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The Progression from Contraction to Contracture in Dupuytren's Derived Fibroblasts: a Study of the Cellular and Molecular Events

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ABSTRACT

Dupuytren's disease is a debilitating fibroproliferative disorder of the palmar fascia affecting hand function. Clinically the appearance of nodules and cords is characterised by the deposition of excess extracellular matrix within the fascia. Progressive shortening of the matrix leads to increased stiffness and permanent tissue contracture. Surgical release of contracture is the only current treatment but despite this, recurrence rates are high. It has been postulated that contracture is a result of two separate processes occurring in parallel: a) Cell mediated contraction of the matrix – whereby fibroblasts act to cause a physical deformation within the resident tissue (Harris *et al* 1981), and b) Continuous matrix remodelling, leading to the permanence of contracture (Flint and Poole 1990; Tomasek *et al* 2002).

A culture force monitor model was used to study the contractile properties of fibroblasts cultured in three dimensional collagen gels. Dupuytren's nodule and cord fibroblasts generated significantly greater forces in comparison to carpal ligament fibroblasts ($p<0.001$), and similar forces in comparison to dermal fibroblasts over a 48 hour period. Carpal ligament and dermal fibroblasts reached tensional homeostasis by 24 hours showing no further increase in contraction. Dupuytren's fibroblasts continued to contract with no plateau at 24 or 48 hours ($p< 0.001$). A reduction in external load applied to these fibroblasts resulted in an increase in cellular contraction by both Dupuytren's and control fibroblasts.

Baseline tissue inhibitor of matrix metalloproteinase (TIMP) expression in both Dupuytren's and control fibroblasts without external mechanical stimulation was significantly greater than that of the matrix metalloproteinases (MMPs). There was no difference in expression between carpal ligament and Dupuytren's fibroblasts. Mechanical stimulation resulted in a significant up-regulation of MMP gene expression by Dupuytren's nodule fibroblasts ($p<0.01$). There was no up-regulation of MMPs by cord or carpal ligament derived fibroblasts. There was a reciprocal significant up-

regulation of TIMP-1 expression by carpal ligament derived cells after mechanical stimulation ($p<0.001$), with a similar response by cord derived cells ($p<0.005$). This response was absent in nodule derived fibroblasts.

The amount of permanent shortening of a collagen matrix, the residual matrix tension (RMT), was quantified over a 48 hour period using the culture force monitor model. Over a short time period residual matrix tension was minimal following disruption of the actin cytoskeleton by cytochalasin-D in all fibroblasts under investigation, indicating that no spatial remodeling of the collagen had occurred. However by 48 hours a permanent shortening of the collagen network was seen which was most marked for Dupuytren's and dermal fibroblasts, and which was significantly greater than that for carpal ligament fibroblasts ($p<0.05$).

In summary, there appears to be a primary abnormality in the process of cellular contraction, leading to the progression of contracture seen in Dupuytren's disease. It is postulated that cellular contraction holds the matrix in a newly shortened state, while concurrently the cells act to remodel the surrounding matrix to hold it there permanently.

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DECLARATION OF ORIGINALITY

I declare that the laboratory research for this thesis is original and that the ideas were developed in conjunction with my supervisors. I performed the experiments myself with the guidance and technical assistance of the laboratory and scientific staff at the Restoration of Appearance and Function Trust (RAFT) Institute, Mount Vernon Hospital, and The Tissue Repair and Engineering Unit at the Royal National Orthopaedic Hospital.

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LIST OF ABBREVIATIONS

CD	cytochalasin-D
CFM	culture force monitor
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl(e)sulphoxide
ECM	extracellular matrix
FCS	fetal calf serum
FPCL	fibroblast populated collagen lattice
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LPA	lipophosphatidic acid
MCPJ	metacarpophalangeal joint
MMPs	matrix metalloproteinases
NGM	normal growth media
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPJ	proximal interphalangeal joint
RMT	residual matrix tension
GT buffer	guanidium thyocyanate buffer
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
TGF-β1	transforming growth factor beta one
TIMPs	tissue inhibitors of matrix metalloproteinases
α-SMA	alpha smooth muscle actin
TEM	transmission electron microscopy

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CHAPTER 1

INTRODUCTION

1.1 HISTORY OF DUPUYTREN'S DISEASE

The name Dupuytren's disease is derived from the French surgeon, Baron Guillaume Dupuytren (1777-1835), who lectured extensively on the subject during the course of 1830's. However this fibroproliferative disorder has been mentioned as far back as the 12th century in a review of the Icelandic sagas (Whaley and Elliot 1993). Four patients appeared to have received miracle cures for their contracted digits, either by rupture, or fasciotomy. It has also been thought that "the Papal hand of Benediction" was adopted due to an early Pope suffering from digital contracture, although this has not been substantiated (Elliot 1999).

The earliest reference to the disease in surgical text is in the writings of Felix Plater of Basel in 1614. He documented the case of a stonemason who presented with contracted ring and little fingers into the palm of the hand, with ridging of the palmar skin. The condition was originally thought to have been due to contracted tendons, which had bowstringed across the palm. It is only retrospective analysis of these findings that has connected Plater's work with Dupuytren's disease.

The legendary "Curse of the McCrimmons" demonstrates the increased prevalence of the disease in the Northern latitudes (Elliot 1999). The clan members were recognized as outstanding bagpipe players; however they were believed to have been cursed with a condition that bent the little finger, making the playing of bagpipes impossible. This curse only seemed to affect the older and more accomplished players, leading to the retrospective assumption that this was due to Dupuytren's disease and not a congenital hand disorder.

Henry Cline, a pupil of John Hunter, is thought to have been the first to dissect 2 hands with the condition in 1777. His notes record the involvement of the palmar fascia and the effects of dividing it as a fasciotomy. Indeed by coincidence this was during the year of Dupuytren's birth. Despite much debate on the origins of the description of this condition, it is Baron Dupuytren's name that has remained associated with the disease

today, and indeed it must be accepted that Dupuytren did much to further the treatment of the condition that bears his name.

1.2 EPIDEMIOLOGY

The prevalence of Dupuytren's disease varies worldwide. McFarlane (1990) looked at over 1000 patients in a study of data from over 12 countries around the world. He found that patients with Dupuytren's disease are of mainly Northern European descent, and that the disease is also relatively common in the Oriental population.

Much variation in the incidence and prevalence of Dupuytren's disease has been reported in the literature. The main problems have been in reporter accuracy in diagnosing the condition. A study by Noble *et al* (1984) found that the prevalence of the disease was 42% when patients were examined by hand surgeons, but only 18% when examined by a physician. Prevalence is defined as the rate of disease in a specific population at a certain time (Prevalence = proportion of population with the disease at one point in time), whereas incidence notes the rate of development of a disease in a group over a period of time (incidence = number of new cases in one year divided by number at risk). Based on these definitions, it is understandable that the majority of data is prevalence based.

A well conducted epidemiological study was performed by Mikkelsen (1972), who reported a prevalence of 9.4% for men and 2.8% for women for Dupuytren's disease. The disease was bilateral in 59% of men and 43% of women. In both sexes the prevalence increases with age. In the UK, prevalence of the disease is estimated at 39% of men and 21% of women in patients over the age of sixty (Lennox *et al* 1993). However a study of 919 patients in an English orthopaedic hospital found that 5% of men and 3.5% of women were affected (Mackenny 1983), while Carson and Clarke (1993) reported a prevalence of 13.75% in a group of elderly ex-servicemen. If a prevalence rate of approximately 5% is extrapolated to the total population of the UK in 2002 of 59.2 million (Office for National Statistics 2003), then a minimum of 2.9 million British

people may be affected by Dupuytren's disease. The resulting economic implications are obvious.

The prevalence of Dupuytren's disease increases with advancing age (Ross 1999), and also the sex distribution changes. Mikkelson (1972) reported that the sex ratio decreases from 8.4:1 (male: female) at the age of 40, to 1.2:1 at the age of 80. Men typically present for treatment one decade earlier than affected women, in the fifth decade (Thurston 2003). Only a few cases in the literature have mentioned the disease appearing in children (Urban *et al* 1996).

Dupuytren's disease has rarely been reported in non-Caucasian races. Within the black population it is extremely rare, and documentation has been limited to only a few case reports (Ross 1999). Saboeiro *et al* (2000) assessed 9938 patients over a 10-year period, looking in particular at racial background. The incidence of Dupuytren's disease was seen to be 0.13% for the black population, 0.07 % for Asians, and 0.24% for the Hispanic population, in comparison to 0.73% in a Caucasian population. Egawa (1985) reported a prevalence of 19% in the Japanese population, but noted that rate of progression appeared to be much slower than in Caucasians.

1.3 AETIOLOGY

The search for a direct causative agent in Dupuytren's disease continues, and although there have been many links to associated diseases or factors, it remains unclear whether the disease / associations have common causes, influence each other or indeed are simply age related.

1.3.1 Smoking

A link between Dupuytren's disease and nicotine use has been documented in the literature. Between 35 – 68% of patients with the disease were reported to be smokers in some research (An 1988; Brenner and Rayan 2003). A study by Burge *et al* (1997)

showed that patients with Dupuytren's disease smoked a significantly greater amount compared to control patients (16.7 vs. 12 pack years). It has been postulated that oxygen free radical generation and local changes in the microcirculation of the hand in smokers may predispose the patient to fibromatous deposits (Yi *et al* 1999).

1.3.2 Alcohol

The role of alcohol in the pathogenesis of Dupuytren's disease is difficult to establish mainly due to the problem that alcoholic patients tend also to abuse other substances including nicotine, and that alcohol excess is also associated with hepatic disease. Noble *et al* (1992) reported findings that the disease was more common in alcoholics in comparison to those with non-alcoholic liver disease, although his study did not reach statistical significance. Burge *et al* (1997) showed evidence that weekly alcohol consumption in patients with Dupuytren's disease exceeded that of control patients. However in contrast separate studies have shown that there was no difference in alcohol consumption between control patients and those with digitopalmar contracture (Hurst *et al* 1990; Brenner and Rayan 2003). With respect to cirrhosis, the prevalence of Dupuytren's disease was seen to be significantly higher in patients with alcohol induced liver disease in comparison to patients with non-alcoholic liver disease, but there was no difference in prevalence between alcoholics with or without liver disease (Attali *et al* 1987).

1.3.3 Occupation

The relationship between occupation and Dupuytren's disease has been extensively debated with no firm conclusions reached. Some studies suggest an increased prevalence in those engaging in hard manual labour (Dupuytren 1832; Mikkelsen *et al* 1978; Liss *et al* 1996), while others have reported that as many non-manual workers are affected as manual workers (Hueston 1987; McFarlane 1991). Recent interest in the condition "vibration white finger" has sparked discussion about vibrating tools and Dupuytren's disease, but no associations have been made (Hueston and Seyfer 1991). It has been suggested however that the severity of Dupuytren's disease is related to manual labour in

two separate studies looking at Tubiana's classification and occupation (Mikkelsen 1990; Brenner *et al* 2001). In both cases disease severity was higher in manual workers.

1.3.4 Hand trauma

The contribution made by repetitive hand microtrauma to Dupuytren's disease is still uncertain. Another relationship which has been discussed is that of an isolated injury to the hand or forearm that initiates contracture. Certainly Dupuytren's disease has been documented occurring after a single distal radius fracture and from simple soft tissue trauma (Hueston 1968; Stewart *et al* 1985; Gordon and Anderson 1961), but opinion remains divided upon whether the insult was the cause, or the factor directing the patient to the disease.

It is felt that a single specific traumatic event to the hand may precipitate the disease, particularly in younger people (Hueston 1968, Millesi 1967). Wilhelm in 1971 stated that a relationship between specific trauma and fibromatous change is present, but only in cases where a hereditary predisposition can be excluded, and no Dupuytren-like changes were identifiable in the preceding 6 months of the injury. However others have stated that a single injury may not cause the disease, but may precipitate its onset in genetically predisposed individuals (Ross 1999).

1.3.5 Epilepsy

Epilepsy has been found to be much more common in patients with Dupuytren's disease with a reported incidence of between 8% and 57% (Yi *et al* 1999). There are two schools of thought on the reasons behind this. Some think that both conditions are hereditary disorders with genetic causes, while others state that the increased incidence of the disease is due to a side effect of anti-epileptic medication, in particular phenobarbitones (Brenner and Rayan 2003). A recent case control study has shown no associations between epilepsy or its medication with Dupuytren's disease (Geohegan *et al* 2004).

1.3.6 Diabetes

An association between diabetes and Dupuytren's contracture has been demonstrated by many workers (McFarlane 1987; Hurst *et al* 1990; Ross 1999). The frequency of the disease in diabetic patients is thought to increase with age and with the duration of diabetes (Ross 1999). What stands out most significantly is the finding that in the majority of cases of Dupuytren's disease affecting diabetics, the disease follows a milder course, with few patients progressing to surgical intervention (McFarlane 1987; Hurst *et al* 1990; Noble 1992). In addition to this the disease appears to affect the radial aspect of the hand to a greater degree than the ulnar side (Brenner and Rayan 2003).

The reasons behind these findings remain unclear. A study by Rosenbloom *et al* (1996) has shown that there is an excess of advanced glycation end products (AGE) in diabetics with Dupuytren's contracture. AGE products are formed as a result of the nonenzymic reaction of glucose with proteins, causing structural protein rearrangement, and dehydration, release of inflammatory cytokines, and increased collagen cross-linking. This may explain the changes seen within the palmar fascia, and also associated changes within the flexor sheath in some patients with diabetes. It has also been hypothesized that these AGE products may be a factor in the limited joint mobility seen in patients with diabetes and Dupuytren's disease.

1.3.7 Autoimmune Disease

An autoimmune disease occurs as a result of a breakdown in either the humoral or cellular immune system, so that antigen cross-reaction occurs, and the body loses the ability to recognize self (Brenner and Rayan 2003). Many have suspected that Dupuytren's contracture may be an autoimmune phenomenon. The first to provide evidence of this however was Menzel (1979) who demonstrated the presence of collagen III antibodies in the sera of patients with the disease. Similarly Pereira (1986) found 69% of patients with Dupuytren's had antibodies to one of the collagen subtypes compared to only 28% of blood donor controls. Other recent work has shown that it may be an

abnormality in the T cell mediated immune response that may be responsible for palmar fibromatosis (Brenner and Rayan 2003).

1.3.8 Links to Neoplasia

Dupuytren's disease is classified as a benign condition. However it does display several features similar to neoplastic disease. These include its strong genetic association with distinct chromosomal abnormalities, the multiple disease stages, and also the high recurrence rate, with invasive phenotypes in some (Varallo *et al* 2003). Indeed recent epidemiological studies have shown an increase in total mortality, and in cancer mortality among men with Dupuytren's contracture, even after adjustments were made for age, and other confounding factors (Mikkelsen *et al* 1999; Gudmundsson *et al* 2002).

Despite a large number of studies over many years, the underlying aetiology of Dupuytren's disease has still not been identified. It is for this reason that experimental investigation into the cellular and environmental factors that may contribute to the switch from normal to diseased fascia must continue.

1.4 GENETICS OF DUPUYTREN'S DISEASE

There are 2 elements that stand out in the aetiology of Dupuytren's disease. The first is the strong association between the disease and Caucasians of Northern European ancestry, and the second is the familial nature of the condition (Burge 1999). Genetic studies have yet to detect a specific gene or set of genes as the primary cause of Dupuytren's disease.

There have been many different hypotheses into the mode of inheritance in Dupuytren's disease, but most workers agree that appears to be a complex oligogenic condition (Burge 1999). Indeed since the development of gene microarray techniques, plus advances in knowledge of the human genome, it has been seen that at least 23 genes are expressed at

consistently different levels in Dupuytren's tissue in comparison with that of normal palmar fascia (Pan *et al* 2003).

Previous work has identified no association between Dupuytren's Disease and TGFβ1 and TGFβ2 polymorphisms (Bayat *et el* 2002). However recent studies by the same authors have shown a new association with a single nucleotide polymorphism in the TGFβRII gene in patients with more severe forms of the disease (Bayat *et al* 2003). Further work has also shown a definite association in Dupuytren's disease with the ZF9 transcription factor gene located on chromosome 10p15. This factor is responsible for the expression of TGFβ1 in tissue, and thus an abnormality of its corresponding gene may be responsible for the increased fibrosis that is pathognomic of Dupuytren's (Bayat *et al* 2003).

1.5 THE ANATOMY OF DUPUYTREN'S DISEASE

1.5.1 Normal palmar fascia

The palmar fascial complex (PFC) consists of the radial aponeurosis (RA), the ulnar aponeurosis (UA) and the central palmar aponeurosis (PA), palmodigital fascia (figure 1.1) and digital fascia (figure 1.2a). Within each digit the fascia is composed of a pretendinous band, a spiral band, and the lateral digital sheet, with additional ligamentous elements of the superficial transverse ligament, natatory ligament, Grayson's ligament and Cleland's ligament. The fascia is described as a three-dimensional interweaving of longitudinal, transverse and vertical fibres (Skoog 1967; McGrouther 1982; 1990). It has been suggested that it provides a protective function to deeper structures of the hand, and aids in the pliant conformation of grasping surfaces to the contours of objects by virtue of its additional dermal connections (Williams *et al* 1989).

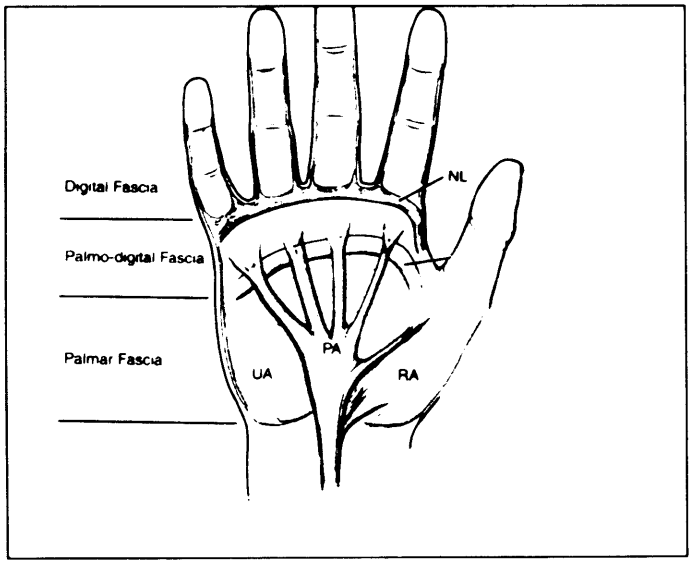


Figure 1.1: The palmar fascial complex, consisting of the palmar fascia (subdivided into radial (RA), ulnar (UA) and palmar aponeuroses (PA)), palmo-digital fascia, and digital fascia. NL represents the natatory ligament. (Adapted from Rayan 1999)

1.5.2 Pathological Anatomy

It has been considered that the nodules and cords in Dupuytren's contracture are a result of pathological changes in normal fascia (table 1.1; figure 1.2b). Nodules usually form superficial to the pretendinous band. Palmar nodules are adherent to the overlying skin, and are fixed to the underlying aponeurosis. Digital nodules may become fixed to other deep structures such as the flexor sheath. It is felt that cords usually form after the development of a nodule (Rayan 1999). The pretendinous cord is most common and is responsible for metacarpophalangeal (MCP) joint contracture. The digital cords are responsible for proximal interphalangeal (PIP) joint contractures, and include the central, spiral and lateral cords. The normal structures in the hand are referred to as bands or sheets. The name of these same structures changes to cords when they are affected by Dupuytren's disease.

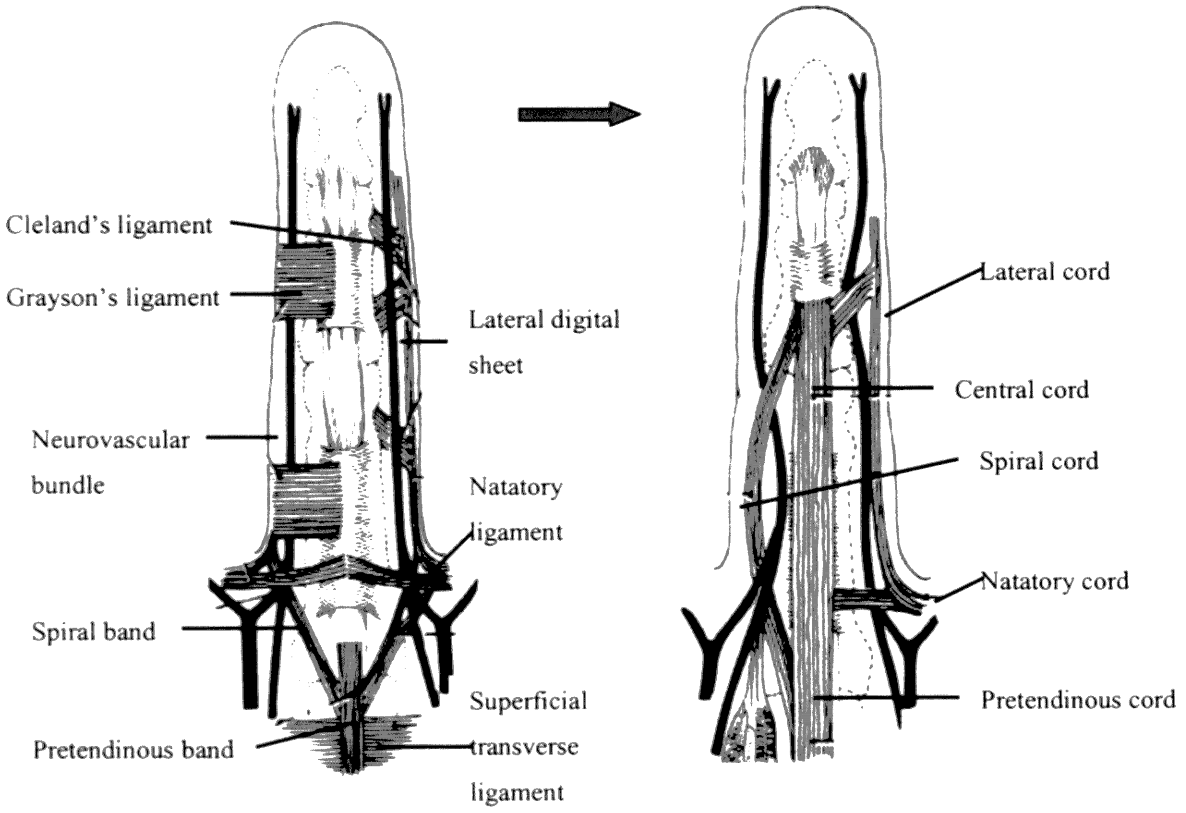


Figure 1.2a Figure 1.2b

Figure 1.2. The normal anatomical structures of the digital fascia on the left (a), and the diseased cords that they become on the right (b). (Adapted from McFarlane 1990)

NORMAL STRUCTURE	PATHOLOGIC CORD	ACTION
Septa of Legueu and Juvara	Vertical cord	Extends deeply between the neurovascular bundle and flexor sheath Contributes to stensosing tenosynovitis
Hypothenar eminence fascia	Abductor digiti minimi cord	Little finger ulnar contracture
Spiral band	Spiral cord	Responsible for displacement of the neurovascular bundle (NV) Originated from the pretendinous cord, passes deep to the NV bundle, then runs lateral to the NV bundle involving the lateral digital sheet, and finally passes superficial to the NV bundle as it joins Grayson's ligament
Natatory ligament	Natatory cord	Converts the U shaped web space fibres into a tight V shape Limits digital abduction
Pretendinous band	Central cord Pretendinous cord	An extension of the pretendinous cord into the digit. Attaches to the flexor sheath or to the periosteum of the middle phalanx
Lateral digital sheet	Lateral cord	Attaches to the skin or the tendon sheath through Grayson's ligament Causes flexion contracture of the PIP joint and rarely the DIPJoint
Abductor digiti minimi tendon	Isolated digital cord / Abductor digiti minimi cord (ADM)	A cord on the ulnar side of the little finger taking origin from the ADM tendon Passes superficially to the NV bundle
Retrovascular band	Retrovascular cord	Present deep to the neurovascular bundle

Table 1.1: The normal anatomical structures and their associated diseased cords seen in Dupuytren's disease. Table adapted from McFarlane (1990).

1.6 CLINICAL PRESENTATION

Early Dupuytren's disease is often not noticed by many patients, until the development of a digital contracture. The disease usually begins with the formation of a subcutaneous nodule in the palm of the hand with associated skin changes of pitting and thickening. Subsequently more visible nodules or cords develop, and over time, which may be months or several years, contracture of either metacarpophalangeal joint, proximal-interphalangeal joint, or both occurs.

In the vast majority of cases the ulnar part of the hand is affected with the ring, little and middle fingers involved in decreasing order of frequency (Rayan 1999). However in rarer cases radial disease of the index, thumb and 1st web may occur. Patients complain rarely of pain or altered sensation. The main problem experienced is that of restricted function with difficulty for example in washing, dressing, donning gloves and work.

Progression of Dupuytren's disease is very variable. In some, fascial disease remains confined to the palm and does not progress to contracture, whereas in others disease progresses rapidly to cause flexion deformity (Rayan 1999). No features have been identified as yet to locate those patients at highest risk of rapid progression.

1.7 CURRENT TREATMENT OF DUPUYTREN'S DISEASE

Despite much research into the aetiology of Dupuytren's disease, very little has changed in the management since Dupuytren's first fasciotomy in the early nineteenth century (Dupuytren 1832). Surgery remains the mainstay of treatment, and although complication rates and rehabilitation have improved, recurrence is still a common occurrence (Rombouts *et al* 1989).

1.7.1 Non-operative Treatment

The search is ongoing for an effective, safe and easy to use non-operative treatment for this disease. Many non-surgical therapies have been used historically including topical creams, massage, radiotherapy, splinting and pharmacological injection (Hurst and Badalamente 1999). Those recently under investigation include mechanical elongation techniques pre-surgery (Messina and Messina 1991), and collagenase injection which is in use within the remits of a phase II clinical trial (Badalamente *et al* 2000; 2002).

