibition of GSK-3β, preserving β-catenin from degradation and in turn promoting profibrotic gene expression via its binding to the transcription factors TCF/Lef (Fig. 5). We found that only palmar fibroblasts from patients with Dupuytren's disease exposed to TNF showed an increase in phosphorylated GSK-3β and TCF/Lef activation that was re- versed by LiCl. Whereas lithium ions inhibit GSK-3β in the majority of cells, they selectively activate GSK-3β in specific cell types such as adipocytes[29] by inhibiting its phosphorylation[50]. LiCl is an ATP noncompetitive inhibitor of GSK-3β and also acts indirectly by activating PKB, which in turn phosphorylates GSK-3β can also inhibit other protein kinases, including casein kinase-2, p38 kinase, and MAPK-activated protein kinase-2[25]. Therefore, it is possible that the apparent reversal of the effects of TNF by LiCl in palmar dermal fibroblasts from Dupuytren's patients may have been due to an activation of a pathway other than that mediated by GSK-3β. However, this is unlikely to account for the entire effect of LiCl in this system because it reduced total GSK-3β at both message and protein level and the level of phosphorylated GSK-3β induced by TNF. The downregulation of GSK-3β by LiCl was accompanied by reversal to baseline of the increased levels of mRNA for α-SMA and COL-1, as well as α-SMA protein. The latter translated to reduced contractility in the culture force monitor. These findings confirm that the TNF-induced development of the myofibroblast phenotype of palmar dermal fibroblasts from patients with Dupuytren's disease is mediated at least in part via the Wnt/β-catenin signaling pathway (Fig. 5). Conversely, LiCl led to increased TOPFlash activity in nonpalmar fibroblasts from Dupuytren's patients and palmar dermal fibroblasts from individuals unaffected with Dupuytren's disease.

It has previously been shown that fibroblasts from different sources may show

divergent responses to the same stimuli[46], and more recently[33], it was reported that Dupuytren's myofibroblasts and fibroblasts from uninvolved palmar fascia from the same patients both expressed genes related to Wnt/β-catenin pathway, unlike fascial-derived fibroblasts from individuals without Dupuytren's disease.

The reason for the differential effects of TNF on the three types of dermal fibroblasts is of interest. It may have been due to the increased expression of TNF receptors by dermal fibroblasts from the palm of patients with Dupuytren's disease. TNF receptor (TNFR)1 is constitutively expressed by most cells, whereas endogenous expression of TNFR2 is restricted to a few cell types, including those of the hemopoietic lineage and mesenchymal stromal cells. TNFR2 can be induced by cytokines such as TNF[51]. The levels of TNFR1 and especially TNFR2 were elevated compared with the other dermal fibroblasts. TNFR2 appears to be crucial in signaling a profibrotic response as intestinal myofibroblasts from WT or TNFR1-deficient mice showed increased proliferation and collagen production on treatment with TNF whereas myofibroblasts from TNFR2−/− or combined TNFR1−/−/TNFR2−/− were unresponsive[52]. These findings would be consistent with our data that only palmar dermal fibroblasts from patients with Dupuytren's disease or Dupuytren's myofibroblasts showed increased expression of TNFR2 at both the message and protein level and may explain the selective effect of TNF on the former compared with nonpalmar Dupuytren's fibroblasts and non-Dupuytren's palmar fibroblasts. Myofibroblasts up to passage 2 in 3D collagen lattices exhibited a dose-dependent inhibition of contraction in the CFM on addition of neutralizing antibody to TNF. Inflammatory cells, in particular classically activated M1 macrophages, are likely to represent the major source of TNF in vivo. However, these cells were not present in the second passage myofibroblasts used in the CFM.We found that, at these early passages, myofibroblasts produced low levels of TNF that may continue to act in an autocrine or paracrine manner on cells expressing high levels of TNF receptors. Furthermore, TNF can up-regulate the expression of TNFR2, and in a murine model of pulmonary fibrosis, exposure to silica or bleomycin led to increased expression of TNFR2 but not TNFR1[53]. Neutralizing antibodies can

bind transmembrane TNF and lead to reverse signaling[54]. The latter can result in apoptosis or modulation of the response to external stimuli[55]. We did not find any evidence of impaired cell viability over 24 h when myofibroblasts were exposed to neutralizing antibody to TNF. Instead, inhibition of myofibroblast contractility by blocking antibodies to TNF was associated with reduced expression of COL1 and α-SMA genes and α-SMA protein, together with disassembly of the α-SMA stress fibers. Therefore, anti-TNF effectively reverses the myofibroblast phenotype.