The use of splintage regimes as an alternative to surgery has never gained in popularity (Abbott *et al* 1987), although splinting post-operatively is commonly used as an adjunct in rehabilitation and in theory as an aid to reducing recurrence rates.

In the early 1990's the continuous elongation technique (TEC) was introduced by Messina and Messina (1991; 1993) as a means of straightening the affected digit, and elongating the contracted palmar fascia via an external fixation device. The method was used in severely contracted PIP joints in particular. The main drawback was the need for immediate surgical intervention after removal of the fixator. If this did not occur, recurrence of contracture became apparent in over 70% of patients, within a period of only days (Messina *et al* 1993). Similar external fixation techniques have been employed by others (Hodgkinson 1994), although long term results are still awaited.

Pharmacological agents have been used to induce "enzymic fasciotomy" of the contracted digits in Dupuytren's disease (Bassot 1965; Hueston 1971; McCarthy 1992). Those recently under investigation were the use of intralesional γ interferon (Pittet *et al* 1994), and clostridial collagenase (Badalamente *et al* 2000). γ interferon is postulated to reduce fibroblast replication, collagen production and myofibroblast expression. Within a pilot clinical trial patients undergoing intralesional injection of γ interferon into Dupuytren's nodules were found to have a reduction in the size of the nodule (Pittet *et al* 1994). However no information on long term follow up is yet available. Dupuytren's cords were tested by loading to failure in an in-vitro study after injection with collagenase

with the result that a dose of 300 units was sufficient to cause rupture (Starkweather *et al* 1996), and following this a phase II clinical trial was successfully performed in 29 patients with clinical contractures (Badalamente *et al* 2000). A randomized placebo controlled double-blind trial (Badalamente *et al* 2002) concluded that this was a safe and effective treatment for contracture, although results of phase III trials and long term recurrence rates are awaited before firm conclusions may be made.

1.1.1 Surgical Intervention

The aim of surgery is to restore hand function with minimal complications. Surgical fasciotomy is performed mainly in patients with metacarpophalangeal joint contractures. It has proved useful in those patients for whom major surgery is contraindicated. Reports have shown that out-patient needle fasciotomy may be performed effectively (Badois *et al* 1993; Lermusiaux *et al* 1997), although this technique has been criticized for its blind approach (Hurst and Badalamente 1999). A problem with simple fasciotomy is the early and high recurrence rate reported in many studies (Luck 1959 (71%); Millesi 1965 (77%); Lermusiaux 1997 (50%)).

Fasciectomy remains the most commonly used surgical procedure for both MCPJ and PIPJ contractures. This may be performed as a limited, segmental regional or rarely as a radical fasciectomy. Limited fasciectomy involves the excision of all macroscopically affected fascia with a small rim of normal appearing fascia (Hueston 1961). Segmental fasciectomy involves the excision of all diseased fascia but with the preservation of the transverse fibres of the superficial transverse ligament (Skoog 1967). Once the diseased tissue has been excised a range of techniques have been employed to close the wound ranging from the "open palm" (McCash 1964), Z-plasty, and full thickness skin graft. Dermofasciectomy has been recommended in younger patients with severe aggressive disease, and in those with recurrent disease (Hueston 1963; Wilson 1997). One advantage is that it has been shown that recurrence with this procedure is significantly reduced (Tonkin *et al* 1984; Brotherston *et al* 1994; Hall *et al* 1997). A curative procedure for this disease is not yet known. Reappearance of the disease may be as a true recurrence (at the site of primary surgery), or as extension (in a new site

within the hand). Recurrence rates are high ranging from 34 – 71% over a 5-10 year follow-up period (Millesi in 1981 stated 39% at 10yrs; Schneider *et al* in 1986 stated 32-48% at 5yrs; Norotte *et al* in 1988 stated 71% at 10yrs; Leclerq in 1986 stated 66% at 10 yrs; McGrouther in 1999 stated 50% at 5 yrs). Recurrence rate increases with the length of follow up time in all cases. A recent report has implicated that tension within a fasciotomy skin wound may result in an increased recurrence rate. It was seen that those patients with a transverse incision showed significantly greater recurrence in comparison to those with a Z-plasty closure, although follow up was for only 2 years (Citron and Hearndon 2003). Similarly Evans *et al* (2002) demonstrated an improved post-operative outcome in patients undergoing a period of rehabilitation without mechanical tension on the digits.

Large numbers of researchers have looked at non-operative alternatives to Dupuytren's surgery, and at recurrence rates post surgical and non-surgical treatment. From this it is clear that at the current time there is no ideal standard procedure for this disease that will improve outcome. These next sections therefore will focus on the histology, cellular biology and cell mechanics underlying the disease, in order to introduce the key elements of this project.

1.8 HISTOLOGY OF DUPUYTREN'S DISEASE

In 1959, Luck classified the progression of Dupuytren's disease into three stages – proliferative, involutional and residual. The first stage was characterized by proliferation and differentiation of fibroblasts with minimal collagen production, the fibroblasts being randomly orientated. This is clinically seen as a nodule. During the involutional stage fibroblasts were postulated to align themselves along lines of tension, and tissue collagen content increased. It was hypothesised that during this involutional phase contraction occured. The final residual stage was characterized by a reduction in cell density, and the presence of a thick almost acellular collagen cord (Luck 1959).

Clinically and morphologically the nodule and the cord are two distinct entities. The nodule is a highly vascularised tissue containing a high population of fibroblasts, with a distinct subset of myofibroblasts. The cord, in contrast, is relatively acellular; relatively lacking in myofibroblasts and has a high proportion of collagen when compared to the nodule. Most theories suggest that the nodule progresses into a cord as the disease progresses with joint contracture occurring at the time of cord formation (Hueston 1985; Moyer *et al.* 2002). Others have suggested that the nodule and cord represent 2 separate entities, and arise independently of each other (Gosset 1985).

Few workers have investigated Dupuytren's disease by studying the nodule and the cord as separate entities (Vande Berg et al 1984; Moyer et al 2002; Bisson et al 2003; 2004). In order to further our understanding of the disease it was elected to use separate nodule and cord cell lines, as there may be a difference in the behaviour of cells from each region.

1.9 THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is a dynamic complex three dimensional environment that is closely connected to its resident cells. It is composed of four major classes of macromolecules – the collagens, proteoglycans, structural glycoproteins and elastin (Haralson and Hassell 1995). It functions not only as a structural support, but also plays a major role in modulating the biology of the cell and the cell's response to growth factors, hormones, and other cell-cell interactions. There are specific cell surface receptors for ECM components which link the matrix to the cell via specific binding domains (Haralson and Hassell 1995). Interactions between the cell, the matrix and growth factors are reciprocal, i.e. a change in mechanics of the matrix will result in altered cellular behaviour, whereas a change in cell growth and biochemical activity will alter the composition of the surrounding matrix (Flint and Poole 1990). ECM production is controlled by cell type, number and many growth factors, and its degradation and

remodeling is controlled by specific proteases (the matrix metalloproteases) that act to cause matrix breakdown (Haralson and Hassell 1995).

The composition of the ECM changes during tissue repair and regeneration. Mutations in matrix genes may result in a variety of pathological changes, and changes in matrix metabolism (both in synthesis and degradation) characterize the changes seen in a number of acquired diseases including Dupuytren's contracture (Tomasek *et al* 2002).

1.10 BIOCHEMISTRY OF DUPUYTREN'S DISEASE

There are major changes between the biochemistry of the collagen found in Dupuytren's disease compared to normal palmar fascia. These changes are very similar to those found in newly healing wounds, granulation tissue, and embryonic tissue (Brickley-Parsons *et al* 1981).

Type III collagen is present throughout the affected and unaffected regions of palmar fascia in Dupuytren's tissue; this collagen type appears virtually absent in normal fascia (Bazin *et al.* 1980; Brickley-Parsons *et al.* 1981). There are increased amounts of total collagen, with an increased proportion of reducible cross-links, both indicating new collagen synthesis (Brickley-Parsons *et al* 1981). The proportion of type III collagen is increased to 20-30% in the nodules and to 30-40% in the cords (Bailey *et al* 1977). In addition to this, 10-15% of all the collagen in the clinically uninvolved regions of the palmar fascia consists of type III collagen. The stimulus for continued type III collagen production in Dupuytren's remains unexplained compared to its disappearance in a normal healing wound. In wound healing these biochemical changes return to that of normal tissue, whereas in Dupuytren's disease the biochemical changes seen are ongoing reflecting disease progression over a long period of time (Bazin *et al* 1980; Murrell *et al* 1991). Collagen production per cell is postulated to fall in areas of high cellularity (Murrell *et al* 1991). A decrease in type I collagen production may raise the ratio of type

III to type I collagen, rather than type III collagen production increasing. This would imply that the cells are acting normally in an abnormal situation (Murrell *et al* 1991).

Hydroxylysine is a functional amino acid residue of collagen. It has been reported that there is an increased concentration of hydroxylysine residues in both the fascia taken from Dupuytren's patients and the clinically uninvolved fascia, with a corresponding increase in the total number of glycosylated hydroxylysine residues (Brickley-Parsons *et al.* 1981). The number of reducible aldimine intermolecular cross links is increased, and hydroxylysinohydroxynorlysine, which is virtually absent in normal palmar fascia was found to be the predominant intermolecular cross-link in cases of Dupuytren's disease. (Brickley-Parsons *et al.* 1981). Water content and glycosaminoglycan contents are also increased.

1.11 THE MATRIX METALLOPROTEINASES

(Taken from reviews by: Parsons *et al* 1997; Massova *et al* 1998; Ravanti and Kahari 2000: Visse and Nagase 2003)

The normal function of a tissue depends upon the interaction of the cells with their surrounding extracellular matrix. Modulation of cell-matrix interactions occurs through the action of proteolytic enzymes, which are involved in the breakdown of matrix components. By the regulation of the structure of the extracellular matrix, these enzyme systems play a major role in the control of signals elicited by matrix molecules, which in turn, regulate cell proliferation, cell differentiation and death. In normal tissues the turnover and remodeling of the extracellular matrix is tightly controlled, but in some pathological conditions characterized by either excessive degradation or lack of degradation (Dupuytren's disease) of ECM components, proteolysis is uncontrolled.

Matrix metalloproteinases (MMPs), also known as matrixins, are a major group of zinc-dependent endopeptidases that function in the degradation of the extracellular matrix.

These proteinases play a central role in many biological processes, both normal and pathological. The major function of MMPs is thought to be the removal of ECM in tissue resorption. However proteolysis of ECM components can lead to release of biologically active molecules such as growth factors. Thus MMP function can act to alter cell behaviours, and phenotypes, demonstrating these enzymes' highly complex role. At the present time there are 23 MMP genes identified in humans. These are subdivided into 6 groups of MMPs seen in the table below (table 1.2).

MMP	Function
Collagenases	MMP-1, MMP-8, MMP-13, MMP-18 These enzymes function to cleave interstitial collagens I, II and III at a specific site three-fourths from the N-terminus. Collagenases can also digest a number of other ECM and non-ECM molecules.
Gelatinases	MMP-2 and MMP-9 These digest the denatured collagens, gelatins These enzymes have 3 repeats of a type II fibronectin domain inserted in the catalytic domain, which bind to gelatin, collagens and laminin MMP-2 digests collagen I, II, III
Stromelysins	MMP-3 and MMP-10 MMP-3 activates a number of pro-MMPs, and its action on proMMP-1 is essential for this MMP to function Act to breakdown collagen IV, V, X, IX, fibronectin, elastin and fibrin
Matrilysins	MMP-7, MMP-26 These are characterized by the lack of a hemopexin domain MMP-7 processes cell surface molecules such as pro-defensin, pro-tumor necrosis factor, and e-cadherin MMP-26 digests a number of ECM components
Membrane-type MMPs	There are 6 of these (MT-MMP) 4 are type I transmembrane proteins (MMP-14, 15, 16, and 24) 2 are glycosylphoshphatidylinositol anchored proteins (MMP-17 and MMP-25) They are all capable of activating pro-MMP-2, and all can digest a number of ECM proteins.
Other MMPs	7 MMPs are unclassified MMP-12 is essential for macrophage migration MMP-19 function uncertain MMP-20 digests amelogenin MMP-22 has an unknown function MMP-23 is thought to be a type II membrane protein mainly expressed in reproductive tissues MMP-28 is mainly expressed in keratinocytes, and is thought to function in wound repair

Table 1.2: The matrix metalloproteinases – classification and functions (from Visse and Nagase 2003)

MMPs produced by fibroblasts are thought to be MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13,

Under normal physiological conditions, the actions of these enzymes are precisely regulated at i) the level of transcription, ii) activation of precursor zymogens, iii) interaction with specific extracellular matrix components, and finally iv) inhibition by endogenous inhibitors. A loss of control at any level may result in the fibrosis commonly seen in Dupuytren's disease. The level of expression of MMPs is very low *in vivo*, but expression can be induced by various exogenous signals, including cytokines, growth factors and chemical agents. The activation of precursor zymogens usually occurs via tissue or plasma proteinases, or other MMPs in the same group forming an effective network in normal physiologic conditions.

Tissue inhibitors of matrix metalloproteinases (TIMPs) are specific inhibitors that act to control the local activities of MMPs in tissues. 4 TIMPs (TIMP-1, 2, 3, 4) have been identified in humans, and their expression is regulated during development and tissue remodeling. They are produced by several cell types including fibroblasts, keratinocytes, endothelial cells and osteoblasts. Under pathological conditions associated with unbalanced MMP activity, changes in TIMP levels are considered to be important because they directly affect the level of MMP activity. TIMPs inhibit all MMPs, although TIMP-3 is different to the others in that it also inhibits the aggrecanases. As well as inhibiting the MMPs, TIMPs have additional wider roles, including cell growth promotion, morphogenesis, and both proapoptotic and antiapoptotic activity. TIMPs are important in establishing a balance between matrix synthesis and matrix degradation caused by MMPs, and are thus present wherever the MMPs are to be found.

1.12 MMPS IN DUPUYTREN'S DISEASE

Few researchers have focused on this area in the study of Dupuytren's disease. Tarlton *et al* (1998) stated that a significant increase in the levels of MMP-2 and MMP-9 were observed after mechanical stretching of Dupuytren's tissue *in- vitro*. These findings were also observed in a separate *in-vivo* study (Bailey *et al* 1994). The success of mechanical stretching such as in the continuous elongation technique of Messina and Messina (1991), was postulated to be due to the detection of stress by resident fibroblasts resulting in the release of degradative matrix metalloproteinases that cause collagen weakening, and thus allow digital extension (Tarlton *et al* 1998). One study has recently looked at the ratios of MMPs and TIMPs (Ulrich *et al* 2003). These workers measured the concentrations of MMP-1,2,9, and TIMP-1 and 2 in the sera and tissue of 22 patients with Dupuytren's disease, and 20 patients with clinically normal palmar fascia. They found that in those patients with the disease, the TIMP-1 concentration was significantly higher in comparison to controls, but there were no significant differences in MMP concentrations between the 2 groups. The MMP to TIMP ratio was also significantly lower in patients with Dupuytren's disease in comparison to the normal group. It was concluded that a decrease in MMP to TIMP ratio may reflect increased collagen synthesis, and decreased breakdown, thus leading to palmar fibromatosis.

1.13 MMP EXPRESSION IN FIBROBLASTS UNDER TENSION

There have been several studies focusing on this area using dermal fibroblast models. No studies have worked on MMP expression by Dupuytren's fibroblasts in a tightly controlled environment. Some work has indicated that fibroblasts can be induced to produce proteases by disruption of the cytoskeleton, or by changes in the mechanical environment (Aggeler *et al* 1984; Unemori *et al* 1986; Mauch *et al* 1988; Lambert *et al* 2001). Lambert *et al* (2001) looked at the expression of MMP-2, 3, 9, 13 and 14 in a free floating circular collagen gel model for human dermal fibroblasts, and found that MMP-2

in particular was significantly up-regulated on disruption of the cytoskeleton by cytochalasin-D. Prajapati *et al* (2000) measured protease activity in three dimensional collagen gels by gelatin zymography after subjecting them to either static or cyclical mechanical loads within the culture force monitor system. Under static loads thirteen times more MMP-2 was produced in comparison to MMP-9, but production of MMP-9 increased significantly thirty-seven fold on cyclical loading while MMP-2 production decreased. The increase in MMP-2 and 9 production occurred to a reduced extent when the cells were stress shielded by using a stiffer surrounding collagen matrix, indicating the mechano-sensitive nature of MMP production. In similar work by the same author (2000), the greatest stimulation of MMP-9 was produced in slow ramp loading regimes over 11 hours, in comparison with loads applied quickly over 10 minutes. Other work (Mudera *et al* 2000) has looked at unidirectional forces applied to cells within a three dimensional collagen lattice, and compared MMP expression by cells in a load aligned zone in comparison to cells in a randomly oriented zone. It was found that cells that were aligned parallel to the mechanical load showed down-regulation of MMP expression, whereas cells in the non-aligned zone significantly increased their MMP expression, in particular MMP-2. It was postulated that non-aligned, mechanically loaded cells are stimulated to move and remodel the surrounding tissue matrix, and utilize MMPs in order to do this.

1.14 THE MYOFIBROBLAST

The myofibroblast was first identified in Dupuytren's disease by Gabbiani and Majno in 1972, although its presence has been documented in many other normal and pathological states (Tomasek *et al* 2002). This cell type exhibited ultrastructural features of both fibroblasts and smooth muscle cells. Morphologically myofibroblasts are characterized by an increase of intracellular actin microfilaments, deep indentations of the cell nucleus, gap junctions connecting one cell to another and hemidesmosomes attaching them to the basal lamina (Bazin *et al* 1980; Badalamente *et al* 1983). Within the cells the large bundles of actin microfilaments are noted to traverse the cell along its long axis

(Gabbiani and Majno 1972). The main distinguishing feature of myofibroblasts is their ability to express alpha smooth muscle actin (α-SMA) in comparison to normal fibroblasts (Schurch *et al.* 1984; Foo *et al.* 1992). Differentiated myofibroblasts in granulation tissue express only α-SMA, whereas a proportion of differentiated myofibroblasts in Dupuytren's disease may also express desmin and smooth muscle myosin (Schurch *et al* 1990; Tomasek and Rayan 1995; Tomasek *et al* 2002). The α-SMA isoform is hypothesized to be instrumental in force generation by myofibroblasts, with significantly stronger contraction noted in collagen gels seeded with cells transfected with α-SMA in comparison to those transfected with cytoplasmic actin (Hinz *et al* 2001). The mechanism by which α-SMA promotes contraction is still unknown, although recent work has identified a specific NH2-terminal peptide that has been postulated to play a role (Hinz *et al* 2002).

Myofibroblasts are capable of sustaining a contractile force that is theorized to be generated by contractile stress fibres (Tomasek 1999). Stress fibres are composed of bundles of actin microfilaments, actin binding proteins and associated non-muscle myosin (Tomasek 1999). Force generation is regulated by myosin light chain phosphorylation and in particular through the rho/rho kinase intracellular pathway (Parizi *et al* 2000; Katoh *et al* 2001). Myofibroblasts are postulated to exert their action via a coordinated multicellular unit (Grinnell 1994), but this has been contraindicated by Eastwood *et al* (1994,1996) who demonstrated early tractional forces in a fibroblast populated collagen lattice in the absence of any myofibroblasts.

The transformation of the fibroblast into the myofibroblast phenotype has been the subject of many studies reviewed by Gabbiani (2003), and is hypothesised to be a result of the combined action of mechanical tension, growth factor stimulation and ED-A cellular fibronectin. Tomasek *et al* (2002) have suggested that fibroblasts differentiate into myofibroblasts, by firstly developing intracellular stress fibres in response to changes in the surrounding connective tissue. This transient change in the cell phenotype has led to the cells being named protomyofibroblasts during this stage. Under the influence of growth factors or a mechanical change in the extracellular matrix, the protomyofibroblast

may evolve into a myofibroblast that is characterized by the *de-novo* expression of α-smooth muscle actin, and large supermature focal adhesion complexes.

An ultrastructural study of the extracellular matrix of the nodules in Dupuytren's disease, and in the granulation tissue of healing wounds reveals that the extracellular material is composed of a mixture of fine fibrils and larger ones which intermingle forming a filamentous complex (Tomasek *et al* 1987). This is associated only with the myofibroblast. These bundles were found to extend from the myofibroblast, connecting these cells to each other, and also to the extracellular matrix (Tomasek *et al* 1987; Tomasek and Haaksma 1991; Tomasek *et al* 1995). The filamentous material is also associated with the large intracellular actin filament bundles, connected via a transmembrane association. It is these specialized transmembrane connections that are thought to translate cellular contraction to the extracellular matrix, and they have been termed "the fibronexus" (Singer *et al* 1984) or "supermature focal adhesions" (Dugina *et al* 2001). In general fibroblasts in vivo are thought to lack this contractile microfilamentous apparatus that is seen in myofibroblasts (Gabbiani 2003).

Myofibroblasts are found primarily in the nodules rather than the cords in Dupuytren's tissue (Vande Berg *et al* 1984; Chiu 1978; Gokel *et al* 1977; Hueston *et al* 1976), although this has been debated by recent work in this laboratory (Bisson *et al* 2003). Their presence has led to the proposal that the myofibroblast may be responsible for digital flexion deformity in Dupuytren's disease (Schultz and Tomasek 1990). In recurrent Dupuytren's disease, degree of recurrence has been related to the number of myofibroblasts present within the dermis (McCann *et al* 1993).

Thus myofibroblast contractility appears to be a key element in the process of the development of a permanent tissue contracture. Many techniques have been used to measure contractility and these will be outlined in more detail below.

1.15 MODELS OF CELLULAR CONTRACTION

Cells exist within a complex dynamic three-dimensional environment *in-vivo* where tension and mechanical loading play a role in each cell's morphology and function. It was Elsdale and Bard (1972) who showed that the phenotypic appearance of fibroblasts cultured in three dimensional collagen gels was much more like that seen *in vivo* in comparison to monolayer cultures. Over several years a multitude of methods have been used to measure tissue, and cell contraction *in vitro* using increasingly advanced techniques. (Table 1.3)

RESEARCHER	EXPERIMENTAL MODEL	ACTION
1956 Ehrman and Grey	Fibroblasts grow within a collagen lattice	
1972 Elsdale and Bard	Fibroblasts within a collagen gel cause contraction of the gel	In a cell filled collagen matrix cell motility results in collapse of the matrix/shrinkage
1982 Stopak and Harris	Wrinkling of a monolayer silicone sheet by fibroblasts	Thought to be due to tractional forces by cell locomotion
1986 Hurst *et al*	Wrinkling of a deformable rubber substratum	Dupuytren's fibroblasts can transmit force to a substratum
1990 Kasugai, Suzuki *et al*	Analogue force transducer of free floating collagen gels	Force generated by dog alveolar periodontal fibroblasts is sufficient to cause tooth eruption
1991 Delvoye *et al*	Analogue system for isometric force measurements	Force is generated by dermal fibroblasts within a rectangular collagen gel
1992 Tomasek *et al*	Stress- release pattern of contraction in circular gels	Measurements of diameter reduction in a circular gel
1992 Kolodney and Wysolmerski	Quantitative measurements of isometric contraction of fibroblasts	Measurement of diameter reduction in a circular gel
1994 Eastwood *et al* 1996 Eastwood *et al*	Culture force monitor (CFM) for measurement of contractile force developed by fibroblasts Tensioning culture force monitor (tCFM)	Force is generated by dermal fibroblasts within rectangular collagen gel. External forces may be applied across the gel to study response to a changing tensional environment

Table 1.3: Experimental models used to investigate cell contractility, and morphology

The earliest of these was the development of a three dimensional cell seeded circular collagen matrix (Elsdale and Bard 1972). These have been studied as free floating gels which are under little mechanical stress, as tethered stressed gels where a tensile stress is applied to the gel via its attachment to a surface, and this provides a reactive tensile force on the resident cells as they contract, or as stress-released gels where cells develop isometric tension while the gel is attached, and this tension is then released to initiate cellular contraction (Grinnell 1994; 2000; 2003). (Table 1.4; figure 1.3) Disadvantages with these systems is that measurement of force generation is only semi-quantitative, and that that manipulation of load is unable to be performed.

MODEL	1) Floating matrix contraction	II) Anchored matrix contraction	III) Stress-relaxed matrix contraction
MEASUREMENT	Reduction in diameter	Reduction in height	Reduction in diameter after release from anchorage
MECHANICS	Isotopic tension distribution Mechanically relaxed tissue	Anisotropic distribution of tension Stressed tissue	Initially mechanically stressed Then mechanically relaxed

Table 1.4: Models for matrix contraction.

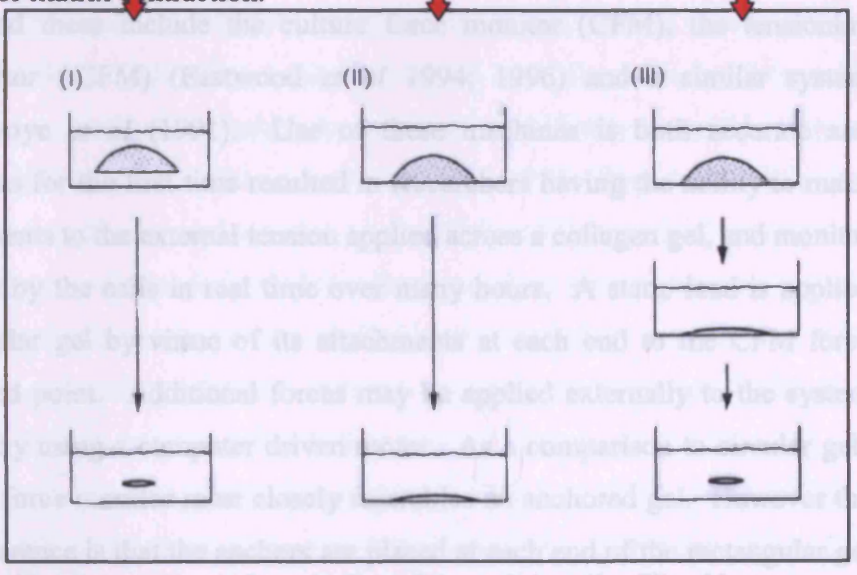


Figure 1.3: Diagrammatic representation of circular models of collagen gel contraction. (Adapted from Grinnell – Fibroblasts, myofibroblasts and wound contraction (1994))

Fibroblasts resident within collagen gels that are floating in culture medium are mechanically unloaded, whereas those in gels that are attached develop isometric tension. These mechanical differences lead to changes in cell phenotype. Fibroblasts that are attached develop stress fibres and may differentiate into myofibroblasts, whereas those that are unstressed or floating will become quiescent, with a subpopulation becoming apoptotic (Grinnell 1994). Morphologically there are marked differences, with cells in floating matrices appearing dendritic, and those in stressed matrices demonstrating a stellate or bipolar appearance (Grinnell 2003).

The mechanism of free floating collagen gel contraction is postulated to be based upon cell attachment to the matrix, with cellular migration or locomotion through it (Grinnell 1994). Tethered gels develop an isometric force as the resident cells contract against the fixed points. Stress-relaxed gels behave as tethered gels initially, and when the gel is released from its attachment, a rapid contraction will occur that is proportional to the isometric force generated by the cells (Grinnell 2003).

Later experimental models have advanced to directly measure cellular forces within a collagen matrix, and these include the culture force monitor (CFM), the tensioning culture force monitor (tCFM) (Eastwood *et al* 1994; 1996) and a similar system developed by Delvoye *et al* (1991). Use of these machines is both accurate and reproducible, and has for the first time resulted in researchers having the ability to make fine precise adjustments to the external tension applied across a collagen gel, and monitor the responses made by the cells in real time over many hours. A static load is applied across this rectangular gel by virtue of its attachments at each end to the CFM force transducer, and fixed point. Additional forces may be applied externally to the system either manually or by using a computer driven motor. As a comparison to circular gels models, the culture force monitor most closely resembles an anchored gel. However the most important difference is that the anchors are placed at each end of the rectangular gel giving a single direction to force generation by the cells whereas within a circular gel forces generated are multidirectional (Eastwood *et al* 1994; 1996). The ability to directly

measure the tensional force on a collagen substrate has led to improved understanding of fibroblast function in both normal and diseased tissue, which is an exciting development in the realm of cellular mechanics.

1.16 FIBROBLAST CONTRACTION

Much research using a variety of the experimental models described above has led to greater understanding of the mechanism by which fibroblasts contract. Several different components to the generation of a contractile force by a single fibroblast have been defined. The process is thought to involve several linked stages, commencing with cell-matrix contact, recruitment of cell contact sites, cytoskeletal organization with cell spreading, generation of cell-matrix tractional force, and finally contraction (Sethi *et al* 2002). Possibly one of the most important of these is physical linking between the cell cytoskeleton and the extracellular matrix. It is thought that cell surface integrins play a vital role in this process by attachment to one or more matrix ligands, including fibronectin, and vitronectin. The integrins are a family of extracellular matrix receptors present on a cell surface which act by linking extracellular macromolecules with the cytoskeleton and promoting cell attachment, migration and shape changes (Burridge *et al* 1988; Clyman *et al* 1990). Cell-collagen attachment has been shown to be fibronectin dependant, with the contractile force being dependant on specifically the $\alpha 2\beta 1$ integrin-matrix interaction (Schiro *et al* 1991; Langholtz *et al* 1995; Xu *et al* 1998), but independent on other integrin interactions such as that of the $\alpha 5\beta 1$ integrin (Tomasek and Akiyama 1992). Recent work has indicated that there may be a short sequential cascade of integrin utilization in the early phases of fibroblast contraction commencing with fibronectin receptors and ending with the collagen integrin subcomponent (Sethi *et al* 2002). Other work has also suggested that TGF-β may act via integrin function and cell attachment during the early phase of cell contraction (Brown *et al* 2002). Indeed TGF-β was seen to dramatically up-regulate vitronectin integrin receptor expression, although it was concluded that TGF-β behaves primarily as a mechanoregulatory growth factor, and that integrin stimulation may be a consequence of altered cell stress.

Forces applied through the membrane bound integrins are transduced into intracellular signals that mediate the redistribution of cytoskeletal proteins, and alter expression of cytoskeletal genes such as actin, filamin and vinculin (D'Addario *et al* 2001). The filamin protein acts to stabilize cortical actin fibres, increasing actin rigidity, and preventing cell depolarization by distortion of the membrane by external force (Kainulainen *et al* 2002). Part of the contractile force generated by the fibroblast is due to stored energy within the cell's actin-myosin motor elements of the microfilaments. This force is proposed to be held as a compressive load that maintains cell shape. This has been termed the residual internal tension (Brown *et al* 1996). In addition to this the microtubules within the cell act as an intracellular framework, which counteracts the pulling force of the microfilaments and maintains the cell morphology (Brown *et al* 1996). Microtubules are thought to affect cell attachment to the extracellular matrix by regulating turnover at adhesion sites (Kaverina *et al* 2002)), and also play a role in modulating mechanotransduction via transcription of the filamin A gene (D'Addario *et al* 2003).

Thus it can be seen that the contraction of a cell seeded collagen gel is an active process. The rate of contraction is dependant on the cell number, the type and concentration of the collagen, the presence or absence of serum (Bell *et al* 1979; Guidry and Grinnell 1985), and is also dependant on all the factors involved in fibroblast action as has been discussed above.

1.17 TENSIONAL HOMEOSTASIS

It has been demonstrated using the culture force monitor that fibroblasts seeded into a three dimensional collagen gel are able to generate substantial contractile forces (Eastwood *et al* 1996). Cellular contraction was described as occurring in three phases. The first phase occurs with in the first three hours, when cells contribute little to the net force recorded by the CFM. The generation of force in this stage was hypothesized to be

due to the stablilsation and intermeshing of the collagen fibrils within the gel. The second phase consists of a near linear increase in force, occurring between 3 and 8 hours that correlates with cell attachment, spreading and extension of cell processes (Delvoye *et al* 1991; Kolodney and Wysolmerski 1992; Eastwood *et al* 1994). The final phase is a flat plateau phase of equilibrium where the force generated was balanced by the tension of the culture force monitor. As tension develops within the system the cells become aligned parallel to the strain along the collagen gel (Eastwood *et al* 1998).

Later experiments using the culture force monitor have demonstrated that fibroblasts respond to changes in mechanical loading in a way, which maintains tensional homeostasis in their surrounding matrix (Brown *et al.* 1998). Fibroblasts seeded in a three-dimensional collagen gel rapidly generate and maintain a tensional force within that matrix. Their response to mechanical stress was deduced by applying precise mechanical loads across the lattice, and then measuring the gross and net force across the substrate. Dermal fibroblasts were seen to increase their contraction in response to underloading of the tensioning culture force monitor, reaching a force close to that prior to unloading. By applying external overloading forces, it was seen that a reduction in cell-mediated tension occurred. The cellular response was always in the opposite direction to the previously applied load. This was defined as tensional homeostasis since resident cells appeared to respond by maintaining a constant endogenous matrix tension. It is still uncertain exactly how fibroblasts monitor tension within the extracellular matrix and by which mechanisms their own internal cellular tension changes. Mechanisms that have been proposed for this phenomenon have included intracellular fluxes of calcium ions in response to stretch (Arora *et al* 1994), and an upregulation of a cyclic AMP secondary messenger system as stress is increased (He and Grinnell 1994).

1.18 CONTRACTION IN DUPUYTREN'S DISEASE

It has been proposed that contractile forces generated by Dupuytren's fibroblasts play an important role in the development and progression of the shortened contracted fascia seen

in this disease (Schultz and Tomasek 1990; Schurch *et al* 1990). The majority of contraction studies on Dupuytren's fibroblasts have utilized stress-relaxed collagen gel models (Rayan and Tomasek 1994; Rayan *et al* 1996; Tarpila *et al* 1996; Sanders *et al* 1999; Vaughan *et al* 2000), with few others utilizing the culture force monitor model (Jemec 1999 MD thesis; Bisson *et al* 2004).

Rayan and Tomasek (1994) examined the organization of the actin cytoskeleton, and extracellular matrix attachments in a stressed collagen gel, and demonstrated that contraction of these gels was completely actin-dependant upon release. These workers noted that normal palmar fascia fibroblasts developed the morphological appearance of myofibroblasts on light microscopy after being seeded into a collagen lattice, plus these cells generated large amounts of contractile force, not significantly different from forces generated by Dupuytren's derived cells. The same team (Rayan *et al* 1996) ran another study to identify specific agents that had the potential to promote or inhibit contraction of Dupuytren's fibroblasts. It was demonstrated that lysophosphatidic acid (LPA) promotes cellular contraction whereas nifedipine, verapamil and prostaglandins inhibit contraction of Dupuytren's fibroblasts. The action of LPA was hypothesized to occur by activation of specific second messenger cascades via decreasing cAMP, and increasing intracellular calcium levels. In other studies using the culture force monitor the application of 5-fluorouracil to a Dupuytren's fibroblast seeded collagen gel resulted in a significant decrease in contraction (Jemec, MD thesis 1999).

A reduction in cellular contraction has been shown to occur in Dupuytren's fibroblasts exposed to interferon-γ in a stress relaxed circular collagen gel model (Sanders *et al* 1999). On a molecular level it was found that interferon down-regulated the mRNA levels of cytoplasmic β and γ actin isoforms. It was postulated that a reduction in these actin isoforms in cytoplasm may impede the formation of filopodia by fibroblasts and thus reduce contractile ability. It has previously been shown that increased expression of α-smooth muscle actin (SMA) is associated with increased contractility of fibroblasts in Dupuytren's disease (Tomasek and Rayan 1995). The expression of α-SMA correlated with the stage of the disease, with the greatest percentage occurring in the proliferative

phase. However of note was the finding that expression of α-SMA is not required for contraction. Enhanced cellular contraction occurs after exposure of Dupuytren's fibroblasts to TGF-β1 in both a stress relaxed model (Vaughan *et al* 2000) and a culture force model (Bisson *et al* MD thesis 2004).

Only two researchers have studied variation in contractility between Dupuytren's nodule and cord derived cells (Moyer *et al* 2002; Bisson *et al* 2004). Moyer *et al* (2002) demonstrated that there was a significantly increased contractile ability for early passage nodule fibroblasts in comparison to cord fibroblasts. Late passage nodules became less effective at contraction, and were equal to both early and late passage cord cells in their ability to contract a collagen gel. It was hypothesized that Dupuytren's nodule fibroblasts change their phenotype after undergoing repeated cell passage acquiring a cord like phenotype, suggesting that the nodule is the early stage of the disease. The work of Bisson *et al* (2004) has also showed increased force generation by nodule derived cells in comparison to cord in a culture force monitor again suggesting functional differences between these different entities.

1.19 FROM CONTRACTION TO CONTRACTURE

It is currently accepted that the contractile ability of fibroblasts is responsible to a great extent for connective tissue remodeling during wound healing or in pathological fibrosis (Tomasek *et al* 2002). Tissue matrices exist under a mechanical tension, while the cells residing within tissue matrices are protected from external forces by the mechanical properties of the tissue itself. This has been described as "stress-shielding" (Tomasek *et al* 2002), and it appears that there is a very close relationship between the normal tension of resident cells, the ability of the matrix to act in "stress shielding" and remodeling of that matrix to keep a tissue in mechanical equilibrium whereby deposition is equal to degradation (Tomasek *et al* 2002). In Dupuytren's disease it appears that the balance has been disturbed towards excess matrix deposition, thus resulting in permanent tissue contracture (Flint and Poole 1990).

1.20 MATRIX REMODELLING AND DUPUYTREN'S DISEASE

There is little data that looks at the measurement of matrix remodeling in the literature. However a vast amount of information has been written on the fact that tissues are able to remodel during embryogenesis, growth, wound healing and disease progression. It is postulated that cells are sensitive to mechanical forces, and can change the extracellular matrix in response to a change in the surrounding environment (Tomasek *et al* 2002). Using a three dimensional collagen gel system allows the creation in vitro of a tissue equivalent material, and this allows fibroblast behaviour to parallel that seen in the in vivo situation with regards migration, morphology, and protein synthesis.

Mechanical tension governs fibroblast proliferation and collagen production in the maintenance of the extracellular matrix. In any tissue there is complex interplay of synthesis and degradation of the extracellular proteins which usually results in a steady state homeostasis of the tissue. In the case of Dupuytren's disease and other fibroproliferative disorders collagen synthesis appears to exceed its degradation resulting in the typical nodules and cords seen within the palmar fascia. It is uncertain however how much remodeling of the matrix contributes to progression of the disease, and how much is due to the cellular fibroblast contraction. Some workers proposed that contracture is a result of physical shortening of matrix, rather than collagen folding or pleating as shown by X-ray diffraction studies (Brickley-Parsons *et al* 1981) and also by electron microscopy (Legge *et al* 1981). Others suggest that contracture is a result of two separate processes occurring in parallel (Guidry and Grinnell 1987; Glimcher and Peabody 1990):-

1. **Cell mediated contraction of the matrix**
   Fibroblasts resident within a tissue matrix contract, pulling on their matrix and causing a physical deformation and shortening of the matrix (Harris *et al* 1981).

2. **Continuous matrix remodeling**

   Remodeling of the tissue matrix in the new shortened position may lead to the permanence of contracture seen in the diseased digits of Dupuytren's disease (Flint and Poole 1990)

By stepwise repetition of these processes over a long period of time it is possible that this may lead to the flexion deformities seen in Dupuytren's disease.

The first major study to measure the ability of cells to transfer force to a tissue matrix was that performed by Grinnell and Ho in 2002. This work using human dermal fibroblasts showed that mechanical load was transferred from fibroblasts incubated with TGF-β1 into a stress-relaxed collagen matrix. It was seen that collagen matrix contraction was completely actin-dependant at culture day one, but by day 6, over 50% of generated force was retained within the gel after disruption of the actin cytoskeleton. It was hypothesised that this retained force involved rearrangement of collagen fibrils, plus deposition of collagen and fibronectin (Fukamizu *et al* 1990; Vaughan *et al* 2000).

Connective tissue contracture tends to be a slow irreversible process, which involves matrix dispersed cells and is dominated by extracellular events such as matrix remodeling (Glimcher and Peabody 1990). The end result is progressive shortening of the tissue involved. It is thought that contracture occurs as a result of local pericellular shortening events initiated by the myofibroblast (Tomasek *et al* 2002). Myofibroblasts within a collagen lattice bind to their extracellular matrix via the fibronexus which is linked to the cells' actin intracellular stress fibres. As the myofibroblast contracts, the local tissue matrix also contracts, and this results in bundling of the pericellular collagen network. New matrix components are then laid down to stabilize the new position of the collagen fibrils. The cell then respreads within the newly remodeled environment, and the process can begin again. Throughout the tissue, this process occurs in a small incremental manner (Ryan *et al* 1974; Tomasek *et al* 2002).

1.21 CYTOCHALASIN-D

Cytochalasin-D (CD) is an alkaloid drug produced by the mould *Metarrhizium anisopliae.* It is one of a group of related fungal toxins discovered in 1964 by Carter *et al.* while working on mould filtrates. It is a potent inhibitor of actin dependant cellular processes, and cellular contractile force. It has been implicated in promoting conditions favourable for depolymerising actin.

Actin is one of the main proteins of eukaryotic cells of which there are 2 forms. G-actin is the monomeric subunit and can polymerize into F-actin. F-actin filaments are organized into higher order fibrous structures by interaction with actin binding proteins. When fibroblasts adhere to a surface, stress fibres of F-actin attach to the inner surface of the plasma membrane at sites called focal adhesion plaques, and also attach from the cell to the extracellular matrix via integrins. Stress fibres are orientated parallel to the direction of movement of the cell (Tomasek and Haaksma 1991). Shortening of the stress fibres occurs via the action of myosin-ATP activity.

After addition of cytochalasin-D, cells become rounded, microvilli and lemellopodia disappear. At high concentrations the actin cytoskeleton is completely disrupted (Wakatsuki *et al* 2000). The total F-actin content of treated cells diminishes as stress fibres and cortical thin filaments are no longer visible under the microscope. Thus, cytochalasin-D is postulated to bind to G actin and prevent polymerization of actin monomers. Existing F-actin fibers then depolymerize as the effective concentration of free G-actin becomes limiting. In some types of cells, binding of cytochalasin-D to G-actin also results in proteolytic degradation of monomeric actin.

Schliwa (1982) proposed that the dramatic effects of cytochalasin-D results from both a direct action of the drug on the actin filament component of the cytoskeletal network, plus a secondary cellular response. The direct action leads to an immediate disruption of the cytoskeletal network that involves breaking of actin filaments. The cellular response

engages network fragments in an energy dependent (contractile) event that leads to the formation of filament foci.

Thus addition of an appropriate concentration of cytochalasin-D to a fibroblast populated collagen matrix will result in a disruption of the cells' cytoskeletal network, loss of stress fibres and loss of contractile ability. Cells will be prevented from further rearranging their collagen fibrils, although those already reorganized are maintained via fibril-fibril interactions stabilized by noncovalent bonds (Guidry and Grinnell 1985). By "knocking out" cellular contraction matrix remodeling may be calculated in the culture force monitor model (Marenzana *et al* 2004 in press).

1.22 SUMMARY

It is appreciated that the current management of Dupuytren's disease is not ideal considering the high recurrence rates following surgery and the lack of a suitable non-surgical option.

Although our understanding of the disease has improved considerably, none of these findings have led to clinical progress. We must therefore continue to improve our knowledge of the cell biology, cell mechanics and molecular biology of the disease.

1.23 INTRODUCTION TO THE THESIS

This thesis focuses primarily on the events that occur from the initial cellular contraction of the Dupuytren's fibroblast through to the shortening and remodeling of the extracellular matrix.

The literature review has summarised the current knowledge of the history, epidemiology and aetiology of Dupuytren's disease. Some background is given to current therapeutic strategies, and the problems associated with these. Further to this the literature specific to the cellular events surrounding Dupuytren's contracture has been evaluated focusing in particular on current theories of cell-mediated contraction, matrix metalloproteinase expression and matrix remodeling.

From this review it was established that there are several gaps in knowledge, from which several key questions were raised:-

1. Do Dupuytren's fibroblasts have the ability to achieve tensional homeostasis over a time course of 48 hours, or it absent in these cells during this period? **Chapter 3.1**
2. Are fibroblast responses to a reduction in mechanical strain altered in Dupuytren's disease in comparison to the responses previously documented in dermal fibroblasts (Eastwood *et al* 1998)? **Chapter 3.2**
3. Does fibroblast morphology change with mechanical stimulation in Dupuytren's disease? **Chapter 3.3**
4. How does the expression of the genes for the matrix metalloproteinases, their tissue inhibitors, and collagen change in response to mechanical stimulation in Dupuytren's fibroblasts? **Chapter 4**
5. Do Dupuytren's fibroblasts display an increased ability to remodel a collagen matrix in comparison to normal fibroblasts? **Chapter 5**
6. Is there any relationship between force of contraction by Dupuytren's fibroblasts and disease recurrence? **Chapter 6**

From the questions raised above the following hypothesis is proposed:-

It is hypothesised that Dupuytren's fibroblasts have increased contractile properties with no evidence of tensional homeostasis in comparison to normal fibroblasts derived from the palmar fascia of the carpal ligament. Dupuytren's fibroblasts will contract in response to an externally applied mechanical load, and this will lead to an up-regulation of matrix metalloproteinase gene expression, with increased matrix remodelling as measured within a three dimensional collagen gel system.

All methods have utilized fibroblasts derived from separate nodule and cord regions from patients with primary Dupuytren's disease only, in order to exclude the influence of previous surgery. Normal control palmar fascia fibroblasts have been taken from patients undergoing carpal tunnel release that have had no history or clinical evidence of Dupuytren's disease.

CHAPTER 2

MATERIALS AND METHODS

2.1. GENERAL CELL CULTURE

Dupuytren's and control carpal ligament fibroblasts were established in culture following local ethical committee approval (Number EC2002-77). Dupuytren's disease tissue was obtained from excised specimens at elective surgical fasciectomy. Skin was obtained from the RAFT tissue bank for use as an additional control tissue. Carpal ligament was selected as control palmar fascia and excised from the incised free edge of the carpal ligament at routine carpal tunnel decompression. These patients showed no clinical evidence of Dupuytren's disease. Several previous investigators have used carpal ligament cells as non-diseased fibroblasts for comparison with Dupuytren's disease derived cells (Badalamente *et al* 1983; Rayan and Tomasek 1994; Tomasek and Rayan 1996). [Cell lines established are detailed in appendix 1]. All cell culture work was carried out in sterile class II laminar airflow hoods, (HERA Safe, No. HS 12, Heraeus Instruments, Hanau, Germany) and flasks maintained in Heraeus (No. BB16, Heraeus Instruments) incubators kept at 37^0C, humidified and with a CO_2 concentration of 5%.

2.1.1. Establishment of Cell Cultures

Fresh tissue was obtained from the plastic surgery theatres wrapped in a saline soaked sterile swab. Dupuytren's tissue excised at fasciectomy was selected to include at least one clinical nodule and a length of pathological cord identified per-operatively as judged by the senior operating surgeon and the primary researcher. All tissue was obtained from patients undergoing primary procedures for Dupuytren's disease; no recurrent cases were included. When cleaned of surrounding fatty and loose connective tissue the specimen would often resemble a "drumstick" shape (figure 2.1). This tissue was sectioned longitudinally with one half being fixed in 10% formal saline for histology and the other half being used to establish cell cultures. An explant method (Jones and Witowski 1979) was used to establish cells in culture with two cell lines obtained from each specimen, one from the nodule and one from the cord (figure 2.1). Tissue from these regions was

macerated using a sterile scalpel and forceps, which had been previously sterilized in 70% Industrial Methylated Spirits (IMS) and allowed to air dry. The tissue was placed on the base of a T_{25} tissue culture flask (No. 690-160, Greiner Labortechnik, Greiner bio one, Germany.) and allowed to adhere for 2 minutes. It was bathed in normal fibroblast growth medium (NGM - see appendix II for composition) and incubated until fibroblasts were observed migrating from the specimen. At this point the media was changed and then further media changes were carried out on every third or fourth day thereafter. Cells were passaged into T_{175} tissue culture flasks (No. 658-170, Greiner bio one.) just prior to confluence.

Carpal ligament tissue was explanted in exactly the same way, but without separation into selected zones. Established dermal fibroblast cell lines were brought up from frozen as detailed in section 2.5.

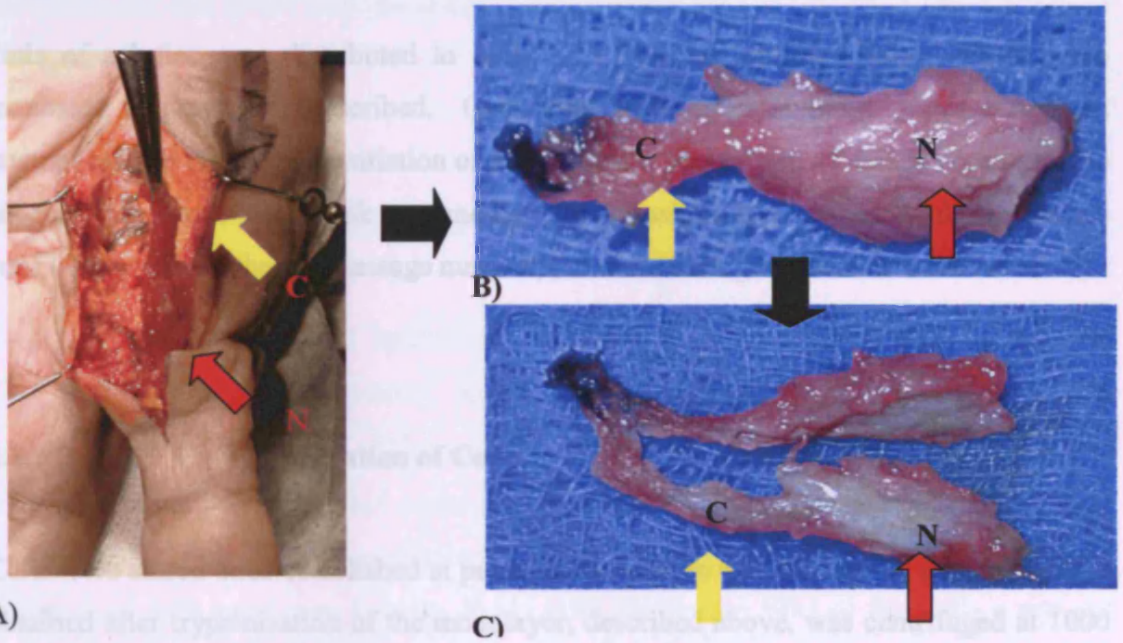


Figure 2.1. A Typical Dupuytren's Disease Specimen.
Dupuytren's disease tissue being excised from a patient undergoing routine primary fasciectomy. b) The defatted specimen illustrating macroscopically identifiable regions of nodule (N, red arrow) and cord (C, yellow arrow). c) The specimen bisected longitudinally. The inked end of the specimen allows orientation of the specimen throughout processing.

2.1.2. Routine Propagation of Cell Cultures

Cells were passaged just prior to confluence by splitting 1:3 in T_{225} tissue culture flasks. Culture medium was aspirated and the cell monolayer washed with 10ml phosphate buffered saline w/o calcium and magnesium and w/o sodium bicarbonate (PBS) (No. 14190-094, Gibco, Paisley, Scotland). This was aspirated and 5ml of 1:10 trypsin : versene (see appendix II for formulation) solution was added. The flask was incubated for 5 minutes and then agitated to obtain a single cell suspension. The trypsin solution was neutralized with 20ml of NGM and the resulting suspension was then centrifuged at 1000 rpm for 5minutes. The supernatant was discarded and the cell pellet resuspended in 9mls of culture medium.