A deeper understanding of the signaling pathways that drive the development of the myofibroblast phenotype should lead to strategies for the treatment of early disease and prevention of further progression. Taken together, our data show that TNF is a rational therapeutic target for the treatment of early-stage Dupuytren's disease or for preventing recurrence. We found that nodules from patients with Dupuytren's disease contain significant numbers of classically activated macrophages, and the cells present in the nodules secrete biologically active amounts of TNF. Unlike TGF-β1, TNF acts specifically on palmar dermal fibroblasts from patients with the appropriate genetic background to induce a myofibroblast phenotype via the Wnt/β-catenin pathway, and these cells show higher expression of TNF receptors, especially TNFR2. The contractility of myofibroblasts was inhibited by anti-TNF and was associated with disassembly of the contractile apparatus. Therefore, we characterized Dupuytren's disease as a localized inflammatory fibroproliferative disorder and proposed TNF as a therapeutic target. It is possible that a similar mechanism pertains to other musculoskeletal fibrotic disorders such as frozen shoulder (adhesive capsulitis), which affects up to 2%of the population[56]. In terms of translating these findings to clinical trials, the ideal anti-TNF agent would be injected locally into the nodules and ideally would remain there to minimize unwanted systemic effects while maximizing efficacy. Systemic inhibition of TNF is now recognized for the treatment of rheumatoid arthritis[57] and inflammatory bowel disease[58,59]. Unlike TGF-β1 inhibition, the safety profile of TNF inhibition is well established. Of the anti-TNF agents approved by the FDA for subcutanoeus administration, the two fully human complete IgG molecules, adalimumab and golimumab,

were the most efficacious in our CFM model in term of inhibiting myofibroblast contractility, and golimumab may be preferred because of a longer half-life, necessitating less frequent injection. Unlike etanercept, both these agents are able to ligate both monomeric and trimeric TNF. Based on our findings, local injection of an anti-TNF to prevent the progression of early stages of Dupuytren's disease or avoid recurrence following surgery, needle fasciotomy, or collagenase digestion is an exciting possibility and is ready to test. If the proof of concept works, second-generation drugs that remain preferentially at the site of disease could be developed.

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Chapter 6

Summary and Discussion

Concluding discussion

Currently the management of established Dupuytren's disease, once digital contractures have developed, is surgical excision of the affected tissue or disruption of the cord with a needle or collagenase; however the problem of recurrence following these procedures has not been resolved. Myofibroblasts are the primary cells that contribute to the pathological processes leading to fibrosis and digital contractures in Dupuytren's disease[1]. Management strategies for treating early disease or for preventing recurrence following excision or division of the cord should be aimed at inhibiting fibroproliferation by targeting the myofibroblast: regulating the myofibroblast phenotype in terms of differentiation, proliferation, ECM production and contractility.

Myofibroblasts play a key role in cell mediated contraction of the extracellular matrix, whereby cells contract to cause physical deformation[2] and secondly, continuous synthesis of extra cellular matrix (ECM), which results in dynamic ECM reorganization, shortening of the matrix and contractures[3]. Tension is know to be a contributing factor in Dupuytren's disease and alterations in the mechanical environment can lead to changes in contractility, matrix remodeling and the production of matrix metalloproteinases (MMPs)[4-6]. There is no animal model for Dupuytren's disease and in vivo conditions are most closely replicated in vitro by populating 3D collagen lattices with cells and maintaining the construct under tension under isometric conditions in a culture force monitor (CFM)[6,7].