3mls of solution was distributed to each of 3 T_{225} cell culture flasks. These were incubated as previously described. Cells used were all at or below passage 5 in an attempt to limit any dedifferentiation of the fibroblast population. (Passage represents the subculturing from one flask to another by trypsinisation, as each flask reaches confluence. The higher the passage number, the older each generation of cells becomes).

2.1.3. Cryopreservation of Cells

Cells were stored once established at passage 2 in culture by freezing. A cell suspension obtained after trypsinisation of the monolayer, described above, was centrifuged at 1000 rpm for 5 minutes to obtain a cell pellet. The supernatant was discarded and the pellet resuspended in 10 mls of NGM prior to being centrifuged again at 1000 rpm for another 5 mins. The supernatant was discarded and the pellet resuspended in 3ml of a solution of 10% DMSO (Dimethylesulphoxide, No. D2650, Sigma Chemical Company, Poole, Dorset) and 90% fetal calf serum (No. 10106-169, Gibco). 1ml was dispensed into each of 3 cryovials labelled with name, cell line, passage and date. The usual cell density for

cryopreservation was at $1x10^6$ /ml). They were wrapped in tissue paper for insulation and then placed in a -80^{0}C freezer for 24 hours. The tissue paper allowed a gradual decrease in temperature during the freezing. Once frozen, samples were transferred to liquid nitrogen for long-term storage.

2.1.4. Raising Cells from Frozen

Cryovials were thawed rapidly in a water bath at 37^{0}C. The cell suspension was transferred to a 15ml Falcon tube and 10ml of NGM was very slowly added whilst agitating gently. The resulting suspension was centrifuged at 1000 rpm for 5mins and the supernatant aspirated and discarded. The cell pellet was washed of remaining cryopreservative with 10ml of NGM and recentrifuged. The supernatant was again discarded, the cell pellet was resuspended in 5ml of NGM and dispensed into a T_{25} flask for incubation.

2.1.5. Determination of Cell Number and Viability

Cells were counted using a haemocytometer (improved Neubauer) and stained with Trypan Blue to determine viability. An aliquot of 50µl of a well-mixed cell suspension was diluted 1 in 2 with 50µl of Trypan Blue (0.4%, Sigma.) and then drawn between the haemocytometer (Nebauer) and cover slip by capillary action. This was examined under an inverted phase contrast microscope (Olympus CK2, Olympus Optical Co., Japan.) where dead cells were seen to stain darkly with the Trypan Blue as live cells pump out the dye. The number of viable cells contained within 1 large grid of the haemocytometer was counted and this repeated in 3 further grids. The mean cell number of all 4 was taken and multiplied by 2 (the dilution factor) and then by 10^4 to give the cell density. (Viable cells/ml)

2.2. HISTOLOGY

The fixed specimens of Dupuytren's tissue were embedded in paraffin blocks with a known orientation maintained by inking of one edge of the sample. This allowed accurate identification of the areas corresponding to those where cell cultures were established from under light microscopy. Representative sections of the embedded tissue were cut and stained with haematoxylin and eosin. Paraffin blocks were sectioned at 4 μm using a Reichert-Jung Microtome (Leica Instruments, Germany) and were mounted on glass slides (No. 00210, Snowcoat Extra; Surgipath, St. Neots, Cambs.). Sections were dewaxed by bathing in xylene (No. 202-422-2, Genta Medical, York, UK) for 10 minutes and were then rehydrated through bathing in a series of ethanol (Hayman Ltd, Essex, UK) dilutions, from 100% then 90%, 70% then to tap water. The sections were stained in Harris Haematoxylin (No. 31945S, BDH, Poole, Dorset, UK.) and Eosin (1% solution; 1034197, BDH) by firstly immersing the slides in Haematoxylin for 1 minute. They were then washed well under running tap water before immersion in eosin for 1 minute. After a further washing sections were dehydrated through the alcohols, cleared and mounted using DPX (No. M81330/C, DiaCheM, London, UK.) and 22 X 30 mm cover slips (Menzel-glazer). Examination at 10x and 20x magnification (Zeiss Axioscope 20, Carl Zeiss, Germany) allowed confirmation of histological differences in the regions used for different cell culture zones. These broadly corresponded to Luck's 1959 classification. The "Nodule" zone was highly cellular with disorganized architecture and minimal collagen deposition correlating to the proliferative phase. (fig. 2.2) The "Cord" was relatively acellular with large amounts of parallel, longitudinally aligned collagen representing the residual phase. (fig. 2.3)

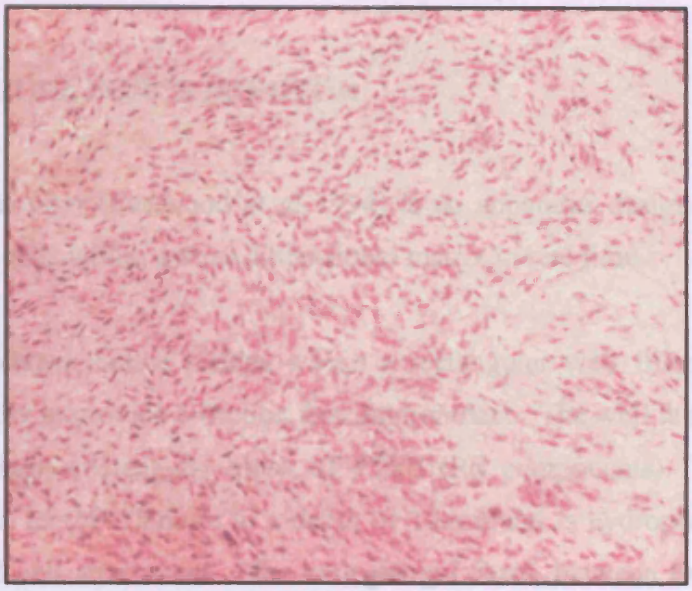


Figure 2.2: Haematoxylin and Eosin section of a Dupuytren's nodule showing cells orientated in a disorganised fashion, and minimal surrounding extracellular matrix (x 200 magnification)

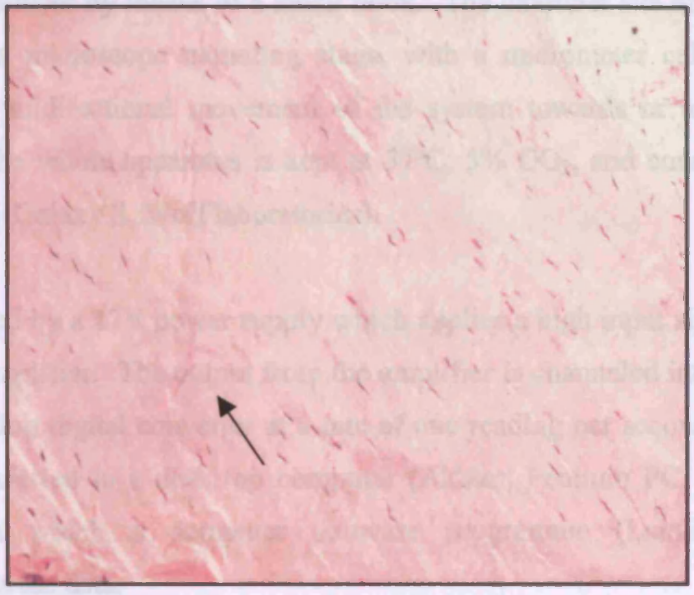


Figure 2.3: Haematoxylin and Eosin section of a Dupuytren's cord showing aligned extracellular matrix, with very few cells aligned in an organised fashion along the matrix fibres (arrow indicates direction of alignment of cells). Note in both pictures the same magnification, but very different cellularity. (x 200 magnification)

2.3. THE CULTURE FORCE MONITOR

The culture force monitor (Eastwood *et al* 1994) is an apparatus designed to measure forces generated by cells within a three dimensional matrix in real time.

The cell seeded collagen lattice resides within a rectangular well filled with normal growth media in order to provide a friction free environment. The well is a standard size situated within a single rectangular block of PTFE (RS components). The well has standard dimensions 75=25=15mmm to a depth of 10mm and is hydrophobic to inhibit collagen gel seeded cell attachment. The collagen gel floats between two hydrophilic floatation bars (appendix III). The floatation bars are connected to the culture force monitor by means of two "A" frames of stainless steel suture wire (appendix III). One frame is attached to a fixed point within the apparatus, and the other is attached to a sensitive force transducer by means of a small hook. The chamber sits on a base stage, similar to that of a microscope mounting stage, with a micrometer calibrated wheel allowing accurate, unidirectional movement of the system towards or away from the force transducer. The whole apparatus is kept at 37°C, 5% CO_2, and constant humidity within an incubator (Galaxy S, Wolf laboratories).

The CFM is powered by a 12V power supply which applies a high input signal increased by a strain gauge amplifier. The output from the amplifier is channeled into a voltmeter, and through an analog digital converter at a rate of one reading per second of force and time. This is transferred to a desk top computer (Akhter, Pentium PC; 48MB RAM; Windows 95) via which a computer software programme (Labview, National Instruments) records the data.

The force transducer is regularly calibrated against a series of known weights (appendix IV) in order to ensure linearity of displacement of the strain guage.

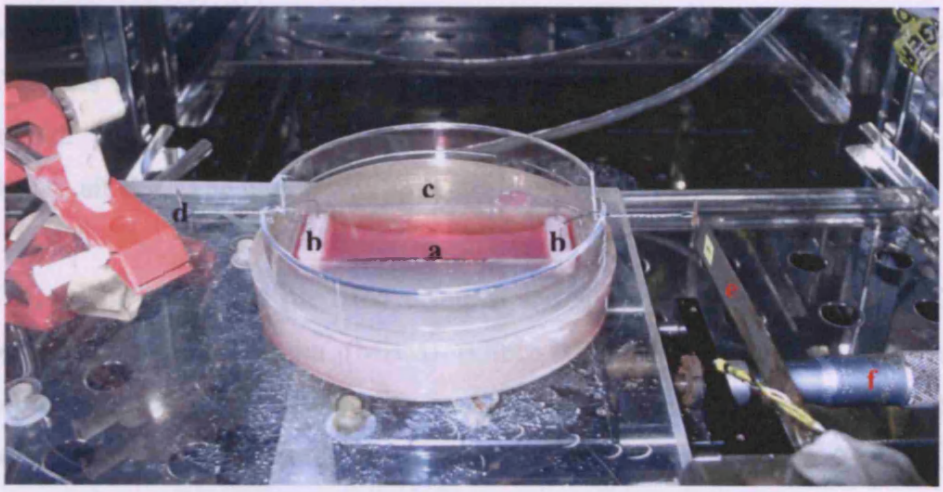


Figure 2.4. The Culture Force Monitor Set Up.
The cell seeded collagen lattice (a) is suspended between two floatation bars (b) attached to "A" frames. It floats within a well of fixed dimensions in a silicone elastomer mould (c), which is filled with growth media. One "A" frame is attached to a fixed point (d), whilst the other is attached to the force transducer (e). The force transducer is connected to a desktop computer. The apparatus sits on a moveable microscope mounting stage, which can be moved towards or away from the force transducer by the micrometer wheel (f).

2.3.1. Preparing the Fibroblast Populated Collagen Lattice

The cells to be used were grown to just sub-confluence in a T225 cell culture flask (Corning, Corning Inc NY). In general two flasks were required to provide sufficient cells at this density. The monolayer was washed twice with 10ml PBS and the cells were then trypsinised off of the flask using 5ml of trypsin/versene solution (1 in 9 dilution). Once in suspension the trypsin was neutralised with normal growth medium and then the cells were centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 10ml of normal growth media. Cell counting and viability confirmation was then carried out as described earlier and the cells were centrifuged once more. The cell pellet was resuspended in normal growth media depending on the viable cell number to reach a concentration of one million viable cells per 100µl of media.

Meanwhile the mould and floatation bars for the culture force monitor gel were prepared. The bars (appendixIII) were checked for symmetry and correct height relative to the mould. They were then immersed in alcohol to sterilise them and left on a sterile petri dish to air dry. The mould was pre-autoclaved and obtained ready for use.

The collagen gel was prepared using 6ml of collagen prep mixed with 700μl of 10x Minimal Essential Media (MEM) (Gibco Cat No. 21430-20). The solution was neutralised using dropwise addition of first 5M NaOH and then with 1M NaOH until a colour change of the solution from yellow to just pink is observed. The liquid was mixed by swirling and 1ml of the solution was quickly dispensed into the lattice of each bar. Five million fibroblasts were then seeded into the remaining solution by adding 500μl of the cell suspension prepared above and mixed trying to avoid bubbles. The bars were placed at each end of the mould well and the gel poured between them and agitated to fill the remaining space before it started to set. This was placed in an incubator at 37°C to gelate using an inverted 9cm petri dish base, with windows cut out to accommodate the bar arms, as a protective lid. (see figure 2.5.) A temperature change from room temperature to 37°C was required in order for the gel to set over a time period of 20 to 30 minutes.

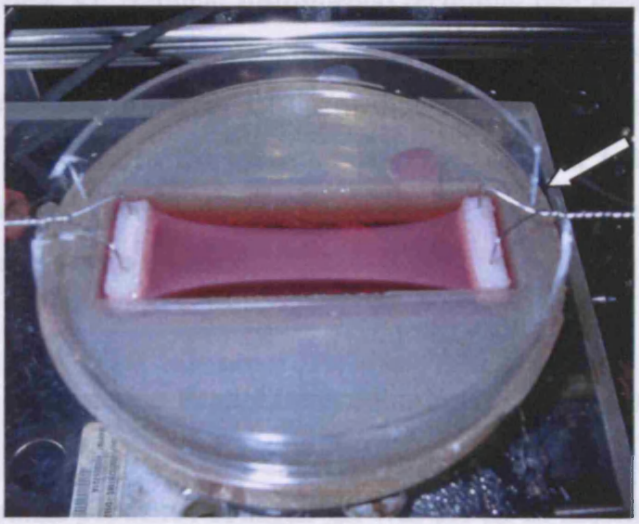


Figure 2.5. A Close up of the Collagen Lattice within the Mould.
The collagen lattice has contracted, indicated by the concave long edges. It is floating freely in the well so that there is no friction on the system and even small changes in force can be accurately measured. The lid (arrow), made out of an inverted 9cm petri dish base, can be see sitting on top of the mould. Windows have been cut out of each side of the lid (arrow head) to allow the "A" frames to extend through.

2.3.2. Setting up the Fibroblast Populated Collagen Lattice on the Culture Force Monitor

Once set, 20ml of normal growth media was added to the mould containing the fibroblast populated collagen lattice. A sterile needle was used to free the edge of the gel from the mould walls and the floatation bars gently moved inwards to release them. The gel then floated to the surface between the buoyant bars. The gel was then transferred to the culture force monitor situated within a humidified incubator at 37°C, and 5% CO_2. The eye of one bar was placed over the fixed strut of the culture force monitor as the container was placed on the mounting stage. The stage was moved in towards the measuring arm until the other bar could be hooked onto it and the system gently altered until the gel, bars and arms were aligned and floating free with no friction (figure 2.5).

The lid was replaced and the force transducer measured a voltage corresponding to the displacement of the measuring arm. The system was linked to a desk-top computer with software (Labview VI, National Instruments) recording one measurement every second, converting the voltage reading into a force measurement in dynes using a pre determined calibration factor (see Appendix IV for method of CFM calibration). Thus a real time graph of the force across the system was generated. At initial set up the force at equilibrium was set at zero and subsequent changes were observed over the following 48 hours with readings once a second.

2.3.3. Contraction Profile Determination (Static loading)

Once the fibroblast populated collagen gel was set up as described above the incubator was closed and data recording begun after 5 minutes of equilibration for temperature and CO_2. The whole system was maintained at 37°C and 5% CO_2 for the duration of the experiment. The gel was left to contract for between 8-72 hours producing a contraction profile for each cell line studied as the attached desktop computer recorded one force reading every second in real time for the duration of the experiment. This data for each experiment was converted to a mean reading per minute (1 minute data points) at the end of the experiment using a DOS macro software program which was able to determine the mean force for each minute from the 60 one second readings recorded. A contraction profile could then be plotted of force against time using Microsoft Excel software (Microsoft corporation).

2.3.4. Determination of Gradients of Contraction

The gradient of contraction at both 24 hours and 48 hours was calculated to give values for contraction rate in dynes per minute. The gradient of contraction at 24 hours was calculated by dividing the difference in the force readings at 23 hours and 24 hours by 60 to give a value of rate of change of force in dynes per minute per 5 million cells. Similarly a similar calculation was made between 47 and 48 hours to determine the 48

hour gradient. The gradient over the whole period from 0-24, and 0-48 was not utilized due to the non-linearity of each contraction profile. Mean maximum force and mean gradients of contraction for Dupuytren's nodule, cord, carpal ligament and dermal fibroblasts overall were calculated using a Microsoft Excel spreadsheet (Microsoft Office 2000).

2.3.5. System Underloading

At the end of an experimental run of 20 hours where the cell seeded collagen gel was allowed to contract on the culture force monitor, a series of underloading forces were applied to the gel. An underload was defined as a reduction in external tension applied to the gel. Each underload was achieved by rapidly turning the micrometer wheel on the CFM mounting stage by 30 micrometers leading to a reduction of longitudinal uniaxial tension on the gel of around 30 dynes. The system was then left for 30 minutes to record the response to this rapid reduction in load. The procedure was repeated a further three times so totaling four rapid underloads and four 30-minute post-underload responses. The experiment was terminated at this point and the gel removed from the machine and processed.

2.3.6. System Overloading

At the end of an experimental run of 20 hours where the cell seeded collagen gel was allowed to contract on the culture force monitor, a series of overloading forces were applied to the gel. An overload was defined as an increase in external tension applied to the gel. Each overload was achieved by rapidly turning the micrometer wheel on the CFM mounting stage by 30 micrometers leading to an increase of longitudinal uniaxial tension on the gel of around 30 dynes. The system was then left for 30 minutes to record the response to this rapid increase in load. The procedure was repeated a further three times so totaling four rapid overloads and four 30-minute post-overload responses. The

experiment was terminated at this point and the gel removed from the machine and processed.

2.3.7. Measurement of Matrix Remodelling by Addition of Cytochalasin-D

Fibroblast populated collagen lattices were set up as described previously (Section 2.31-3). The lattices were allowed to set for 30 minutes at 37°C in 5% CO_2 prior to floatation in 20ml of normal growth media and insertion into the culture force monitor. In half of the test cases of Dupuytren's fibroblasts the media was supplemented by the addition of ascorbic acid to give an overall concentration of 50μg/ml. Three separate experiments were conducted.

In the first the collagen lattices were left to contract over a period of 8 hours. At this point the maximum generated force was noted, and then a single dose of cytochalasin-D (Sigma, Poole, Dorset, UK), 20μl of 60mM in 0.5ml of normal growth media was added to the normal growth media filled chamber to give an overall concentration of 60 μM within the chamber. This was added rapidly in order to minimise disruption to the incubator temperature and CO_2 levels. Throughout this period force measurements were continuously recorded by the culture force monitor in real time, and these were continued for at least 4 hours after addition of cytochalasin-D. The above process was followed in separate experiments occurring at 24 hours and at 48 hours in order to investigate the effects of incubation time on matrix remodelling.

2.3.8. Limitations of the Culture Force Monitor Model

The culture force monitor set up is in an open chamber within an incubator, and thus the environment is not sterile. Infection of the media or collagen gel becomes more frequent by 72 hours, and indeed many specimens analysed at this time point under light

microscopy showed microscopic evidence of infection. This would be likely to cause error in results. All cell seeded collagen gels were examined under light microscopy after removal from the CFM in order to exclude those from the study that showed evidence of contamination or infection.

2.3.9. Removal and Processing of gels from the CFM

At the conclusion of each experimental run the data was saved. The collagen gel was removed from the CFM mould under tension by holding the floatation bars in a fixed position using a sterile needle at each end attached to a polystyrene block. The culture media was removed with a pipette and the gel washed 3 times with sterile phosphate buffered saline solution. The collagen gel was removed from between the floatation bars. The gel was cut into 2 and processed according to figure 2.6. One half was snap frozen in liquid nitrogen before being stored at –80μ°C. This half was used for molecular processing. The other half of the gel was processed for histological purposes in order to study cell morphology and extracellular matrix appearance. This section of gel was fixed in 4% paraformaldehyde solution and stored for 48 hours at 4°C.

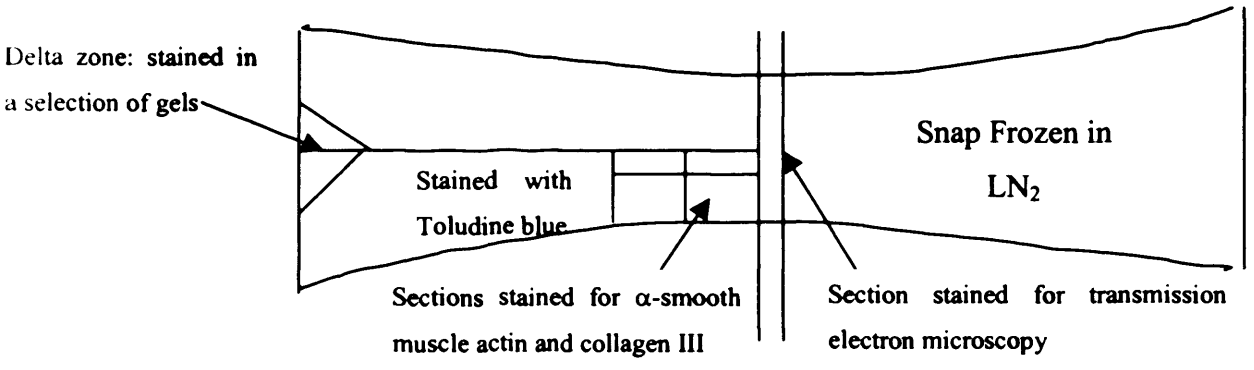


Figure 2.6. Processing of CFM gel at termination of runs.
Diagram indicating the whole collagen gel at the conclusion of experimental runs and the fate of different regions of the gel.

2.3.10. Measurement of Cell Viability within collagen gels on the CFM

Cell viability within the three dimensional collagen gels for separate nodule (n=4), cord (n=4) and carpal ligament (n=4) cell lines was determined at the end of 24 hours and 48 hours on the culture force monitor in order to ensure that there was no significant cell death over the time that experiments were run using trypan blue. In all cell types the number of viable cells remained high at greater that 95% viability at both 24 and 48 hours, with no significant difference in percentage viability between cell types. Thus variation in contraction profiles over time or between cell types cannot be attributable to differential cell death within the collagen gels.

2.4. STAINING FIBROBLAST POPULATED COLLAGEN LATTICES FOR LIGHT MICROSCOPY

Basic cellular morphology within FPCLs run on the culture force monitor was assessed by staining with Toluidene blue. One quarter of each gel was soaked in a 1% solution of Toluidene blue (No. G298, Gurr's Ltd, London, UK) for 15 seconds and then washed thoroughly with three changes of PBS for 15 seconds each. It was then observed under a light microscopy (Zeiss Axioscope 20). Digital images were taken and comparisons in morphology and alignment were made between Dupuytren's nodule, cord and carpal ligament cell lines. The degree of alignment of the cells was calculated by measuring the mean angle of deviation from the long axis of the collagen gel of the bipolar fibroblasts that were in focus in a random 2-D micrograph at 200 times magnification. 8 randomly selected fibroblasts were measured from each field of view, with three fields of view used for each gel.

2.4.1. Staining Fibroblast Populated Collagen Lattices for α-Smooth Muscle Actin

To assess myofibroblast content and orientation within the collagen gels small rectangular sections of the gel previously fixed and stored in PBS at 4^0C were cut. The position of origin within the gel was noted as this has a bearing on the lines of stress and hence cellular orientation (Eastwood *et al* 1998). In each case a portion from the middle of the gel was used (figure 2.6).

The segment of gel to be stained was soaked in ice-cold methanol in a 6 well plate on an orbital shaker (Luckham R100 Rotatest Shaker) for 1hr to permeabilise the cells. The gel was then washed with three changes of PBS for a total of 1hr again on the orbital shaker

(1 wash lasting 20 minutes each). The gel was incubated overnight in the dark at 4°C bathed in 500µl of the primary antibody, a mouse monoclonal anti α-smooth muscle actin antibody (Sigma) at 1in 1000 dilution in PBS. A second piece of gel to be used as a negative control was placed in a separate well and incubated in the same way with only 500µl of PBS.

The following morning the gels were transferred to separate wells of a new 6 well plate and washed with three changes of PBS on an orbital shaker for a total of 45 minutes (1 wash for 15 minutes each). They were then placed in another well of the 6 well plate and each incubated with 500µl of the secondary antibody solution covered in foil on orbital shaker for 1hr. The secondary solution was a FITC conjugated rabbit anti-mouse monoclonal antibody (Dako) at 1 in 400 dilution in PBS with Propidium Iodide at 1 in 50 dilution as a nuclear counterstain. Finally the gels were again washed with three changes of PBS for 1hr in a Universal container wrapped in foil to keep them in the dark and prevent degradation of the fluorescence.

The pieces of gel were placed on a glass slide, two drops of DABCO (see appendix V for composition) added and then covered with a cover slip for viewing. Stained pieces of gel were viewed under ultraviolet light on a microscope at x 20 and x 40 magnification. Myofibroblasts positive for α-smooth muscle actin demonstrated intra cellular fibres that fluoresced bright green. Negative cells showed a red nucleus with diffuse pale red or orange cytoplasmic staining.

2.4.2. Assessment of Cell Alignment within Collagen Gels

Digital images of the gels stained with both toluidine blue and smooth muscle actin were captured at x 20 and 40 magnification using a Zeiss axioscope with a Leica DC200 mounted camera and software (Leica DC Viewer, Leica Miscrosystems Ltd). By focusing in a specific plane a 2-D slice of the gel was captured and these were analysed using image analysis software (Sigma Scan).

By correct orientation of the gel on the microscope slide, the overall orientation of the whole gel and its long axis was known. The long axis of the cells in focus was drawn and the angle of this from the long axis of the gel was calculated from the slope of the line. The mean angle of orientation of the in-focus cells was then calculated for each gel. 0° would indicate perfect cell alignment along the gel axis, whereas 90° indicated cell orientation at right angles to the direction of gel alignment and force.

2.4.3. Staining Fibroblast Populated Collagen Lattices for Collagen Type III

To assess for the presence of collagen type III within the collagen type I gels, small rectangular sections of the gel previously fixed and stored in PBS at 4^0C were cut. The position of origin within the gel was noted as this has a bearing on the lines of stress and hence cellular orientation (Eastwood *et al* 1998). In each case a portion from the middle of the gel was used (figure 2.6).

The segment of gel to be stained was soaked in ice-cold methanol in a 6 well plate on an orbital shaker (Luckham R100 Rotatest Shaker) for 1hr to permeabilise the cells. The gel was then washed with three changes of PBS for a further 1hr again on the orbital shaker. The gel was incubated overnight in the dark at 4°C bathed in 500µl of the primary antibody, an anti-human collagen type III monoclonal antibody (ICN) at 1in 10µg/ml dilution in PBS. A second piece of gel to be used as a negative control was placed in a separate well and incubated in the same way with only 500µl of PBS.

The following morning the gels were transferred to separate wells of a new 6 well plate and washed with three changes of PBS on an orbital shaker for a total of 40 minutes. They were then placed in another well of the 6 well plate and each incubated with 500µl of the secondary antibody solution covered in foil on orbital shaker for 1hr. The secondary solution was a FITC conjugated rabbit anti-mouse monoclonal antibody

(Dako) at 1 in 400 dilution in PBS with Propidium Iodide at 1 in 50 dilution as a nuclear counterstain. Finally the gels were again washed with three changes of PBS for 1hr in a Universal container wrapped in foil to keep them in the dark and prevent degradation of the fluorescence.

The pieces of gel were placed on a glass slide, two drops of DABCO (see appendix V for composition) added and then covered with a cover slip for viewing. Stained pieces of gel were viewed under ultraviolet light on a microscope at x 20 and x 40 magnification. Cells showed a red nucleus with diffuse pale red or orange cytoplasmic staining. The presence of collagen III was seen by the appearance of fibres or a haze staining green.

2.5. FIXATION AND STAINING OF GELS FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

Post fixation the gel was cut in half. One central sliver was processed for transmission electron microscopy (figure 2.6). This sliver was placed in a sterile petri dish and fixed for a further 24 hours in 2.5% glutaraldehyde buffered with 0.1M sodium cacodylate (320 mosmol pH 7.4 at room temperature) (Appendix VI).

After 24 hours the small sliver of gel was cut into small portions of approximately 1-2mm^3. These sections were placed into a container and washed for 5 minutes in 0.1M sodium cacodylate buffer. They were then soaked in 1% osmium tetroxide buffered with 0.1M sodium cacodylate for 90 minutes (Appendix VI). The specimens were then washed three times for 10 minutes each in 0.1 M sodium cacodylate.

The tissue blocks were progressively dehydrated with increasing concentrations of ethyl alcohol (Electron Miscroscopy Sciences Ltd Cat 15058) (2×10 mins 70%; 2×10 mins 90%, 2×10 mins 96% ethyl alchohol and 2×20 mins 100% ethyl alcohol dried with

sodium sulphate). The blocks were left for a final 30 minutes in 100% alcohol (dried with sodium sulphate).

The blocks were transferred to a fresh container and immersed in a 1:1 resin: alcohol mixture for an hour (Appendix VI). From now after each stage blocks were placed in fresh containers. The specimens were then immersed in pure resin for one hour with vacuum infiltration, and then a further hour in fresh resin, and finally they were left overnight in a final immersion of fresh resin. The next morning the blocks were placed in labelled cylindrical embedding moulds with fresh resin and cured overnight at 65 °C for a total of 18 hours.

The resin blocks were rough trimmed using a hacksaw. Thick 1 micrometer sections were cut using a glass knife on a Reichart ultracut microtome. 2 –3 sections were placed onto a glass slide and stained with methylene blue to confirm depth and location of the sections, prior to cutting ultrathin sections.

The microtome was then adjusted to cut using a diatome diamond knife (Gilder). Ultrathin sections from each block were cut and placed on to copper grids (Gilder 200 mesh, 3mm diameter). The sections were transferred to a plate of dental wax surrounded by fresh crystals of sodium hydroxide in order to minimize CO_2 degradation of stain. The sections were stained for 15 minutes with 2% aqueous uranyl acetate, followed by rinsing three times in deionised water. They were then stained for a further 15 minutes with Reynold's lead citrate (Reynolds 1963), followed by washing three times in deionised water. Grids were stored in a glass petri dish on the surface of a piece of blotting paper.

Sections were viewed on a Phillips CM12 electron microscope at 80KV at a magnification of 3,400 to 50,000 times. Photographs were taken using a 35mm roll film camera housed within the microscope.

2.5.1. Development of TEM Negatives

All procedures were carried out in a dark room. A stock solution of Kodak D19 was prepared by taking 700ml and making it up to 2000ml volume with water at 20°C. This solution was poured into a developing tank. A working solution of the fixative was also prepared and placed into a second fixing tank.

The negatives were carefully removed from the electron microscope under dark room conditions, and transferred into a negative carrier. The negative carrier was transferred into the developing tank for 4 minutes agitating every ten seconds. After this time the cassette was removed from the tank and washed for 20 seconds in water. The cassette was transferred into the fixing tank for 3 minutes, again agitating regularly. The carrier was then washed under running water for 10 minutes, and then a single drop of Kodak wetting agent was added to the water filled tank. The carrier was agitated briefly before removal and transfer to a drying room. Once dry each negative was checked and placed into separate bags and stored.

2.5.2. Printing of TEM Photographs

All processes were carried out within safe dark room conditions. In a standard dark room, the fixative (Kentmere photographic – high speed fixative) was made up to a volume of one litre, 1:9 with water at 20°C and poured into a developing tray. The developer (Kentmere photographic – variable contrast developer) was similarly prepared using 100ml of developer in 900 ml of water, and again was poured into a separate developing tray keeping the mixture at 20°C. Two trays were filled with running tap water for the wash stages.

The negative to be processed was placed on a Devere 514 photographic machine. The negative was placed into an emulsion. An appropriate filter was selected from a book of

Ilford multigrade filters and placed into the machine. Magnification and filter setting were adjusted accordingly, as were exposure time and light aperture.

The negative was exposed onto Kentmere 6 by 8 inch glossy mid weight photographic paper. The paper was then immersed fully into the developer and this tray was gently agitated for between 30-90 seconds until development was complete. The paper was washed once in water for 15 seconds, and transferred to the fixative for a total of 3 minutes. Following this the photograph was placed in a tray of running water for 10 minutes, and then dried using a Durst RCD 3200 photographic drier. The photograph was then ready to be viewed.

2.6. RNA EXTRACTION

2.6.1. Harvesting of Cell Monolayers

An 80% confluent tissue culture flask was trypsinised by the usual method (section 2.12). The resulting cell pellet was then washed with sterile PBS and cells counted. This cell mixture was then centrifuged at 1000rpm for 5 minutes; the supernatant was removed leaving a single cell pellet of known cell number. 2ml of GT buffer at 4°C was added to the cell pellet within the universal container (See Appendix VII for RNA extraction reagents). The mixture was divided into 0.5ml aliquots in sterile eppendorf tubes. To each aliquot in a fume cupboard 500µl of water saturated phenol, 100µl of (24:1) isoamylalcohol ($CHCl_3$), plus 50µl 2M sodium acetate was added. This mixture was vortexed and incubated on ice for fifteen minutes. These aliquots were then spun in a precooled centrifuge (4°C) at 13,000rpm for 15 minutes.

After the aliquots were removed it was seen that there were two layers of fluid within the tube. The top layer was removed by sterile pipetting into a new eppendorf tube, taking care to avoid picking up any DNA which lies at the phase interface. The bottom layer was discarded. An equal volume of isopropranolol (approx 500-600µl) was added to the new eppendorf contents at room temperature, and mixed using a vortex. The tubes were then left at -20°C overnight before proceeding to the next stage.

After removal from the freezer the eppendorfs were spun in a precooled centrifuge for 30 minutes at 13,000rpm. The supernatant was removed and the residual pellet was gently washed with 300µl of 70% ethanol at 4°C (see appendix VII). The residue was removed with a fine tip. The pellets were dried slowly in a 37°C heating block for 10 minutes, and then dissolved in 20-40µl of DEPC (diethylpyrocarbonate) water (appendix VII) by repetitive aspiration, until the viscosity increased. The RNA was then pooled into a

single eppendorf, incubated at 65°C for 5 minutes and then either used immediately for preparation of cDNA or stored in a freezer at -80°C.

2.6.2. Harvesting RNA from collagen gels

Each collagen gel was removed from the -80°C freezer and allowed to defrost slowly on ice. Once defrosted the gel, within a universal container, was centrifuged at 2500rpm for 2 minutes to remove any excess water. To the gel, 3ml of GT extraction buffer was added. The contents of the tube were mixed thoroughly by vortexing, and once the gel had dissipated within the buffer, the fluid was aspirated several times through a 20 guage needle, to facilitate cell break up. The procedure documented above for cell monolayer extraction was then followed.

2.6.3. Determination of RNA yield and quality

A 2µl aliquot of the extracted RNA was diluted with 998µl of DEPC (diethylpyrocarbonate) treated water (1:500 dilution factor), and its absorbance determined spectrophotometrically. The spectrophotometer (ComSpec M330) was zeroed by making a reference against DEPC water alone, and absorbence readings were taken at 260 nm (A_{260}) and at 280 nm (A_{280}). 1 absorbance unit (A_{260}) equals 40µg of single stranded RNA per ml (Sambrook *et al* 1989).

The purity of the extracted RNA was estimated by comparing the ratio of absorbences A_{260} : A_{280}. Pure RNA gives a ratio of 2.0, however sample ratios between 1.7 and 2.1 were deemed acceptable (Sambrook *et al* 1989).

The concentration and subsequently the total yield of RNA were calculated from the A_{260} reading : -

$$\text{mRNA concentration } (\mu g/\mu l) = A_{260} \times 40 \times \text{dilution factor (500)}$$

mRNA yield (μg) = mRNA concentration × total volume of pooled RNA

2.6.4. Determination of RNA Integrity

The integrity of the RNA was checked using denaturing agarose gel electrophoresis. It was checked that the ratio of 28S to 18S eukaryotic ribosomal RNAs was approximately 2:1 by ethidium bromide staining indicating that no gross degradation of the RNA had occurred.

The gel equipment was assembled (Gibco BRL Electrophoresis power source (model 250Ex), plus Biorad gel tank) and a 0.8% agarose gel solution was prepared (0.8g agarose, with 100ml 1×TAE) (Appendix VII). The gel mixture was microwaved (Proline microchef 950W, category E, at power level 6) for 2-4 minutes, stirring frequently, until fully dissolved, and then left to cool to approximately 60°C. To the solution 5μl of ethidium bromide (Invitrogen Cat 15585-011, 10mg/ml) was added and mixed well by swirling. The gel was then poured into a mould of dimensions 6x7cm, and an 8 well cone added and the gel was then allowed to set for 30 minutes (dimensions of each well = 5x1x10mm). The gel was then placed into an electrophoresis tank filled with 1×TAE buffer. 10 μl of each RNA sample was loaded into a gel well mixed with 2μl blue dye ("all Blue" Biorad Cat 161-0373). A 1 kilobase DNA ladder was loaded into a separate well as a marker (Invitrogen Cat 15615-016). The gel was run for 30 minutes at 100V. Analysis was performed under an ultraviolet light source (UVP-dual intensity transilluminator), with eye protection using a UVP face shield. Bands were viewed on a Sony black and white (SSM 121CE) monitor and printed images taken using a Sony Video graphic printer (UP890 CE).

2.7. OBTAINING CDNA FROM RNA (REVERSE TRANSCRIPTASE (RT) REACTION)

All reagents were thawed on ice except for reverse transcriptase (RT) (See appendix VIII for reagent make up). The heating block was set to 65°C. The RNA from the stock solutions was first diluted in DEPC water at a ratio of 5μg / 8μl in fresh sterile PCR grade eppendorf tubes. The samples were heated at 65°C for 10 minutes and then placed on ice for a further 5 minutes.

In a separate tube the RT working mix was prepared (4μl 5× RT buffer, 2μl 0.1M DTT, 1μl oligo-dT primer, 1μl DEPC water, 2μl 10mM dNTP, per RNA sample) (see appendix VIII). 10 μl of the working mix was added to each sample and mixed by pipetting. 1μl of RNA guard (an RNAse inhibitor) and 1μl of Reverse transcriptase (RT) (200IU/ml – fresh from the freezer) was then added to the samples and mixed by pipetting. The samples were then pulse spun in a centrifuge for 10 seconds and left at 37°C for one hour in a water bath. The reaction was terminated by heating samples to 75°C with a heating block (Techne DRI) for 10 minutes. cDNA samples were then spun at 13,000 rpm for 1 minute at 4°C and stored at -80°C or used immediately for PCR.

2.8. THE POLYMERASE CHAIN REACTION (PCR) METHOD

All reagents were stored on ice, except DNA polymerase (Appendix IX). Both a positive and a negative control (DEPC water) were used for each primer. The positive control used was cDNA from a dermal fibroblast cell line. 2μl of each cDNA sample was aliquoted into a fresh 0.5ml PCR grade eppendorf tube. In a separate tube the PCR working mix was prepared (2μl 10×PCR buffer, 2μl 2mM dNTPs, 2μl of forward and reverse housekeeping primer (10pM) (GAPDH), 2μl of forward and reverse test primer

(10pM), 1µl DMSO, 4.75µl DEPC-water and 0.25µl 5U/ml DNA polymerase) (Appendix IXa). Full details of each primer sequence used are given in Appendix IXb. This 18µl mix was added to each aliquot of cDNA. The samples were pulse spun in a centrifuge for 10 seconds. 2 drops of mineral oil were then added to each sample to ensure the PCR working mix remained at the bottom of the tube, and the PCR was set up to run overnight. The PCR machine used was a Techgene (Techne, Jenkons PLS), and a total of 30 PCR cycles were utilized for each experiment (See appendix IXa).

2.8.1. PCR Gels

2% agarose gels were made by dissolving 2g agarose in 100ml of 1×TAE buffer and heating in a microwave for 2-4 minutes stirring frequently as discussed previously in section 2.64. The gel solution was then allowed to cool to approximately 60°C before adding 5µl of ethidium bromide and casting the gel. The gel was left to set for 30 minutes. At this stage the gel was gently inserted into an electrophoresis tank filled with 1×TAE buffer. 10µl of each PCR sample was mixed with 2µl of blue dye on a sheet of paraffin, mixing thoroughly. Each sample was loaded onto the gel along with an EZ Load precision molecular Mass Standard DNA ladder (Biorad) (see overleaf figure 2.7), and a 1,2,3, DNA ladder (Invitrogen-Cat 15613-029). The gel was run at 100V for 30 minutes, or until the dye front had migrated down to two thirds of the gel. The gel was then viewed under an ultraviolet lamp.

2.8.2. Oligonucleotide Primers

Specific human oligonucleotide primers were used to amplify the various variable portions for collagen I, collagen III, MMP-1,2,3,9,13 and TIMP-1 and 2 (appendix IX). GAPDH was the housekeeping gene used as an internal cellular control. All primers were ordered and synthesised commercially by MWG Biotech. The internal control is

required to compare the changes in mRNA against the primers to be tested. It was important that the mRNA level of the housekeeping gene did not change with the alterations in tension given to the gel. It has previously been reported that GAPDH is mechano-insensitive (Mudera *et al* 2000; Cheema *et al* 2003; 2004; Jemiolo *et al* 2004; Spofford *et al* 2003; Tan *et al* 2004) hence its use as a housekeeping gene for these experiments. The level of expression of the test mRNAs was compared to the internal housekeeping gene.

2.8.3. Image Analysis

Gel analysis was performed using the Labworks Image Acquisition and Analysis Software system (UVP Laboratory Products). The gels were scanned within a UVP epichemII darkroom using an ultraviolet source and a digital camera. Bands were identified using the computer software above, and intensities quantified. The net intensities for GAPDH were quantified and then compared to net band intensities for the test genes. This resulted in relative band intensities.

Using the EZ precision molecular mass marker, quantification of bands was made possible via the Labworks system. When 5µl of marker was used in a lane, the quantification of each band intensity is seen in figure 2.7.

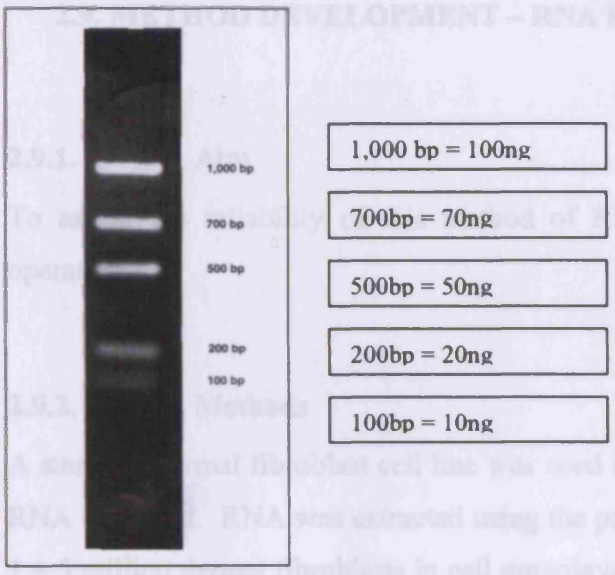


Figure 2.7:- Molecular mass marker used for the analysis of bands in the PCR reaction

2.84 STATISTICAL ANALYSIS

The mean and standard error of the mean were calculated for each gel type analysed for the Dupuytren's derived fibroblasts, the dermal fibroblasts and the carpal ligament control fibroblasts. Either a paired students t-test or a Mann-Whitney Rank Sum test for unpaired data was used to compare the groups, using Sigma Stat statistics software package (Jandel Corporation). Statistical significance was taken as a p value of <0.05.

2.9. METHOD DEVELOPMENT – RNA EXTRACTION

2.9.1. Aim

To assess the reliability of this method of RNA extraction utilized by the primary operator.

2.9.2. Methods

A standard dermal fibroblast cell line was used in order to assess the yield and purity of RNA extracted. RNA was extracted using the protocol documented above from between 1 – 5 million dermal fibroblasts in cell monolayer. For collagen gels 3 dermal fibroblast gels were made, and after 24 hours snap frozen in liquid nitrogen as per the Dupuytren's tissue and stored at -80°C for at least one week. These were then defrosted and RNA extracted as per the documented policy.

2.9.3. Results

Figure 2.8 demonstrates the total RNA yield calculated for each specimen for the dermal fibroblast cell line. It is seen that as cell number increases so does the yield.

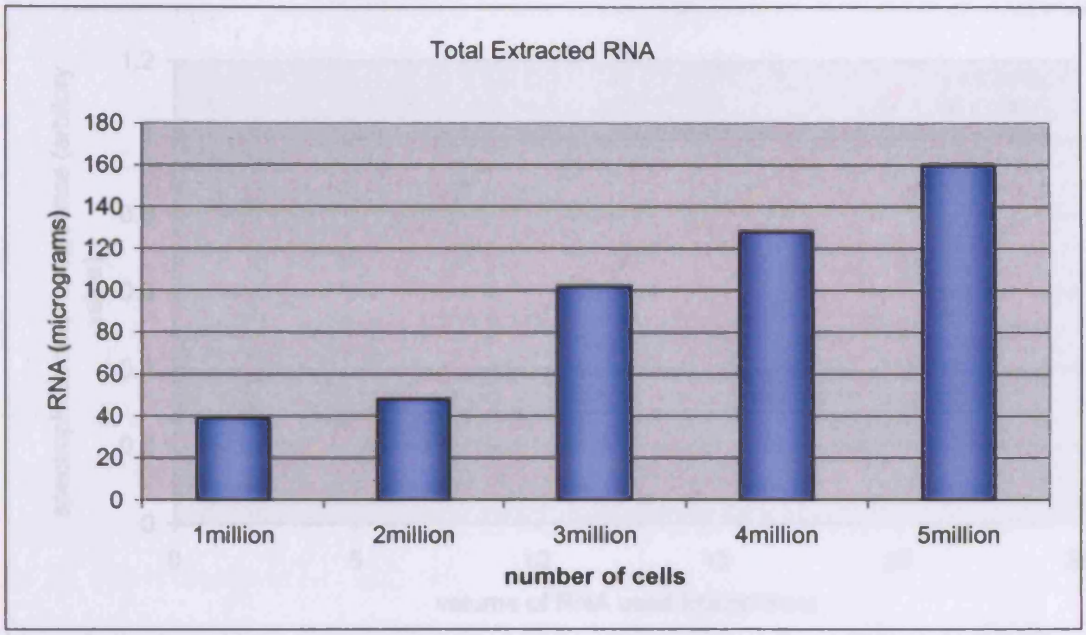


Figure 2.8 :- A histogram demonstrating total yield of RNA in micrograms for a series of 1-5 million dermal fibroblasts. Note the increasing yield with increasing cell number.

For each specimen the ratio of A260 to A280 was calculated and in all cases this was between 1.7 – 2.0.

In order to assess the reliability of the dilutions used within the spectrophotometer, a further test was performed. For a single dermal fibroblast RNA specimen, the absorbance at 260nm was measured using a range of dilutions of the specimen with DEPC water. It was hypothesized that the relationship should be linear within the range of dilutions anticipated for experimental use. Absorbance was measured using dilutions of between 1in 1000 to 1in 50. Results are shown in figure 2.9 .

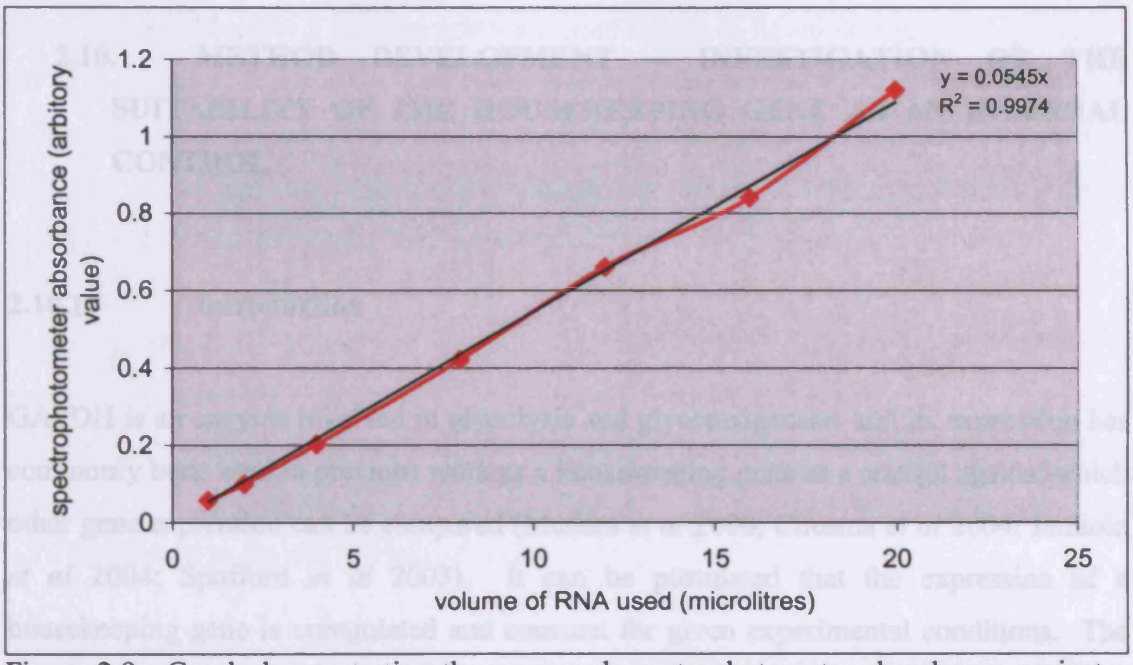


Figure 2.9:- Graph demonstrating the measured spectrophotometer absorbance against a range of concentrations of RNA. Note the near linear relationship demonstrating reliability of method in the range to be used experimentally.

For three dimensional collagen gels, 3 separate gels were made of 3 different cell lines each containing 2.5 million cells per gel. The RNA extraction protocol was followed and RNA yield calculated using spectrophotometric analysis. An average of 240μg of total RNA was extracted (range 192 – 288), confirming the suitability and reliability of this technique.

2.9.4. Conclusions

It can thus be summarized that the materials and methods used for extracting RNA are reliable and can be applied to the experimental specimens.

2.10. METHOD DEVELOPMENT – INVESTIGATION OF THE SUITABILITY OF THE HOUSEKEEPING GENE AS AN INTERNAL CONTROL

2.10.1. Introduction

GAPDH is an enzyme involved in glycolysis and glyconeogenesis and its expression has commonly been used in previous work as a housekeeping gene as a control against which other gene expression can be compared (Mudera *et al* 2000; Cheema *et al* 2004; Jemiolo *et al* 2004; Spofford *et al* 2003). It can be postulated that the expression of a housekeeping gene is unregulated and constant for given experimental conditions. The use of GAPDH as a housekeeping gene in Dupuytren's disease has been reported in one previous publication (Alman *et al* 1996), but validation of GAPDH as a housekeeping gene in Dupuytren's disease or in normal palmar fascia has not previously been reported in the literature.