Using this model we compared the contraction profiles of Dupuytren's nodule, cord and control flexor retinaculum cells and, in line with previous studies[5,6], we found that flexor retinaculum cells reached a plateau, unlike Dupuytren's cells that kept generating force and continued to contract[8]. Furthermore, we demonstrated that nodule cells are more contractile and play a greater role in matrix turnover and matrix remodeling compared to cord cells[9]. These findings are consistent with the in vivo observation and in line with previous studies[10,11], whereby nodules are comprised of contractile myofibroblasts

and cord represents mainly residual or burnt out disease[12].

In Dupuytren's disease the mechanical environment is dynamic as it is subjected to frequent loads applied to the hand. Therefore, we examined cell contractility in response to altered mechanical load. We showed that nodule cells differ from cord cells by further increasing contractility[6] and related MMP mediated matrix remodeling[9] in response to externally applied loads. Therefore, therapeutic targets should be aimed primarily at regulation of the myofibroblast rich nodules to prevent matrix synthesis and contraction. This would aim to prevent disease progression, and hence would be ideal treatment before there is established contracture.

Myofibroblasts exist in aggregates in Dupuytren's disease, where they represent the vast majority of the cells in the histological nodules of early or active disease[12]. These cells primarily communicate via three distinct means, adherens, mechanosensitive and gap junctions. Adherens junctions are composed of cadherins and at these junctions stress fibers of contacting myofibroblasts are directly connected[13]. We demonstrated that Dupuytren's myofibroblasts expressed more OB-cadherin mRNA and protein whilst dermal fibroblasts expressed higher levels of N-cadherin, consistent with previous reports[13,14]. Myofibroblast development is associated with upregulation of cadherin expression, and OB-cadherin junctions exhibit higher adhesion strength than N-cadherin[14,15]. Mechanosensitive channels open and calcium ions influx through when force is transmitted by tension applied via adherens junctions and adjacent cells can also communicate electrochemically via gap junctions to allow passage of molecules such as calcium ions.

We inhibited each individual form of intercellular junction using blocking peptides, neutralizing antibodies or silencing their RNA and found inhibition of contractility and α-smooth muscle actin (α-SMA) at both message and protein levels[16]. We showed for the first time that mechanical and electrochemical cell-cell junctions are responsible for approximately 50% of the coordinated activity of myofibroblast development and contractility. In addition to cell-cell junctions, myofibroblast develop cell-matrix junctions, which regulate force transmission

and contractile force to promote contraction of the ECM, which is then remodeled to a shortened position, a mechanism termed 'lock-step'[17]. Our data suggests that myofibroblast activity may significantly be downregulated by inhibiting cell-cell junctions and this may offer a new treatment strategy to inhibit myofibroblast activity in fibrosis.

An alternative to inhibiting intercellular junctions would be to reduce myofibroblast cell density and therefore indirectly reduce cell-cell contact. Based on findings in other fibrotic disease, mesenchymal stem cell therapy has been shown to be a potential treatment strategy in fibrosis[18-21]. Therefore, our group has investigated the injection of lipoaspirates containing adipose-derived stem cells following extensive percutaneous needle aponeurotomy as a novel treatment approach to treat Dupuytren's contracture[22]. We examined the biological effect of this novel approach by co-culturing Dupuytren's myofibroblasts with adipose-derived stem cells on contractility and associated levels of α-SMA at both message and protein level. To test whether the effect was specific to adipose-derived stem cells we also compared co-cultures with bone marrow-derived stem cells and dermal fibroblasts.

We demonstrated that myofibroblasts co-cultured with adipose-derived stem cells and dermal fibroblasts led to a dose dependent decrease in contractility and the myofibroblast phenotype, most likely to be due to serial dilution of myofibroblasts, and in turn reduced overall force generation[7]. In direct contrast to our findings with adipose-derived stem cells, we found a dose dependent increase in the myofibroblast phenotype when bone marrow-derived stem cells were co-cultured with myofibroblasts. Here we demonstrate that the source of mesenchymal stem cells should be considered when planning their use in the clinical setting as they may have similar characteristics, but they can exhibit differential effects on myofibroblast contractility[23,24].