2.10.2. Aims

- To investigate the expression of the housekeeping gene GAPDH (glyceraldehydes-3-phosphate dehydrogenase) by Dupuytren's fibroblasts to establish whether it can be used as an internal control gene for competitive PCR reactions

2.10.3. Methods

Fibroblast seeded collagen gels were set up on the culture force monitor and allowed to contract over a 20 hour period (*Section 2.3*). At this time point they were either allowed to contract for a further 4 hours (static load), or exposed to a series of uniaxial underloads, or uniaxial overloads (*Section 2.3.3- 2.3.6*). After removal from the culture

force monitor (*Section 2.3.9*) the gels were snap frozen in liquid nitrogen and stored at -80°C in a freezer immediately until being used for RNA extraction as outlined previously in section 2.6-2.8. There were 3 groups composed of Dupuytren's nodule, Dupuytren's cord and carpal ligament derived gels. Each group was subdivided into statically loaded gels (n=4-5), overloaded gels (n=4-6), and underloaded gels (n=4-6).

5μg of extracted RNA was taken from each cell line, and used for the RT reaction to make cDNA (Section 2.7). 2μl of cDNA per cell line was utilised for the PCR reaction (*section 2.8*), with the exception that the only primers used in the working mix were those for GAPDH. The volume of working mix was made up to 20μl with the addition of 4μl of DEPC water to replace the test primers.

2.10.4. Results

Figure 2.10 demonstrates the electrophoretic separation of GAPDH PCR products in a 2% agarose gel by a selection of Dupuytren's fibroblast cell lines exposed to a variety of stimuli. A single band appears in each lane, with the 462 base pair (bp) product corresponding to GAPDH. The figure indicates that there was no difference in expression of GAPDH between cell lines, and stimuli, and this was quantified as outlined earlier using a UVP image analysis system (*Materials and Methods 2.83*). A similar protocol was followed for all remaining Dupuytren's cell lines and all carpal ligament fibroblast cell lines with similar findings. Following densitometric scanning this data was plotted graphically (figure 2.11). There was no significant difference observed between cell type or mechanical stimulus in the expression of GAPDH. It was therefore concluded that GAPDH could be utilized as a control against which all other gene expression could be compared.

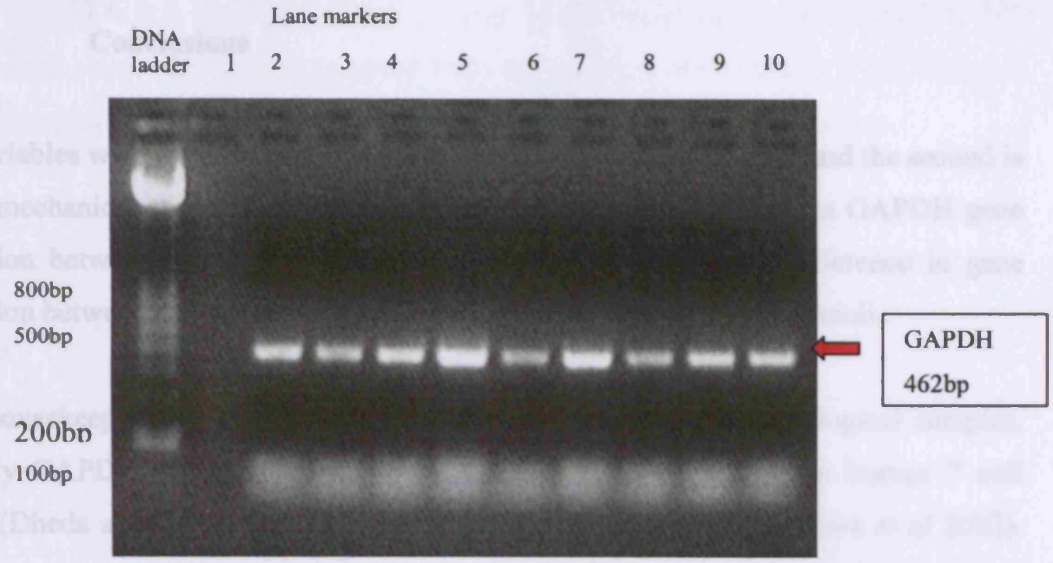


Figure 2.10:- A photograph depicting GAPDH expression by a representative section of Dupuytren's nodule cell lines, exposed to static load (lane2,3), overload (lane4,5), underload (lane 6,7), and overload with TGFbeta (lane 8,9,10). The 2% agarose gel stained with ethidium bromide shows a 462bp band representing GAPDH. A 100 bp DNA ladder marker is placed on the left of the gel.

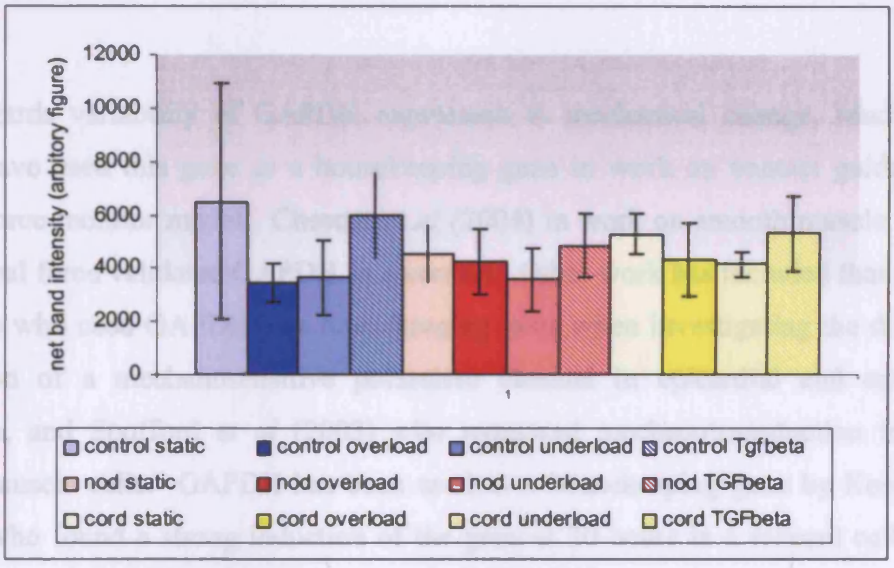


Figure 2.11:- The expression of GAPDH as net band intensity by carpal ligament (blue), Dupuytren's nodule (red), and Dupuytren's cord (yellow) derived fibroblasts exposed to either a static load, and underload, an overload, or exposed to TGFbeta. Error bars represent standard errors of the mean. There was no significant difference in gene band intensity between cell type or mechanical stimulus.

2.10.5. Conclusions

Two variables were tested within this investigation, the first is cell type and the second is that of mechanical stimulus. There was no significant difference seen in GAPDH gene expression between cell types. Similarly there was no significant difference in gene expression between the various types of externally applied mechanical stimuli.

Some housekeeping genes may vary considerably between certain biological samples. Certainly GAPDH has been shown to be variable in its expression in human T cell culture (Dheda *et al* 2004; Bas *et al* 2004) and in asthmatic airways (Glare *et al* 2002). However when GAPDH was investigated in work on human skeletal muscle at rest and after exercise, stable expression was found in both cases (Jemiolo *et al* 2004; Mahoney *et al* 2004). In addition work on myeloid cell lines has demonstrated GAPDH to be a good control (Ullmannova *et al* 2003), as has research on embryonic stem cells (Murphy *et al* 2002).

With regards variability of GAPDH expression to mechanical change, Mudera *et al* (2000) have used this gene as a housekeeping gene in work on contact guidance in a culture force monitor model. Cheema *et al* (2004) in work on smooth muscle cells and mechanical force validated GAPDH as a control. Other work has included that of Tan *et al* (2004) who used GAPDH as a housekeeping gene when investigating the differential expression of a mechanosensitive potassium channel in epicardial and endocardial myocytes, and Spofford *et al* (2003) who measured mechanotransduction in arterial smooth muscle cells. GAPDH has been used as a housekeeping gene by Kessler *et al* (2001), who found a strong induction of the gene at 20 hours in a relaxed collagen gel system, and these authors did not recommend its use as a control for the quantification of RNA levels in this type of system.

As stated earlier this is the first time that GAPDH expression has been validated for use in Dupuytren's fibroblasts, and from this it can be concluded that this gene was a suitable

control against which the expression of other genes could be compared within this experimental setup. However it is noted from the evidence above that it is important to ascertain a gene's response to stimulation before it is used in a control situation.

CHAPTER 3

THE CONTRACTILE PROPERTIES OF DUPUYTREN'S FIBROBLASTS AND THEIR RESPONSE TO MECHANICAL STIMULATION

3.1 THE CONTRACTION OF DUPUYTREN'S FIBROBLASTS IN THE ABSENCE OF MECHANICAL STIMULATION

3.11 INTRODUCTION

Researchers have used a variety of models of cellular contraction in order to assess contractile force generation in Dupuytren's disease (Schultz and Tomasek 1990; Rayan and Tomasek 1994; Rayan *et al* 1996 Tarpila *et al* 1996; Sanders *et al* 1999; Vaughan *et al* 2000; Moyer *et al* 2002; Bisson *et al* 2004). (For full details on models of cellular contraction – see Section *1.14-1.16 Introduction*).

Previously Dupuytren's fibroblasts and palmar fascia fibroblasts have been shown to generate equal amounts of contractile force measured by a reduction in diameter of circular collagen gels as a stress relaxed model, and in both cases this was proposed to be entirely actin-dependant (Rayan and Tomasek 1994). Later work by the same team (Rayan *et al* 1996) has also demonstrated that lipophosphatidic acid (LPA) promotes cellular contraction whereas nifedipine, verapamil and prostaglandins inhibit contraction of Dupuytren's fibroblasts. A reduction in cellular contraction has been shown to occur in Dupuytren's fibroblasts exposed to interferon-γ (Sanders *et al* 1999), and in those exposed to 5-fluorouracil (5-FU) (Jemec, MD thesis 1999). Enhanced cellular contraction occurs after TGF-β1 stimulation of Dupuytren's fibroblasts in both a stress relaxed model (Vaughan *et al* 2000) and a culture force model (Bisson *et al* 2004).

Bisson *et al* (2004) revealed differences between the contraction profiles of normal palmar fascia in comparison to Dupuytren's fibroblasts. Using the same experimental set up as described here (*Materials and Methods* section 2.3), it was seen that Dupuytren's derived cells exhibited a rapid production of force within the first few hours, and a failure of these fibroblasts to plateau at the end of the experimental time course at 20 hours. These results contrasted with those of previous work on other normal cell types in the same model that did reach a plateau of force generation by 20 hours (Eastwood *et al*

1994; 1996; Brown *et al* 1998). The plateau in force generation was defined as tensional homeostasis by Brown *et al* (1998) as it was postulated that cells generate and subsequently maintain a constant endogenous matrix tension. It was hypothesized that failure of Dupuytren's fibroblasts to plateau in force generation may be a result of an abnormality in these cells ability to reach tensional homeostasis, or an escape from normal homeostatic controls, and that this may be a cause for the slow tissue shortening seen in a typical flexion deformity (Bisson *et al* 2004). A limitation to the research was that contraction profiles were obtained over a period of time of just 20 hours. This work raised some questions which the following section aims to answer: - 1) Tensional homeostasis – is it delayed in Dupuytren's fibroblasts? 2) Does homeostasis persist in normal cell lines?

3.12 HYPOTHESIS

- Tensional homeostasis is absent in Dupuytren's derived fibroblasts over a period of 48 hours
- Tensional homeostasis persists in normal cell lines derived from dermal fibroblasts and carpal ligament over a 48 hour time period.

3.13 METHODS

Fibroblasts derived from Dupuytren's nodule (n=12), Dupuytren's cord (n=15), and carpal ligament derived palmar fascia from non-Dupuytren's patients (n=7) were seeded into three dimensional collagen gels and allowed to contract on the culture force monitor over a period of 24 hours (as detailed in section 2.3 *Materials and Methods*). A similar procedure was followed as above over 48 hours for 7 Dupuytren's nodule, 7 Dupuytren's cord, 3 carpal ligament and 4 dermal fibroblast cell lines. Average gradients of contraction were calculated for each cell type investigated as outlined in section 2.34 (*Materials and Methods*).

3.14 RESULTS

24 Hour Profiles

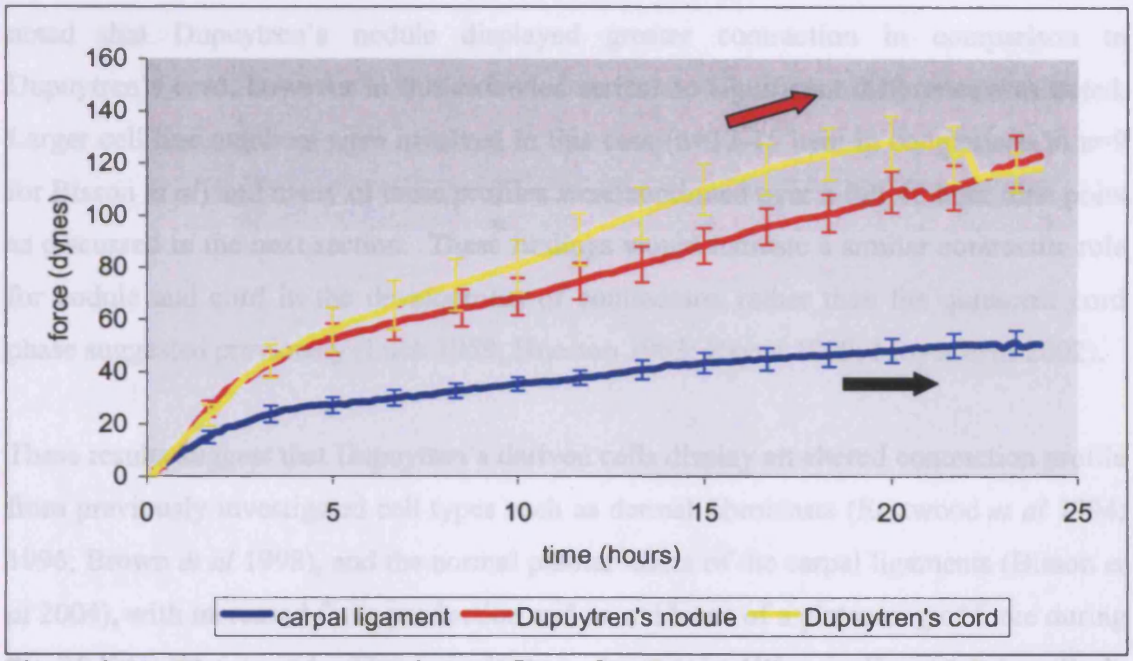


Figure 3.11: The mean contraction profiles of n=7 carpal ligament, n=12 Dupuytren's nodule, and n=15 Dupuytren's cord fibroblast cell lines. The error bars represent standard errors of the mean. Black arrow outlines the plateau of force generation by the carpal ligament cell lines, with the red arrow showing the continued increased force generated by Dupuytren's cells

The mean contraction profiles of the cell lines investigated are shown in figure 3.11 above, for n = 7 carpal ligament, n = 12 Dupuytren's nodule and n = 15 Dupuytren's cord specimens. Carpal ligament fibroblasts demonstrate an early rise in force generation over the first 5 hours to just over 20 dynes, with an increase in measured force after 15 hours to 40 dynes. The force then changed little or reached a plateau during the next 9 hours, with maximum force at 24 hours recorded at 45 dynes (black arrow). In contrast to this is the rapid rise in force generation of the Dupuytren's derived cell lines (red arrow). There is very little difference in contraction profile between the nodule and cord derived cells, with no significant difference in force generation ($p > 0.38$ (t-test)) at 24 hours. The Dupuytren's cell lines show a rapid rise in force over the first 5 hours to 55 dynes, with a

continued increase in force generation over the next 20 hours with no evidence of a plateau. By 24 hours Dupuytren's nodule fibroblasts generate a force of 121 dynes (SEM ± 9.6 dynes) and cord generate a force of 135dynes (SEM ± 9.8 dynes). There is significantly greater force produced by these cell lines in comparison to the carpal ligament derived controls at 51.8 dynes (SEM ± 4.1) ($p<0.002$). Bisson *et al* (2004) noted that Dupuytren's nodule displayed greater contraction in comparison to Dupuytren's cord, however in this extended series, no significant difference was noted. Larger cell line numbers were involved in this case (n=12-15 here in comparison to n=9 for Bisson *et al*) and many of these profiles were continued over a full 48 hour time point as discussed in the next section. These findings would indicate a similar contractile role for nodule and cord in the development of contracture, rather than the quiescent cord phase suggested previously (Luck 1959; Hueston 1963; Rayan 1999; Moyer *et al* 2002).

These results suggest that Dupuytren's derived cells display an altered contraction profile from previously investigated cell types such as dermal fibroblasts (Eastwood *et al* 1994; 1996; Brown *et al* 1998), and the normal palmar fascia of the carpal ligaments (Bisson *et al* 2004), with increased force production and no evidence of a plateauing of force during the 24 hour time period. These results may represent a delay in Dupuytren's cells in reaching tensional homeostasis or that these cells have lost or do not have the ability to achieve homeostasis. No work has examined contraction profiles using a culture force monitor model over an extended period of time beyond 24 hours. Experiments were set up to over 48 hours, in order to establish whether Dupuytren's derived cells continue to contract or begin to plateau in force generation during this time period. In view of the finding that carpal ligament fibroblasts generate a significantly lower force in comparison to Dupuytren's fibroblasts, an additional control, the dermal fibroblast, was used. These fibroblasts have been investigated using the culture force monitor model in the literature, and were thought to generate similar amounts of force in comparison to Dupuytren's fibroblasts (Eastwood *et al* 1994; 1996; Brown *et al* 1998; Bisson *et al* 2004). Tarpila *et al* (1996) found dermal fibroblasts to be more contractile than Dupuytren's fibroblasts in a stress-relaxed circular gel model, although the mechanics of circular gels differs from

that of a semi-constrained gel used here (see *Intoduction section 1.14*) which makes comparison of data difficult.

48 Hour Profiles

The graph below demonstrates the mean contraction profiles for n = 3 carpal ligament, n = 7 Dupuytren's nodule, n = 7 Dupuytren's cord and n = 4 dermal fibroblast cell lines over a 48 hour time course (Fig. 3.12).

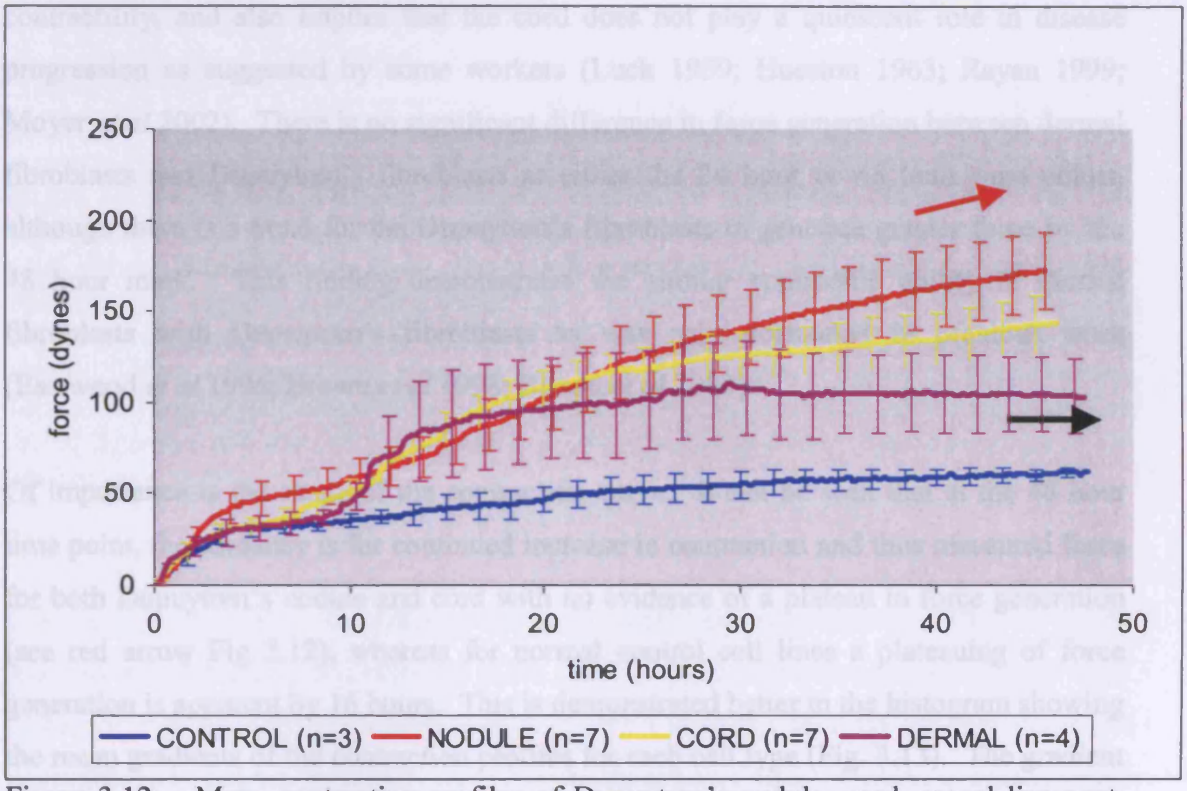


Figure 3.12: - Mean contraction profiles of Dupuytren's nodule, cord, carpal ligament, and dermal fibroblast cell lines over a 48 hour period. Error bars represent standard errors of the mean. The black arrow outlines the flattening or plateauing of force generation by the control cell lines, whereas the red arrow demonstrates that for both Dupuytren's nodule and cord cell lines force generation continues to increase.

The appearances of the graph over the first 24 hours are as discussed earlier for the Dupuytren's and carpal ligament derived cells. The dermal fibroblasts generated a contraction profile of a similar shape to that of the carpal ligament, albeit at a higher measured force. There was an early rise in force generation over the first 15 hours to 95

dynes. From 15 to 48 hours the measured force altered little (horizontal arrow parallel to x axis) indicating force generation had plateaued. Measured force at 15 hours was 95 dynes and at 48 hours was 100 dynes. Similarly for carpal ligament the force generated at 24 hours of 50 dynes was not significantly different from force measured at 48 hours of 55 dynes. By 48 hours Dupuytren's nodule demonstrated greater force generation at 172 dynes (SEM ± 21 dynes). For cord force generation rises to 143 dynes (SEM ± 16 dynes). Again there is no significant difference in force between the nodule and the cord ($p>0.2$). This indicates that both nodule and cord have a very similar level of contractility, and also implies that the cord does not play a quiescent role in disease progression as suggested by some workers (Luck 1959; Hueston 1963; Rayan 1999; Moyer *et al* 2002). There is no significant difference in force generation between dermal fibroblasts and Dupuytren's fibroblasts at either the 24 hour or 48 hour time points, although there is a trend for the Dupuytren's fibroblasts to generate greater force by the 48 hour mark. This finding demonstrates the similar contractile ability of dermal fibroblasts with Dupuytren's fibroblasts as was only postulated in previous work (Eastwood *et al* 1996; Brown *et al* 1998; Bisson *et al* 2004).

Of importance is the shape of the contraction curve. It can be seen that at the 48 hour time point, the tendancy is for continued increase in contraction and thus measured force for both Dupuytren's nodule and cord with no evidence of a plateau in force generation (see red arrow Fig 3.12), whereas for normal control cell lines a plateauing of force generation is apparent by 16 hours. This is demonstrated better in the histogram showing the mean gradients of the contraction profiles for each cell type (Fig. 3.13). The gradient of contraction at the 24 and 48 hour time points were calculated as described in section 2.3.4.

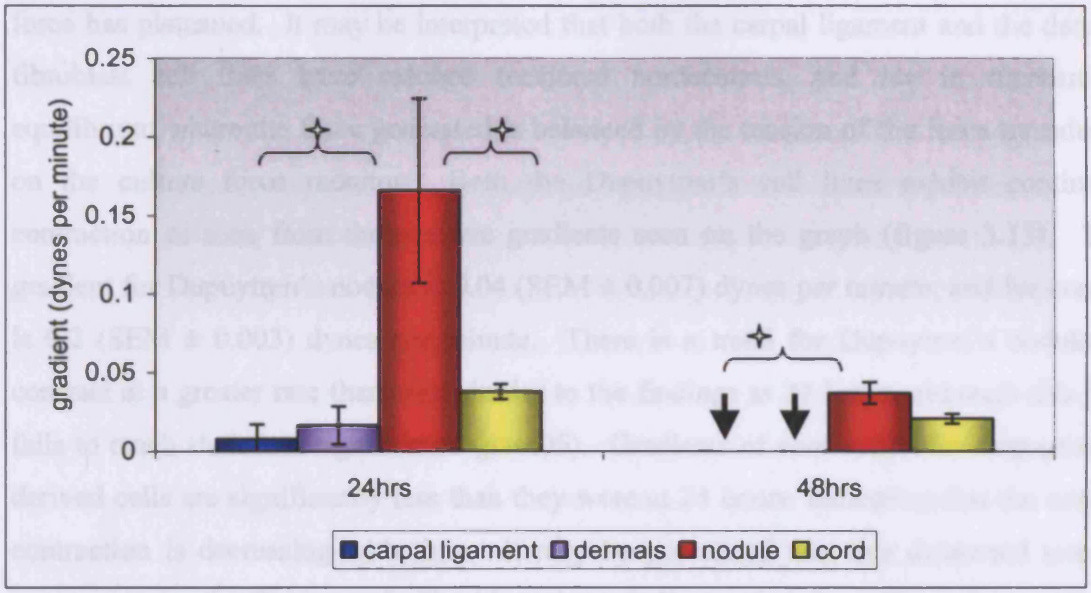


Figure 3.13: - Histogram demonstrating the gradient of contraction profile at 24 and 48 hours for n = 3 carpal ligament, n = 7 Dupuytren's nodule, n = 7 Dupuytren's cord, and n= 4 dermal fibroblast cell lines. Error bars represent the standard error of the mean. There is a statistically significant difference ✧ between both Dupuytren's cell lines and the carpal ligament and dermal fibroblast cell lines ($P<0.05$ at 24 hours; $p<0.01$ at 48 hours). There is a statistically significant difference ✧ between Dupuytren's nodule and cord at 24 hours ($p<0.02$), but not at 48 hours ($p=0.06$). Note the absence of bars for carpal ligament and dermal fibroblasts at the 48 hour time point – for both of these the gradient was measured at zero.

It is seen that at 24 hours the gradients for both carpal ligament and dermal fibroblasts are minimal at 0.008 (SEM ± 0.008) and 0.002 (SEM ± 0.01) dynes per minute respectively, indicating that although the force generated by these cells is rising, the increase is almost negligible. In contrast for Dupuytren's nodule the gradient of contraction is significantly greater at 0.15 (SEM ± 0.05) dynes per minute, as it is for cords with a gradient of 0.04 (SEM ± 0.004) dynes per minute ($p<0.05$). Additionally there is a trend for nodules to be contracting at a greater rate than cords at 24 hours that is significant ($p<0.02$). Thus at 24 hours, although the nodule and cord generate a similar amount of force, it is the nodule that is contracting more quickly at this time point.

At 48 hours the gradient of contraction for both control cell lines is zero (arrowed on figure 3.13), indicating that there is no further increase in contraction and that generated

force has plateaued. It may be interpreted that both the carpal ligament and the dermal fibroblast cell lines have reached tensional homeostasis, and are in mechanical equilibrium where the force generated is balanced by the tension of the force transducer on the culture force monitor. Both the Dupuytren's cell lines exhibit continued contraction as seen from the positive gradients seen on the graph (figure 3.13). The gradient for Dupuytren's nodule is 0.04 (SEM ± 0.007) dynes per minute, and for cord it is 0.2 (SEM ± 0.003) dynes per minute. There is a trend for Dupuytren's nodule to contract at a greater rate than cord similar to the findings at 24 hours, although this just fails to reach statistical significance ($p>0.06$). Gradients of contraction for Dupuytren's derived cells are significantly less than they were at 24 hours, indicating that the rate of contraction is decreasing with time. It may be postulated that this decreased rate of contraction by the Dupuytren's fibroblasts is an indicator that the cells are beginning to reach a plateau phase, and thus will achieve tensional homeostasis.

3.15 DISCUSSION

Dupuytren's disease has been defined as a fibrocontractive disorder. It is hypothesised that digital flexion contracture is caused by a combination of cell mediated contraction and matrix remodelling (Brickley-Parsons *et al* 1981). This study has provided a means of identifying the contribution of cellular contraction to this process using a defined collagen gel model on the culture force monitor in real time.

In previous work using the culture force monitor it has been shown that dermal fibroblasts demonstrate a three-phase contraction profile over a 24-hour period (Eastwood *et al* 1996). In the first phase there is a rapid increase in force generation over 8 hours thought to be secondary to cellular locomotion and attachment throughout the collagen matrix. The second phase is related to cellular traction on the matrix and represents a plateauing of force generation over the 8-12 hour period. The final phase represents the steady state of force generation where the cells are in tensional

homeostasis with the collagen matrix and the forces of cellular contraction are balanced by matrix tension (Brown *et al* 1998).

These results are the first to compare dermal fibroblast contraction with that of Dupuytren's disease specimens in the culture force monitor. The majority of work using this model previously has examined dermal fibroblast contraction only. The mean force generated at 24 hours of 120 dynes for nodule derived cells, and 118 dynes for cords is not significantly different than that for dermal fibroblasts at 101 dynes. These values for dermal fibroblasts correspond with previous work using the culture force monitor model (Eastwood *et al* 1996; Brown *et al* 1998), although a study using a free floating collagen gel model has demonstrated that dermal fibroblasts contract to a greater degree than Dupuytren's nodule cells at 36 hours (Tarpila *et al* 1996). The free floating model as discussed earlier is based on slow sustained cellular contraction, in contrast to the tethered collagen gels utilised in the culture force monitor, so that these studies cannot be directly compared based on their different mechanics. However by 48 hours although the force generation by Dupuytren's nodules of 172 dynes is not significantly greater than that of the dermal fibroblast cell lines, it is the different shape of the curves that is significant. Force generation has plateaued for dermal fibroblasts with a gradient of zero, but gradient continues to rise for Dupuytren's fibroblasts indicating continued cellular contraction. This plateau of force was defined by Brown *et al* (1998) as being the stage of tensional homeostasis where the force generated by the cells is equal to the resistance of the force transducer, and it was hypothesised to be the preferred level of tension at which the cells exist within a matrix. This stage is absent in Dupuytren's derived fibroblasts within the time period of these experiments.

Previous work looking at the contraction profiles of nodule and cord derived fibroblasts have shown that nodules display a higher level of contraction in comparison to cord. This has been demonstrated in both the culture force monitor model (Bisson *et al* 2004) and in a free floating collagen lattice model (Moyer *et al* 2002). This work is contrary to the above. At both 24 and at 48 hours there is no significant difference noted in force generation between nodule and cord. What is important here is the large number of

specimens investigated in comparison to the other authors, providing a more accurate representation of the contraction profiles of nodule and cord (Moyer *et al* 2002; Bisson *et al* 2004). Moyer *et al* (2002) from work on free floating collagen gels proposed that nodules progressed to cords as the disease progressed. This was based on the finding that nodule derived fibroblasts contracted a collagen gel to a greater extent than cord, but that this contraction decreased to cord like levels with increasing cell passage. Bisson *et al* (2004) postulated that the nodule represented the most active stage of the disease. It may be hypothesised from this that nodule and cord derived cells are similar in phenotype, and indeed that the cord is not the quiescent phase of the disease as stated by many (Luck 1959; Hueston 1963; Rayan 1999; Moyer *et al* 2002).

The altered contraction profiles of Dupuytren's derived fibroblasts in comparison to those of both dermal fibroblasts and carpal ligament fibroblasts may be highlighted here. Certainly the early phase rapid generation of force remains similar corresponding to cell attachment and migration throughout the matrix. However even by 48 hours the Dupuytren's fibroblasts exhibit continued contraction without evidence of a plateau, the nodules to a greater extent than the cords. These findings indicate that Dupuytren's fibroblasts show a delay in reaching tensional homeostasis in view of the progressive slowing in rate of force generation at 48 hours in comparison to 24 hours, but it can be speculated that these cells may never reach homeostasis within their surrounding tissue matrix. Experiments could not be continued much beyond 48 hours in order to elucidate whether a plateau of force generation is reached over longer time periods due to the limitations of the CFM system. The culture force monitor set up is in an open chamber within an incubator, and thus the environment is not sterile (*see Material and Methods section 2.3.7*). Other theories for loss of contraction with time may include cell utilisation of growth factors within the bath of growth media, and build up of cell metabolites. However there was no change in cell viability over the 48 hour time course and no significant cell death as shown from assays taken at the end of the experimental time period (see *Materials and Methods section 2.3.10*). Other workers have also studied cell viability and numbers within collagen gels. Within tethered systems such as used

here both Kolodney and Wysolmerski (1992) and Greco and Ehrlich (1992) found no proliferation of human fibroblasts during a three day time period.

Carpal ligament fibroblasts taken from the normal palmar fascia exhibit a lower level of force generation plus evidence of homeostasis in a similar manner to dermal fibroblasts. It is postulated therefore that tensional homeostasis persists over the period tested for these control fibroblasts. There is little data on other cell types in the literature using this experimental model and thus it is difficult to determine whether all cells will show this sort of behaviour within a three dimensional collagen gel. Indeed work with human myoblasts and smooth muscle cells (Cheema *et al* 2003) has shown an absent early phase of contraction, with later plateauing of force, and studies on rat tendon fibroblasts have shown continued cellular contraction over a 48 hour time period in a similar manner to Dupuytren's cells (Wilson-Jones *et al* MSc thesis 2003). The significantly lower level of contraction of carpal ligament fibroblasts has been documented previously (Bisson *et al* 2004), and was proposed to be due to these cells location in a stress-shielded area of matrix in-vivo. In contrast to the results presented here, and those of Bisson *et al* (2004), Rayan and Tomasek (1994) used a stress-relaxed circular gel model that found similar levels of gel contraction by both carpal ligament and Dupuytren's nodule fibroblasts. As discussed earlier, a direct comparison of these results in not possible due to the mechanical differences of the models used.

This data suggests that Dupuytren's fibroblasts have a delayed ability to reach tensional homeostasis, and will generate higher levels of tension within a tissue matrix than control fascial fibroblasts. An increase in cellular contraction may over time result in local fascial shortening, and this may be a significant factor in the progressive contracture seen in the digits of Dupuytren's disease patients.

3.16 SUMMARY

- Dupuytren's fibroblasts generate significantly greater forces in a culture force monitor model in comparison to control fibroblasts derived from carpal ligament.

- Dupuytren's fibroblasts generate similar forces to dermal fibroblasts within a culture force monitor model at both 24 hours and 48 hours.

- There is no significant difference in force generation between Dupuytren's nodule and cord at 24 or 48 hours in a series of 12 specimens. This is in contrast to previous work utilizing smaller numbers using this model and a circular collagen gel model (Moyer *et al* 2002; Bisson *et al* 2004).

- Dupuytren's derived cells continue to contract over 48 hours with an absence of the plateau seen in dermal fibroblasts or carpal ligament fibroblasts. The rate of contraction falls significantly after 48 hours. It is postulated from this that tensional homeostasis is delayed rather than absent.

- Tensional homeostasis persists in control cell lines over a 48 hour time period.

This work has demonstrated a difference in contractile force generation by Dupuytren's fibroblasts in comparison to control fibroblasts with the absence of a plateau phase of force generation. Previous work has established that Dupuytren's fibroblasts contract in response to an externally applied mechanical load (Bisson et al 2004) in contrast to dermal fibroblasts which act by relaxation (Brown et al 1998). The effects of a reduction in externally applied load on Dupuytren's fibroblasts have not been elucidated, and this was investigated further.

3.2 MECHANICAL UNDERLOADING OF DUPUYTREN'S FIBROBLASTS

3.21 INTRODUCTION

Distinctive nodules and cords aligned in a longitudinal direction within the palm of the hand and digits are characteristic features of Dupuytren's disease (Rayan 1999). The arc of flexion and extension at the MCP and PIP joints occurs along the longitudinal axis, as does the transmission of any externally applied load to the hand. This section concentrates on unidirectional forces applied to cells as this simulates the situation seen *in-vivo*.

Cells are highly sensitive to external mechanical forces that are transmitted through the extracellular matrix. Cellular responses to tension include changes in morphology, synthesis of hormones, changes in matrix synthesis, and release of regulatory enzymes (Jones 1992; Ohno *et al* 1995; Butt *et al* 1995; Eastwood *et al* 1996; Kain and Reuter 1995). Fibroblast contractility has been studied for many years, mainly as a model of wound healing, and usually in circular three dimensional collagen gel experiments (Elsdale and Bard 1972; Bell *et al* 1979; Delvoye *et al* 1991; Tomasek and Hay 1984; Eastwood *et al* 1994; 1996; Kolodney and Wysolmerski 1992; Grinnell and Ho 2002). Using a circular model it is not possible to ascertain cellular responses to external unidirectional changes in mechanical tension, and it was not until the development of the tensioning culture force monitor (Eastwood *at al* 1996) or it's equivalent (Delvoye *et al* 1991) that these responses have been measured.

Bisson *et al* (2004) have demonstrated that rapid overloading of control carpal ligament fibroblast lattices results in a gradual reduction in force over a 30-minute period, which is consistent with the theory of tensional homeostasis seen in dermal fibroblasts (Brown *et al* 1996). However overloading of lattices seeded with Dupuytren's fibroblasts resulted

in an abnormal contractile response during the first overload period, and this effect was enhanced by pre-incubation of these cells with TGF-β1 (Bisson MD thesis 2003).

This section investigates the application of unidirectional mechanical loads to Dupuytren's fibroblasts using a culture force monitor model. Previously extension or overloading forces have been discussed, but no earlier work has looked at the application of underloading or a reduction in external load as would occur in passive flexion of the digits, to this model. The only clinical reports that have looked at mechanical unloading of the palmar fascia in Dupuytren's disease are those debating the use of fasciotomy, where incising the Dupuytren's cord suddenly reduces tension within the fascia. In some of these cases it has been observed that the disease regresses after tension is released (Moermans 1981; Andrew and Kay 1991). However conversely it has also been demonstrated that simple fasciotomy leads to rapid disease recurrence (Luck 1959; Millesi 1965; Lermusiaux 1997).

3.22 AIMS

- To test the effects of mechanical underloading on Dupuytren's nodule, cord, and carpal ligament fibroblasts.

3.23 HYPOTHESIS

- Mechanical underloading of Dupuytren's fibroblasts will result in greater cellular contraction in comparison to normal controls

3.24 METHODS

Fibroblast populated collagen lattices were set up as described previously, and allowed to contract (*Materials and Methods 2.33)*. At 20 hours a series of uniaxial mechanical underloads were applied to the gel (*Materials and Methods 2.34)*. This was achieved by rapidly turning the culture force monitor mounting stage towards the force transducer by manually turning the stage micrometer wheel through 30 micrometers. This led to a reduction in uniaxial load on the gel of 30 dynes. The experiment was then left to run for a further 30 minutes while any change in force was recorded in real time. In total 4 underloading forces were applied per gel.

6 acellular control gels, 4 Dupuytren's nodule, 6 Dupuytren's cord and 3 carpal ligament cell lines were investigated. The gradient of the contraction profile over the 30-minute post underload period was calculated as the rate of change in force (dynes per minute).

3.25 RESULTS

Acellular control gels

A control acellular contraction profile is demonstrated below (figure 3.21). Each underload is characterized by a sudden decrease in force recorded by the force transducer. There is a steady increase in force recorded after each underload. After each underload the force increases, but never returns to the pre–underloading level of force, so that with each underload there is a step-wise decrease in measured force. Of note is the finding that after the first underload, the remainder of the underloads occur with negative force i.e. with the force transducer being pushed away from its normal resting position (figure 3.22).

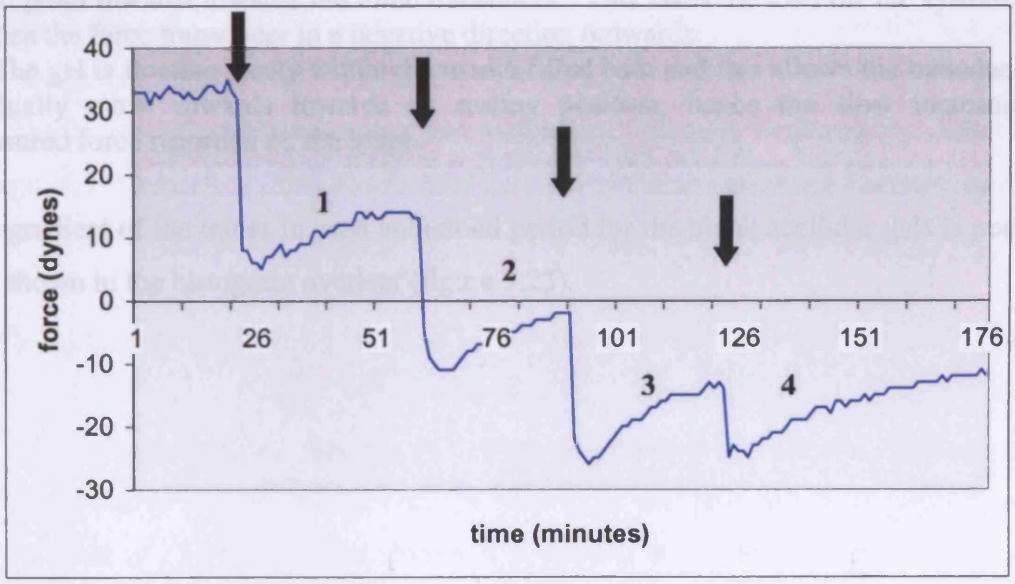


Figure 3.21: - Contraction profile of a control acellular blank gel undergoing a series of 4 uniaxial tensional underloads (arrows) after 20 hours on the CFM. The subsequent post underload periods are numbered 1-4.

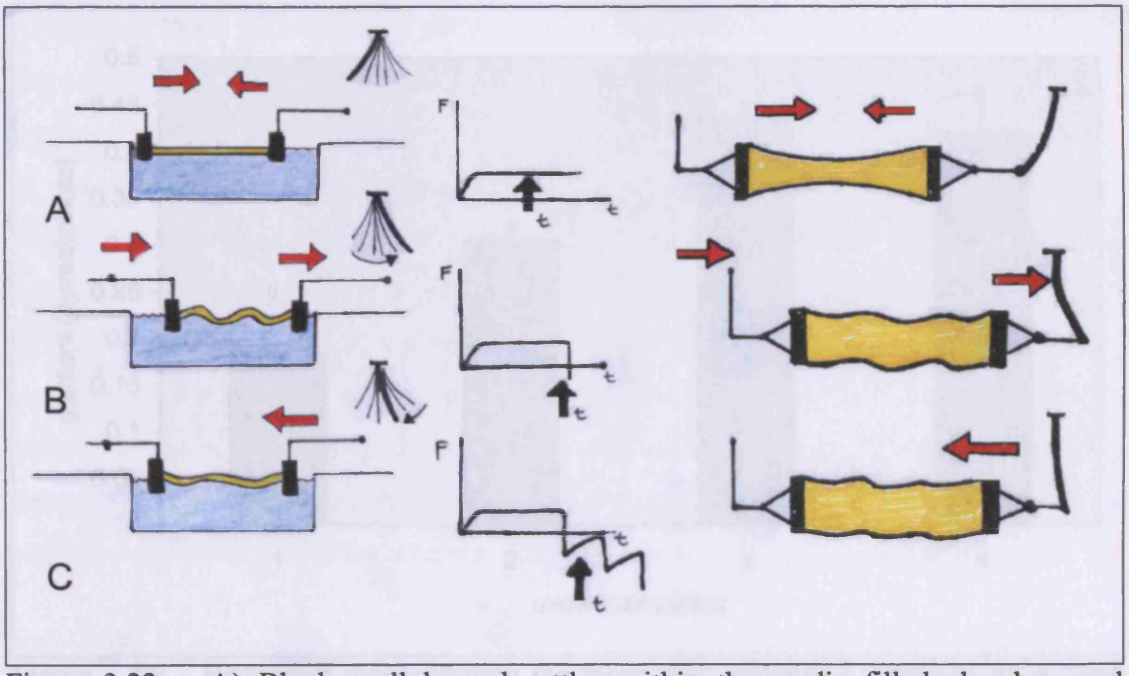


Figure 3.22: - A) Blank acellular gel settles within the media filled chamber, and contracts to a resting tension of 20 dynes, pulling the force transducer inwards.
B) The system is underloaded by rapidly turning the micrometer screw which pushes the fixed point inwards towards the force transducer. This takes the load off the system, and pushes the force transducer in a negative direction outwards.
C) The gel is floating freely within the media filled bath and this allows the transducer to gradually move inwards towards its resting position, hence the slow increases in measured force recorded on the graph.

The gradient of the traces in each underload period for the blank acellular gels is positive and shown in the histogram overleaf (figure 3.23).

zero value. It is therefore only valid to utilise data from the 1st underload period with any accuracy.

In order to test this hypothesis, a further set of experiments was carried out. After insertion of the acellular gel into the CFM system, the external force applied to the system was increased rapidly to over 200 dynes. The gel was then left to equilibrate for 20 hours, and at this time 4 sequential underloads were applied as previously. There was a minimal increase in contraction of the gel after each underload period of 0.18 dynes per minute in each case (figure 3.24). There was no significant difference in gradient between each underload period. It was noted that each period in this preloaded sequence was very similar to that of the 1st underload period in the original acellular gels tested.

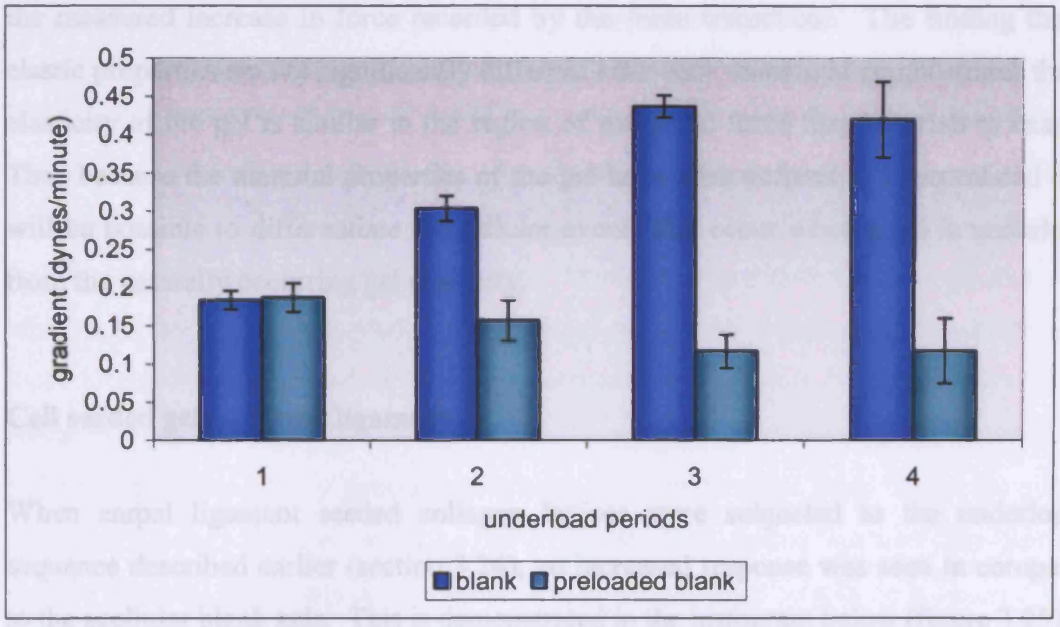


Figure 3.24: - Histogram showing the mean post underload gradients for control acellular blank gels (n=3), and preloaded acellular blank gels (n=3), after a series of 4 uniaxial tensional underloads. Error bars represent standard errors of the mean. Note that for the preloaded blank gels there is a similar positive gradient.

In view of these findings, the results of underloading cell-seeded gels were compared to those of the preloaded blank gels. This avoided the utilization of data that involved

negative deflection of the force transducer, and was thus not validated. The use of preloaded gels was reproducible, and accurate as demonstrated above, was and therefore a suitable control model for this experimental model.

Of note is that an acellular collagen gel has non-linear mechanical characteristics. Unpublished data from this laboratory (Marenzana, personal communication 2003) has shown that increasing external tension by overloading will result in increased matrix stiffness. Gels manufactured from type I collagen are hypothesised to possess non-linear viscoelastic properties when axial strain is applied, and will not return to their original position once tension is increased. (Ozerdem *et al* 1995). It is seen here that within acellular gels that have been loaded to increase matrix stiffness, an underloading regime will not act to allow the force to return to pre-underloading levels. After application of a reduction in external load it may be postulated that the gel does display some elasticity by the measured increase in force recorded by the force transducer. The finding that the elastic properties are not significantly different after each underload demonstrates that the elasticity of the gel is similar in the region of measured force that we wish to examine. Thus because the material properties of the gel have been defined, it is postulated that it will be possible to differentiate the cellular events that occur when a gel is underloaded from the naturally occurring gel elasticity.

Cell seeded gels – carpal ligament

When carpal ligament seeded collagen lattices were subjected to the underloading sequence described earlier (section 3.24), an increased response was seen in comparison to the acellular blank gels. This is demonstrated in the histogram below (figure 3.25).

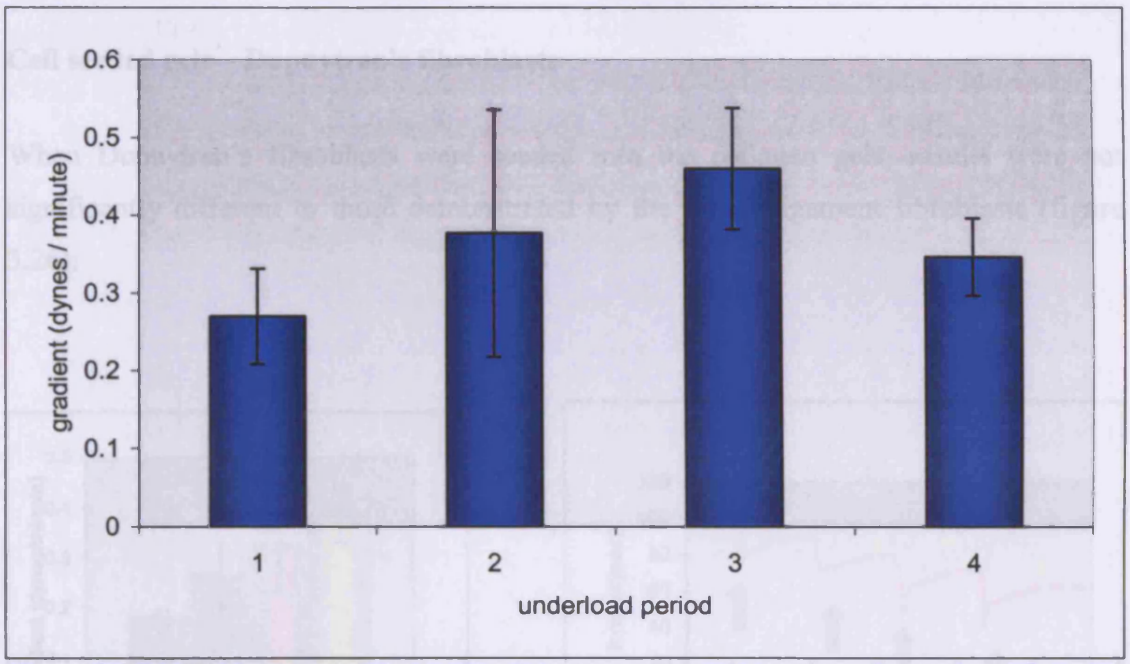
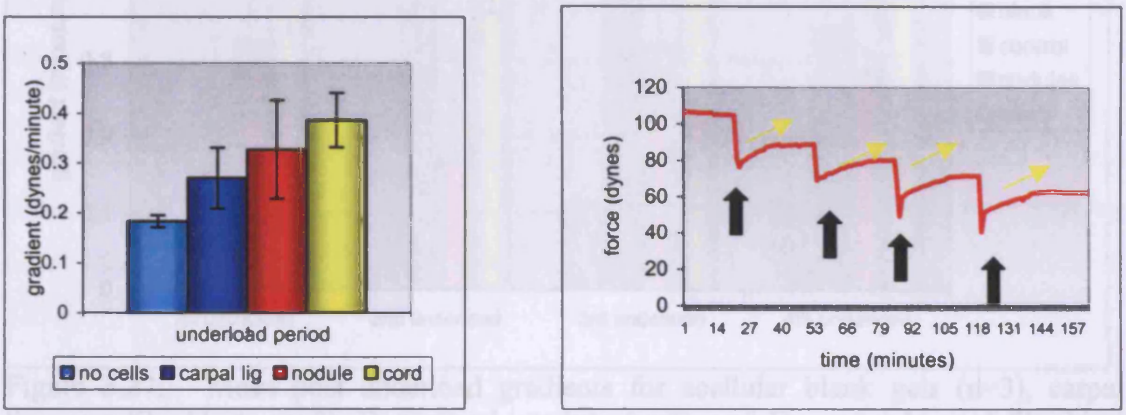


Figure 3.25: - Histogram demonstrating the mean post underload gradients for n=3 carpal ligament cell lines. Error bars represent the standard error of the mean.