Mesenchymal stem cells may mediate their anti-fibrotic effects through their inhibition of proliferation as demonstrated in various tumour and non-tumour cells, including cardiac and renal fibrosis[21,24,26]. In line with these studies we demonstrated a decrease in proliferation of Dupuytren's myofibroblasts

co-cultured with either adipose or bone marrow-derived stem cells. Several lines of evidence suggest that mesenchymal stem cells may in part mediate their inhibitory effect on proliferation by paracrine mechanisms[27]. We examined whether proliferation of myofibroblasts was inhibited in response to soluble factors produced by adipose-derived stem cells, by culturing myofibroblasts in conditioned medium from adipose-derived stem cells and myofibroblasts separated by either a transwell insert to prevent cell-cell contact, or by allowing direct cell-cell contact. We found that Dupuytren's myofibroblast proliferation was inhibited by adipose-derived stem cells under all conditions, and greatest inhibition was seen when direct cell-cell contact between adipose-derived stem cells and myofibroblasts was permitted. This inhibition of proliferation by soluble factors, influenced by cell-cell contact dependent signalling, has been reported by others in other forms of fibrosis and cancer models[21,28].

Inflammation has been shown to contribute to the development and progression of fibrosis[29]. Mesenchymal stem cells have been shown to downregulate inflammatory cytokines, inhibit proliferation and reduce α-SMA protein expression in fibrosis[19,26,30]. This lends support to the potential benefit of lipografting in conjunction with percutaneous needle aponeurotomy in the treatment of Dupuytren's disease.

The link between inflammatory cytokines and myofibroblast development in Dupuytren's disease remain largely unknown. We examined the signaling mechanisms responsible for the development and persistence of the myofibroblast by studying freshly isolated cells. Using flow cytometry and immunohistology, we demonstrated that whilst the majority of the cells in Dupuytren's nodules were myofibroblasts, ~87%, interestingly ~7% were macrophages of which the classical M1 proinflammatory phenotype predominated. These classical M1 macrophages have previously been shown to be associated with fibrosis[31].

Furthermore, we found that cells freshly disaggregated from Dupuytren's nodules released appreciable amounts of proinflammatory cytokines TNF and TGF-β1. We tested the effect of these proinflammatory cytokines on dermal

fibroblasts from palmar and non-palmar sites from the same group of patients, using palmar dermal fibroblasts from individuals without Dupuytren's disease as controls. It is well established that TGF-β1 induces the myofibroblast phenotype[32]. We demonstrated that TGF-β1 increased the contractility in a dose-dependent manner in dermal fibroblasts from all three sources at concentrations of 1–10 ng/ml, which is in excess of the range we found released by freshly disaggregated cells from Dupuytren's nodular tissue. We have also described that SD208, a small molecule inhibitor of TGF-β1R1/Smad2/3 interactions, led to a dose-dependent reduction in the myofibroblast phenotype. However, whilst there have been limited clinical trials to inhibit TGF-β1 in fibrosis, no late-phase studies to date have demonstrated efficacy of inhibiting TGF-β1 pathways in fibrotic disorders[33]. Furthermore, we demonstrated that proinflammatory cytokine TNF led to a dose-dependent increase in contraction in palmar fibroblasts from patients with Dupuytren's disease, with associated increased expression of α-SMA and COL1 message and α-SMA protein. This effect peaked at 100 pg/mL, a concentration similar to that observed in freshly disaggregated Dupuytren's nodular tissue. In contrast, contraction of palmar fibroblasts from individuals unaffected by Dupuytren's disease and non-palmar fibroblasts from patients with Dupuytren's disease was inhibited by TNF, consistent with a previous report[34].

The differential response to TNF of dermal fibroblasts from different anatomical sites reflects the localization of Dupuytren's disease to the palm in susceptible patients. Unlike TGF-β1, TNF acts specifically on palmar dermal fibroblasts from susceptible patients to induce a myofibroblast development. Wnt/β-catenin signaling has been shown to be intimately involved with fibrosis[35], and a recent genomewide association study showed that this pathway is likely involved in Dupuytren's disease[1]. We found that only palmar fibroblasts from patients with Dupuytren's disease exposed to TNF showed an increase in phosphorylated GSK-3β and TCF/Lef activation that was reversed by LiCl. These findings confirm that the TNF-induced development of the myofibroblast phenotype of palmar dermal fibroblasts from patients with Dupuytren's disease is mediated at least in part via the Wnt/β-catenin signaling pathway. It

has previously been shown that fibroblasts from different sources may show divergent responses to the same stimuli[35], and more recently[36], it was reported that Dupuytren's myofibroblasts and fibroblasts from uninvolved palmar fascia from the same patients both expressed genes related to Wnt/β-catenin pathway, unlike fascial-derived fibroblasts from individuals without Dupuytren's disease. The reason for the differential effects of TNF on the three types of dermal fibroblasts is of interest. We have demonstrated that only palmar dermal fibroblasts from patients with Dupuytren's disease or Dupuytren's myofibroblasts showed increased expression of TNF receptor 2 at both the message and protein level and this may explain the selective effect of TNF.

Finally, the contractility of myofibroblasts was inhibited by anti-TNF and was associated with reduced expression of COL1 and α-SMA genes and α-SMA protein, together with disassembly of the α-SMA stress fibers. TNF acts specifically on palmar dermal fibroblasts from patients with the appropriate genetic background to induce myofibroblast development via the Wnt/β-catenin pathway, and these cells show higher expression of TNF receptors, especially TNF receptor 2. Therefore, we characterized Dupuytren's disease as a localized inflammatory fibroproliferative disorder and proposed TNF as a therapeutic target[37].

Future work

A deeper understanding of the signaling pathways that lead to the development and persistence of the myofibroblast phenotype should ultimately lead to strategies for the treatment of early disease and prevent recurrence. Areas of future work leading on from this thesis should include a more detailed investigation of the interaction of Dupuytren's myofibroblasts and adipose-derived stem cells, examining the soluble factors produced by adipose-derived stem cells inhibiting proliferation and how this is mediated by cell-cell contact. These anti-proliferative effects may include inhibition of inflammatory

cytokines, changes in anti-proliferation-related genes and blocking of cellular communication. Furthermore, it will be interesting to examine the anti-inflammatory potential of adipose-derived stem cells in the prevention of fibrosis at the early inflammatory and later stages of Dupuytren's disease.

Clinically, long term results following extensive percutaneous aponeurotomy followed by lipografting as a new treatment for Dupuytren's disease are awaited, and may prove beneficial in reducing Dupuytren's recurrence through reduced myofibroblast proliferation.

Current efforts to treat Dupuytren's disease have been directed towards excision or disruption of the affected tissue. However, these fibrotic cords have resulted following initial myofibroblast proliferation, ECM production and contraction, and therefore, primarily targeting the myofibroblast would serve as a better treatment strategy. Our data suggest that TNF inhibition may be real therapeutic options worth investigating in the clinical setting.

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Summary

Research into treatment strategies for Dupuytren's disease should be targeted at inhibiting fibroproliferation by modulating the myofibroblast, the cell responsible for both extracellular matrix deposition and contraction. Taken together, we examined what factors regulate the myofibroblast phenotype in terms of differentiation, proliferation, extracellular matrix production and contractility.

In **chapter 2** we showed that changes in the mechanical environment in Dupuytren's disease lead to increased myofibroblast contractility and matrix metalloproteinases mediated matrix remodeling. In turn this may lead to permanent matrix shortening and progress to cause digital contracture. We confirmed that nodule cells are more contractile and play a greater role in matrix remodeling compared to cord cells, these findings are consistent with the in vivo observation, whereby nodules in active disease are comprised of contractile myofibroblasts and cord represents mainly residual or burnt out disease.

Dupuytren's nodules are comprised of densely packed myofibroblasts that communicate through adherens, mechanosensitive and gap junctions. In **chapter 3** we confirmed that Dupuytren's myofibroblasts expressed more OB-cadherin at both message and protein levels, consistent with higher contractile force, compared to fibroblasts expressing higher levels of N-cadherin. Furthermore, by using an in vitro model of contraction we have described that myofibroblasts act in concert to contract. By blocking cell-cell contact we also demonstrated this coordinated activity is responsible for approximately 50% of the overall contractile effect, the remainder being through cell to matrix interactions.

By reducing the cell density and hence cell-cell contact by co-culturing contractile myofibroblasts with adipose-derived stem cells in **chapter 4**, we decreased overall myofibroblast contractility in a dose-dependant manner. Furthermore, adipose-derived stem cells inhibited myofibroblast proliferation. These effects were mediated by soluble factors and influenced by cell-cell contact dependent signalling. This lends support to the potential benefit of lipografting in

conjunction with extensive percutaneous needle aponeurotomy in the treatment of Dupuytren's disease. We also demonstrate that MSCs isolated from different tissues have similar characteristics, but they can exhibit differential effects on myofibroblast contractility.

Finally in **chapter 5**, we characterized Dupuytren's disease as a localized inflammatory fibroproliferative disorder in which TNF induced development of the myofibroblast phenotype in palmar dermal fibroblasts from patients with Dupuytren's disease. This is mediated at least in part via the Wnt/β-catenin signaling pathway. In contrast, myofibroblast development in palmar fibroblasts from individuals unaffected by Dupuytren's disease and nonpalmar fibroblasts from patients with Dupuytren's disease was inhibited by TNF. The differential response to TNF of dermal fibroblasts from different anatomical sites reflects the localization of Dupuytren's disease to the palm in susceptible patients, where these cells specifically express higher levels of TNF receptors, especially TNF receptor 2. Anti-TNF treatment in vitro inhibited the contractile activity of myofibroblasts derived from Dupuytren's patients. Based on these findings, local injection with an anti-TNF agent to prevent the progression of early Dupuytren's disease and help reduce recurrence has reached the threshold for trials in the clinical setting.

Samenvatting

Onderzoek naar nieuwe behandelingsmogelijkheden voor de ziekte van Dupuytren zou gericht moeten zijn op het inhiberen van myofibroblasten. Myofibroblasten zijn verantwoordelijk voor zowel de productie als voor de contractie van de extracellulaire matrix. We onderzochten welke factoren de myofibroblast reguleren op het niveau van de differentiatie, proliferatie, extracellulaire matrix productie en de contractiliteit.

Hoofdstuk 2 toont aan dat mechanische veranderingen in de extracellulaire matrix in de ziekte van Dupuytren kan leiden tot verhoogde contractiliteit van de myofibroblasten en matrix metalloproteinase gemedieerde matrix remodeling. Dit kan vervolgens leiden tot permanente matrix verkorting met als gevolg contracturen aan de vingers. Bovendien bevestigden we dat cellen van de nodus contractieler zijn en een grotere matrix remodeling capaciteit hebben in vergelijking met cellen van de streng. Deze bevindingen komen overeen met de in vivo observatie, waarin de nodus in actieve ziekte bestaat uit contractiele myofibroblasten en de streng voornamelijk uit restant of uitgebluste ziekte.

Dupuytren's noduli bestaan uit dicht op elkaar gepakte myofibroblasten die communiceren via adherens, mechanosensitive junctions en gap junctions. In **hoofdstuk 3** bevestigden we dat Dupuytren's myofibroblasten meer OB-cadherin tot expressie brengen, op zowel het gen en als op het proteïne niveau. Dit is in overeenstemming met een hogere contractiekracht, in vergelijking met fibroblasten die meer N-cadherin tot expressie brengen. Door middel van een in vitro model van contractie toonden we aan dat myofibroblasten samenwerken bij de contractie door cel-cel contact. Door het blokkeren van dit cel-cel contact demonstreerden we dat deze samenwerking verantwoordelijk is voor ongeveer 50% van de totale contractiekracht, en de resterende 50% waarschijnlijk door cel en matrix interacties.

Door het verminderen van de dicht op elkaar gepakte myofibroblasten en subsequent de vermindering van cel-cel contact door het kweken van contractiele myofibroblasten met stamcellen verkregen uit vetweefsel in **hoofdstuk 4**, hebben we de contractiliteit van de myofibroblast verminderd op een dosis-afhankelijke wijze. Bovendien inhibeerden stamcellen de proliferatie van myofibroblasten. Deze inhiberende effecten werden bewerkstelligd door oplosbare factoren die geproduceerd werden door stamcellen en werden beïnvloed door cel-cel contact. Deze bevindingen ondersteunen het potentiële voordeel van het injecteren van vetweefsel na extensieve percutane naald aponeurotomie in de behandeling van de ziekte van Dupuytren. Ook toonden we aan dat stamcellen die geïsoleerd zijn van verschillende weefsels overeenkomstige eigenschappen hebben, maar dat deze andere effecten op de contractiliteit van myofibroblasten bewerkstelligen.

Tenslotte hebben we in **hoofdstuk 5** de ziekte van Dupuytren als een gelokaliseerde inflammatoire fibroproliferatieve aandoening gekarakteriseerd, waarin TNF myofibroblastdifferentiatie induceert in palmaire huidfibroblasten van patiënten met de ziekte van Dupuytren. Dit werd tenminste gedeeltelijk gemedieerd via de Wnt/β-catenin signaling pathway. Echter, TNF inhibeerde myofibroblast differentiatie in palmaire fibroblasten van individuen zonder de ziekte van Dupuytren en in niet palmaire fibroblasten van patiënten met de ziekte van Dupuytren. Het verschil in respons op TNF in huidfibroblasten van verschillende anatomische lokaties weerspiegelt de lokalisatie van de ziekte van Dupuytren in de handpalm van vatbare patiënten, waar deze cellen een hogere expressie van TNF receptoren hebben, vooral van TNF receptor 2. Behandeling van myofibroblasten van patiënten met de ziekte van Dupuytren met anti-TNF inhibeerden de contractiliteit in vitro. Voortbouwend op deze bevindingen, is lokale injectie met anti-TNF ter preventive van progressie in een vroeg stadium van de ziekte van Dupuyten en ter reductie van recidief na behandeling klaar om getest te worden in de klinische setting.

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Curriculum Vitae

Jennifer Susanna Noëlle Verhoekx was born on December 25th 1983 in Alphen aan den Rijn, the Netherlands. After graduating from the Groene Hart Lyceum in Alphen aan den Rijn she went on to study Medicine at The University of Leiden in 2004. Her inspiration for Plastic & Reconstructive Surgery began when assisting in the operating theatre as a medical student and led to her BSc programme in Stanmore under the supervision of Dr. Mudera, at Univeristy College London. Intrigued by the basic science research in to fibrosis and the pathobiology of Dupuytren's disease, her study was extended in to a PhD thesis to investigate new therapies and regulate fibroproliferation in Dupuytren's disease. This work was carried out over four years in 3 large research facilities both in Holland and the UK. In Holland she worked at the department of Plastic, Reconstructive and Hand Surgery at the Erasmus Medical Centre in Rotterdam, with Professor Hovius. In The UK she was based at The Tissue Repair and Engineering Centre, University College London with Dr. Mudera, and at The Kennedy Institute of Rheumatology, University of Oxford with Professor Nanchahal. This work was funded by the Esser Foundation and has been published in high impact science journals. It was awarded prizes for best presentation at a national and an international meeting, and presented internationally as an invited lecture. Following completion of her Doctorate of Philosophy, Jennifer will expect to complete her MD training in June 2014.

PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student: Jennifer Verhoekx
Erasmus MC, University Medical Centre
Department: Plastic, Reconstructive and Hand Surgery
PhD period: January 2009 – January 2014
Promotor: Prof. dr. S.E.R. Hovius
Copromotor: Dr. E.T. Walbeehm

PhD training	**Year**	**Workload (hours/ECTS)**
General courses		
• Principles of Research in Medicine (ESP01)	2011	20 / 0.7
• The why and how of readable articles (ESP60)	2011	14 / 0.5
• Biostatistics for Clinicians (EWP22)	2011	20 / 0.7
Specific courses		
• Microsurgery; Skillslab Mevr. J.M. Hekking	2011-2012	36 / 1.3
Presentations		
• Oral presentation at the annual scientific meeting of the Rijnland hospital in Leiden, The Netherlands. De behandeling van Morbus Dupuytren met plaatjes rijk plasma.	2008	20 / 0.7
• Oral presentation at the European Conference of Scientists and Plastic Surgeons (ESCAPS) meeting in Rotterdam, The Netherlands. The synergistic effect of TGF-β1 and PDGF-BB on inhibition of myofibroblast induction in Dupuytren's fibroblasts.	2009	20 / 0.7

• Oral presentation at the annual research meeting of the Dutch Society of plastic Surgery (NVPC) in Maastricht, the Netherlands. Inhibitie van Dupuytren's contractuur door hoge concentraties TGF-β1 en PDGF-BB.	2009	20 / 0.7
• Oral presentation at the annual scientific meeting of the Rijnland hospital in Leiden, The Netherlands. Inhibitie van Dupuytren's contractuur door hoge concentraties TGF-β1 en PDGF-BB (**awarded with best oral presentation**).	2009	20 / 0.7
• Oral presentation at the London-Cardiff Mesenchymal Cell Biology meeting in Bedford, the United Kingdom. The effect of cytokines on profibrotic fibroblasts in Dupuytren's disease.	2010	20 / 0.7
• Poster presentation at the Tissue and Cell Engineering Society (TCES) at the University of Leeds, the United Kingdom. Cytokines modulated myofibroblast induction and force generation in Dupuytren's fibroblasts; lessons for tissue engineering?	2011	20 / 0.7
• Oral presentation at the European Conference of Scientists and Plastic Surgeons (ESCAPS) meeting in Munich, Germany. Adipose derived stem cells inhibit proliferation and contractility (**awarded with best oral presentation**).	2012	20 / 0.7
• Oral presentation at the British Association of Plastic, Reconstructive and Aesthetic Surgeon (BAPRAS) winter meeting combined with the American Society of Plastic Surgeons. Adipose derived stem cells and Dupuytren's Contracture (**invited guest lecture**).	2012	20 / 0.7
• Oral presentation at the European Association of Plastic Surgeons (EURAPS) meeting in Antalya, Turkey. Adipose derived stem cells inhibit the contractile myofibroblast in Dupuytren's disease.	2013	20 / 0.7

Organisation

• Helping organise the 17th Esser Course: Dupuytren's contracture with live surgery in Rotterdam, The Netherlands	2011	40 / 1.4

Teaching

Supervising practicals

• Organising and supervising tissue culture practical's for BSc students at TREC	2010-2011	20 / 0.7
• Organising and supervising tissue culture practical's for MSc students at TREC	2010-2011	20 / 0.7
• Organising and supervising Journal Club	2011-2012	18 / 0.6

Supervising

BSc thesis

• Supervising BSc thesis by Maaike Hermans: Matrix remodelling in Dupuytren's disease on the nanoscale.	2010	80 / 2.9
• Supervising BSc thesis by Edward Bayliss: Quantifying matrix remodelling by Dupuytren's fibroblasts under the influence of fixed or varied concentrations of transforming growth factor β1.	2012	80 / 2.9

MSc thesis

• Supervising MSc thesis by Alex Bucknor: Dupuytren's fibroblasts extend and retract their processes with altered concentrations of TGF-β1 (prize for best research project).	2011	80 / 2.9

PhD thesis

• Supervising 2 PhD students, Darren Player and Neil Martin, on building the t-CFM and setting up research methodology at Bedford University.	2010	60 / 2.1
• Supervising 2 PhD students, Prasad Sawadkar and Katerina Stamati, on t-CFM methodology at UCL.	2013	60 / 2.1

• Supervising 1 PhD student, David Izadi, on tissue culture, CFM, PCR, Western blot, immunofluorescence staining at Oxford University.	2013	140 / 5.1

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• Esser foundation (€ 30.000)

• Rijnland hospital (€ 5.000)

Total	**908 / 32.3**

Publications

Verhoekx JSN, Beckett KS, Bisson MA, McGrouther DA, Grobbelaar AO, Mudera V. The mechanical environment in Dupuytren's contracture determines cell contractility and associated MMP mediated matrix remodeling. J Orthop Res. 2013; 31:328-34.

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Verhoekx JSN, van der Werff JFA, Koch RA, Bucknor A, Mudera V. PDGF inhibits TGFβ1 induced myofibroblast differentiation and force generation in Dupuytren's disease. Submitted.