Gradients are positive after each underload period (0.27 ± 0.06; 0.37 ± 0.16; 0.46 ± 0.08 and 0.34 ± 0.05 dynes per minute respectively). There is no significant difference between the gradients in each period. These results are significantly greater than those for the acellular gels ($p<0.001$), indicating a cellular response to the change in external tension. If the mechanical properties of the gel are removed as shown earlier, it remains that a positive gradient persists. It can be postulated that this can be attributed to the cells seeded within the gel. The fibroblasts have responded to the reduction in external load applied across the gel, by generating an increase in contractile force, hence the positive gradient. As shown in the previous section (chapter 3.14) carpal ligament fibroblasts will rapidly generate an endogenous tension within the collagen matrix as measured by the culture force monitor, and by 15 hours this measured force will have plateaued. When endogenous tension is reduced as performed here, the cells respond in a manner that tends to increase the force back towards this level of endogenous tension.

Cell seeded gels – Dupuytren's fibroblasts

When Dupuytren's fibroblasts were seeded into the collagen gels, results were not significantly different to those demonstrated by the carpal ligament fibroblasts (figure 3.26).



a) b)

Figure 3.26: - a) Histogram on left demonstrates the mean underload gradients for n=3 acellular gels, n=3 carpal ligament cell lines, n=4 Dupuytren's nodule and n=5 Dupuytren's cord cell lines after the first underload. Error bars represent standard errors of the mean. There is no significant difference between cell types, but all gradients for cell seeded gels are significantly greater than that of the blank acellular gels ($p<0.05$).
b) Graph on right demonstrates a typical Dupuytren's cell line undergoing a series of 4 uniaxial tensional underloads after being left to contract for 20 hours. Note the increase in generated force after each underload period (block arrows in black = underload applied to the gel; arrow in yellow = increase in measured force as shown).

After the first underload period the mean gradient for nodule was 0.32 dynes per minute (SEM ± 0.09) and for cord 0.39 dynes per minute (SEM ± 0.05). There was no significant difference between gradients for nodule and cord (figure 3.26a). This pattern continued in the later underload periods; all gradients were positive with cord demonstrating a slightly increased response in comparison to nodule (figure 3.27). Once again there was no significant difference between cell types or between underload periods. Values for nodule were 0.23 ± 0.05, 0.32 ± 0.09 and 0.38 ± 0.1 for 2nd to 4th

underload period, and those for cord were 0.39 ± 0.05, 0.42 ± 0.06, 0.49 ± 0.04, and 0.43 ± 0.05 dynes per minute respectively.

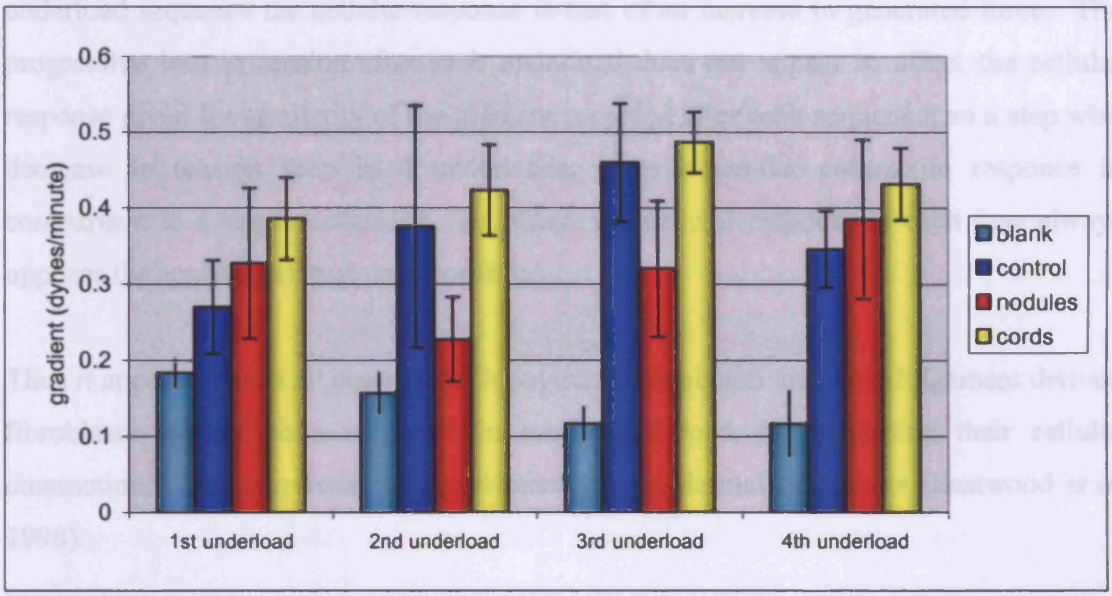


Figure 3.27: - Mean post underload gradients for acellular blank gels (n=3), carpal ligament fibroblasts (n=3), Dupuytren's nodule (n=4), and Dupuytren's cord fibroblast (n=6). Error bars represent standard errors of the mean.

Thus there is a significant increase in force generated by all the cell seeded gels ($p<0.001$) in comparison to the blank acellular gels, with control carpal ligament fibroblasts showing a gradient of 0.33 dynes per minute (SEM ± 0.14), a gradient of 0.39 dynes per minute (SEM ± 0.08) for cords and a gradient of 0.32 dynes per minute (SEM ± 0.11) for the nodules. There is no significant difference seen between the gradients in any of the cell seeded gels. All of the cell lines investigated here demonstrate a positive response to the reduction in externally applied load, by increasing cell generated force. This follows tensional homeostasis, whereby fibroblasts will establish a tension within the collagen matrix, and act to maintain the level of tension against the opposing influence of mechanical unloading (Brown *et al* 1998). The Dupuytren's derived fibroblasts responses are similar to those for carpal ligament within the remits of this experiment.

In the second to fourth underload periods it is seen that for each cell seeded gel, there is a positive gradient of contraction. There is no significant difference in gradient between the cell lines investigated or between underload periods. Thus after each progressive underload sequence the cellular response is that of an increase in generated force. The progressive loss in tension after each underload does not appear to affect the cellular response given the similarity of the gradient recorded after each sequence; so a step wise decrease in tension seen in 4 underloads, gives a similar contractile response in comparison to a single underload. As before the cellular response in each case always opposes the applied external underloading.

Thus it appears that in all cases both Dupuytren's fibroblasts and carpal ligament derived fibroblasts respond to a reduction in mechanical load, by increasing their cellular contraction. This correlates with previous work on dermal fibroblasts (Eastwood *et al* 1998).

3.26 DISCUSSION

Tensional homeostasis in dermal fibroblasts was first described by Brown *et al* in 1998. The fibroblast response to mechanical stress was deduced by applying precise mechanical loads of between 15 and 120 dynes across a collagen lattice and then observing cellular response to these loads in real time on the culture force monitor. Dermal fibroblasts were seen to increase their contraction in response to underloading (a reduction in externally applied load), reaching a force close to that prior to underloading. By applying external overloading forces, it was seen that a reduction in cell-mediated tension occurred. The cellular response was always in the opposite direction to the previously applied load. This was defined as tensional homeostasis since resident cells appeared to respond by maintaining a constant endogenous matrix tension. It was hypothesised from this work that fibroblasts are able to monitor and regulate endogenous tension in a manner which reduces or minimises the externally applied tensional loading.

Bisson *et al* (2004) applied a series of overloading forces to Dupuytren's fibroblast seeded collagen gels using a similar system to Brown *et al* (1998), and the responses of the resident cells within the collagen matrix were measured in real time. After an overloading force was applied Dupuytren's fibroblasts tended to increase their contraction, rather than reducing it as seen in the case of dermal fibroblasts and carpal ligament fibroblasts. This phenomenon was enhanced by the pre-incubation of cells with TGFβ-1. It was postulated from this that Dupuytren's fibroblasts do not achieve tensional homeostasis as proposed by Brown *et al* (1998), given the increase in contractile force generation after an increase in externally applied load.

It has been demonstrated here, that after application of an underload all cell types tested responded by increasing contraction, and thus the measured force within the system. There was no significant difference seen between nodule, cord or carpal ligament. It can therefore be concluded that no matter which external mechanical stimulus is applied, the response by Dupuytren's fibroblasts remains the same i.e. an increase in cell-generated force.

This study was limited by the fact that the cellular response to each underload period was observed for only 30 minutes. It would be anticipated that in carpal ligament cell lines the increased contraction would eventually plateau to a steady state of force as the cells return to homeostasis. However in Dupuytren's fibroblasts the contraction in response to an underload may be speculated to increase to a level above that measured before external forces were changed, and indeed the force may continue to increase, as it appears that Dupuytren's fibroblasts never reach tensional homeostasis and indeed continue to contract within the time frame investigated here.

It was observed that carpal ligament fibroblasts demonstrated a definite response to a reduction in external load. Previous work (Bisson *et al* 2004) has demonstrated that there was no significant difference in response between the acellular blank collagen gels and carpal ligament seeded gels to an overloading force. At this time it was concluded that this cell type exhibited a low level of responsiveness to mechanical overloading in the

environment and they were hypothesised to be mechano-insensitive. However this work provides evidence to the contrary. Carpal ligament cell lines responded to a reduction in external load, and there is a significant difference between the gradient of contraction calculated for an acellular blank gel in comparison to that for the carpal ligament fibroblast seeded gels, which indicates a cellular response to the change in force once the mechanical properties of the collagen gel alone are subtracted. It can be concluded that this cell type does exhibit some features of tensional homeostasis as shown by its typical plateau type contraction profile, and its response to underload stimuli in a similar manner to dermal fibroblasts (Brown *et al* 1998). However the lack of response to an overloading stimulus as demonstrated by Bisson *et al* (2004) is important to consider. Bison *et al* (2004) hypothesised that this lack of response was due to the low level of force generated by these cells when allowed to contract alone in the absence of stimulation, such that cellular relaxation after a mechanical overload was indistinguishable from the elastic properties of a blank acellular gel.

It may be postulated that the low level of force generated by carpal ligament fibroblasts can explain the lack of response to an overload. A blank acellular gel has been demonstrated to produce a force of 20 dynes on average, whereas for carpal ligament an average force of 50 dynes is generated (Bisson *et al* 2004). It may be speculated that the gels mechanical properties do not change, and that there is a minimal increase in matrix stiffness at this low level of force based on previous work in this area (Eastwood *et al* 1994; Tranquillo 1999). Due to this carpal ligament fibroblasts may be able to perceive by their deformation a reduction in load because they are not "stress-shielded" by a stiffer matrix, and thus respond in a manner similar to dermal fibroblasts by increasing tension. Dupuytren's derived cells generate higher baseline forces within a collagen gel, and will thus cause the gel to become stiffer as it deforms. This has been demonstrated previously using rat tendon fibroblasts within the culture force monitor model (Marenzana *et al* 2004 in press). The fibroblasts will therefore be "stress-shielded" to a greater extent, and this may explain why these cells did not respond to a decrease in load to any greater degree than the carpal ligament fibroblasts.

Another limiting factor in this work and that of Bisson *et al* (2004) is that these mechanical loads were applied to Dupuytren's cells while they were still contracting and not in the plateau phase of the contraction profile. It may be that this contractile response to both overloading and underloading is simply an exaggeration of the events already occurring if the cells were simply exposed to a static load, as occurs within the culture force monitor model, and left to contract as seen earlier in section 3.11-3.16. It would be useful to apply mechanical underloads and overloads to cell seeded collagen gels at the time Dupuytren's fibroblasts reach tensional homeostasis, if in fact this ever occurs. A proposal for future work would be setting up the culture force monitor within a sterile closed environment to allow the measurement of collagen gel contraction to proceed over time courses beyond 48 hours, with the avoidance of bacterial contamination and infection.

These findings are important in several respects. From this and previous work it is postulated that Dupuytren's fibroblasts will contract irrespective of the mechanical load applied. In the case of a reduction in external load however, it does appear that these cells do respond to some mechanical cues in a similar manner to control dermal and carpal ligament fibroblasts. If these findings are combined with the significantly greater contraction and force generation these cells exhibit in comparison to carpal ligament fibroblasts, this goes some way to explaining why progressive digital contracture continues or recurs. It may be speculated that surgical intervention such as fasciotomy will reduce the external load detected by the resident fibroblasts, and they will respond by increasing contraction to return to a level of homeostasis. If the cells' homeostatic equilibrium is set at a very high level, then contraction and contracture are likely to recur. If these cells display equilibrium at a lower level of force, then contracture may be less likely to recur. Similarly overloading forces such as external splintage regimes will result in an increase in cell mediated contraction, leading again to progressive contracture.

3.27 SUMMARY

- Rapid underloading of pre-loaded acellular gels results in a very gradual increase in force over 30 minutes due to the gels' visco-elastic properties.

- Underloading of control carpal ligament fibroblasts results in a slow increase in cell generated force over a 30 minute period which corresponds with the theory of tensional homeostasis in dermal fibroblasts proposed by Brown *et al* (1998)

- Underloading of Dupuytren's fibroblasts results in a response not significantly different to that of carpal ligament fibroblasts. Combining this with previous work (Bisson *et al* 2004) it is postulated that Dupuytren's fibroblasts will contract irrespective of the mechanical stimulus. In the case of a reduction in external load however, it does appear that these cells will respond to this mechanical cue in a similar manner to control dermal and carpal ligament fibroblasts.

In conclusion, Dupuytren's fibroblasts will contract in response to a decrease in the externally applied load in a manner similar to control dermal and carpal ligament fibroblasts. Perhaps *in-vivo,* it can be speculated that an external extending force applied to the finger will be transmitted to the cells within the matrix of the palmar fascia, and this will result in cellular contraction, rather than a reduction in cell mediated tension in Dupuytren's disease. A flexing force will tend to take load off the palmar fascia, and it is speculated that this may have little effect on disease progression or even disease induction as both Dupuytren's and control palmar fascia responded to this type of stimulus by contraction.

3.3 THE CELLULAR MORPHOLOGY OF DUPUYTREN'S FIBROBLASTS WITHIN COLLAGEN GELS

3.31 INTRODUCTION

The observation of cell morphology within three dimensional gels has allowed a much greater understanding of the relationship between cellular orientation and contractile function (Bellows *et al* 1981; Ehrlich and Rajaratnam 1990; Eastwood *et al* 1996; 1998; Vaughan *et al* 2000). Using toluidine blue staining to observe overall cellular morphology it has been observed that fibroblasts change from a rounded appearance when first seeded into a gel, to the extension of cell processes as the cell attaches and contraction begins, and proceeding to alignment of the cells along the lines of isometric tension within the gel in a culture force monitor model (Eastwood *et al* 1996; 1998). It was postulated that the early generation of force by fibroblasts was due to the extension of cell processes, attachment to the matrix and tractional remodelling (Eastwood *et al* 1996).

Within a dermal fibroblast seeded three–dimensional collagen gel as used here previous work (Eastwood *et al* 1998) has demonstrated 2 zones where the measured force is different (figure 3.31). The delta zone has minimal strain due to the stiffness imparted into the collagen gel by its proximity to the rigid floatation bars. The remainder of the gel has a high strain gradient, aligned along the gel's long axis. Morphologically it was seen that cells were aligned with bipolar morphology along the gel's long axis, with the exception of the delta zone where the cells were non-aligned with a mixture of stellate and bipolar morphology. This work indicated that fibroblasts are sensitive to mechanical load and will orientate in a manner to reduce the perceived strain across the cell, thus the long bipolar morphology parallel to the principal strain, whereas in areas with no strain the cells are randomly orientated.

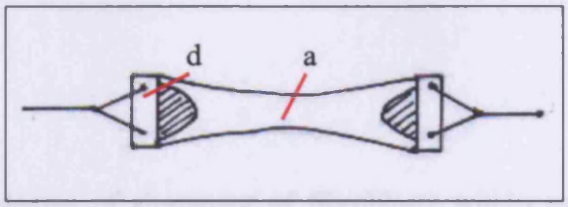


Figure 3.31: - The delta (d) and aligned (a) zones of a three dimensional collagen gel. Note the shaded areas of the delta zone which are protected from strain by the attached floatation bars (Derived from Eastwood *et al* 1998).

Other methods have been employed to look at fibroblast orientation, including immunohistochemical staining for α-smooth muscle actin (Vaughan *et al* 2000; Tomasek *at al* 2002), and fluorescent staining of actin using phalloidin (Ehrlich and Rajaratnam 1990; Rayan and Tomasek 1994; Tarpila *et al* 1996). Recently a novel method using time-lapse photography has been employed to measure the relationship between cell traction and migration (Shreiber *et al* 2003) in mechanically strained collagen gels.

Bisson (MD thesis 2003) used both immunohistochemical staining for α-smooth muscle actin, and toluidine blue staining to measure cell alignment, and postulated that immunohistochemical stains gave better visualization of alignment. Other work has looked at the quantification of cellular alignment. Umeno and Ueno (2002) measured cell orientation caused by magnetic fields, and composed a scale of investigated cell orientation related to the mean total orientation of the cells. Both Bisson (2003) and Harding *et al* (2000) utilised the technique of constructing a line through bipolar fibroblasts and measuring the deviation of this line from the long axis of the gel in Bisson's case and fibronectin cables in Harding's case.

3.32 AIMS

- To quantify the degree of alignment of fibroblasts within collagen gels following static loading and underloading in order to compare cell type.

- To relate changes in cell morphology to molecular changes in the same cells as shown in the next chapter.

3.33 METHODS

At the conclusion of the experimental runs on the culture force monitor, the collagen gels were processed for light microscopy as detailed in section 2.4 (*Materials and Methods*). A combination of both staining with toluidine blue, and immuno-histochemical staining of alpha smooth muscle actin was undertaken for each gel. The degree of alignment was calculated by measuring the mean angle of deviation from the long axis of the collagen gel of the bipolar fibroblasts that were in focus in a 2-D micrograph at 200 times magnification (for n = 4 statically loaded gels, and n = 4-6 underloaded gels; all separate cell lines). The results were compared and statistical analysis performed using a student's t-test (Sigma Stat, Jandel Corps).

3.34 RESULTS

The following pages demonstrate representative examples of micrographs of the aligned zone from the three cell types under investigation. Both staining techniques for each specimen are shown (figures 3.32-7). The long axis of the three dimensional collagen gel in all cases lies across the page (or at 0°).

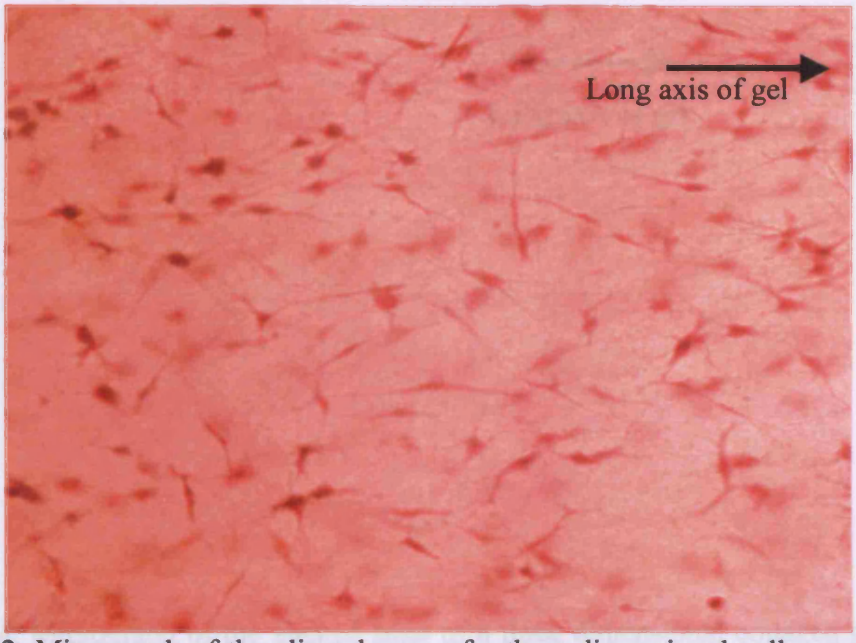


Figure 3.32: Micrograph of the aligned zone of a three dimensional collagen gel seeded with carpal ligament fibroblasts (x 200 magnification). At the conclusion of the experiment on the culture force monitor the gel was fixed in 10% formal saline, and then stained with 1% toluidine blue. Note random cell alignment.

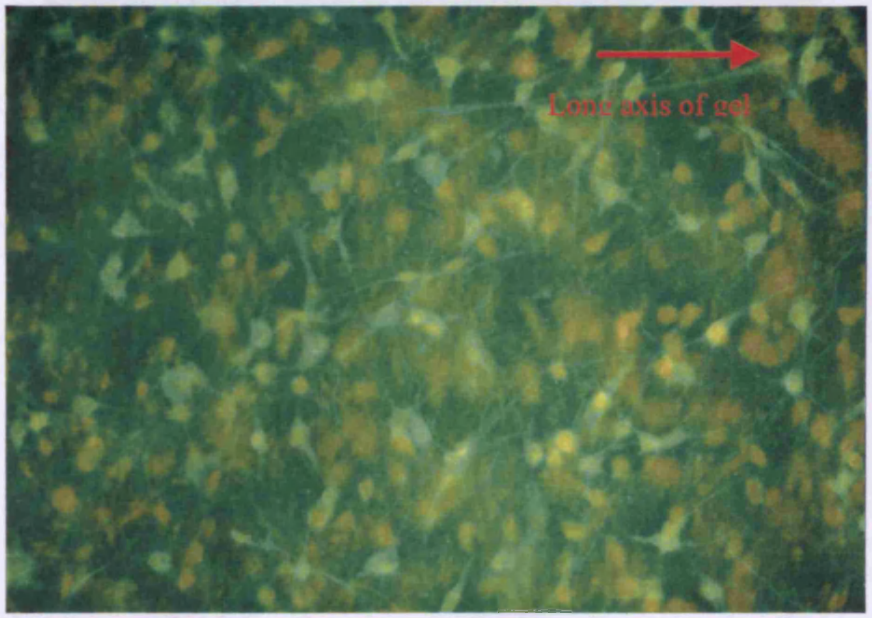


Figure 3.33: Micrograph of the aligned zone of a three dimensional collagen gel seeded with carpal ligament fibroblasts (x 200 magnification). At the conclusion of the experimental run on the culture force monitor the gel was fixed in 10% formal saline, and then stained immunohistochemically for alpha smooth muscle actin. Note random cell alignment.

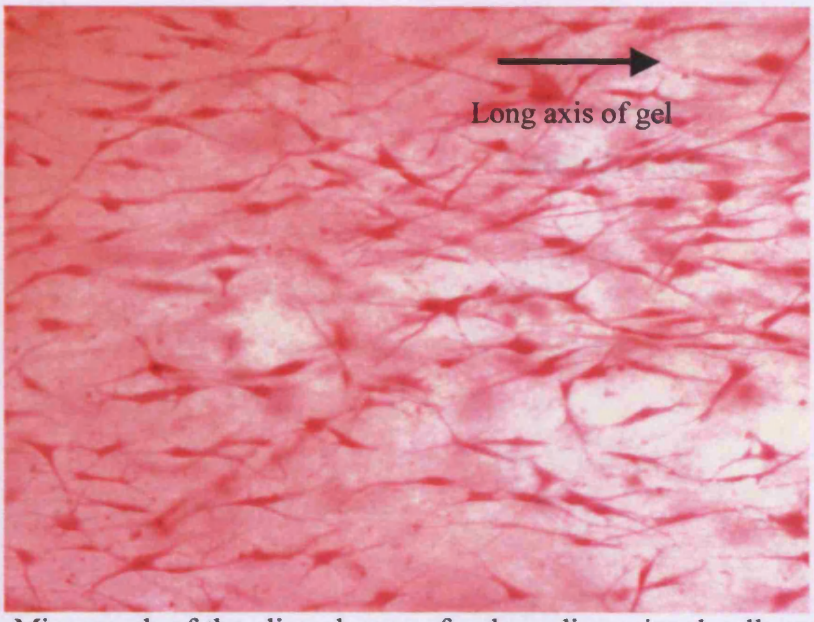


Figure 3.34: Micrograph of the aligned zone of a three dimensional collagen gel seeded with Dupuytren's nodule fibroblasts (x 200 magnification). At the conclusion of the experimental run on the culture force monitor the gel was fixed in 10% formal saline, and then stained with 1% toluidine blue. Note alignment of cells along the long axis of the gel.

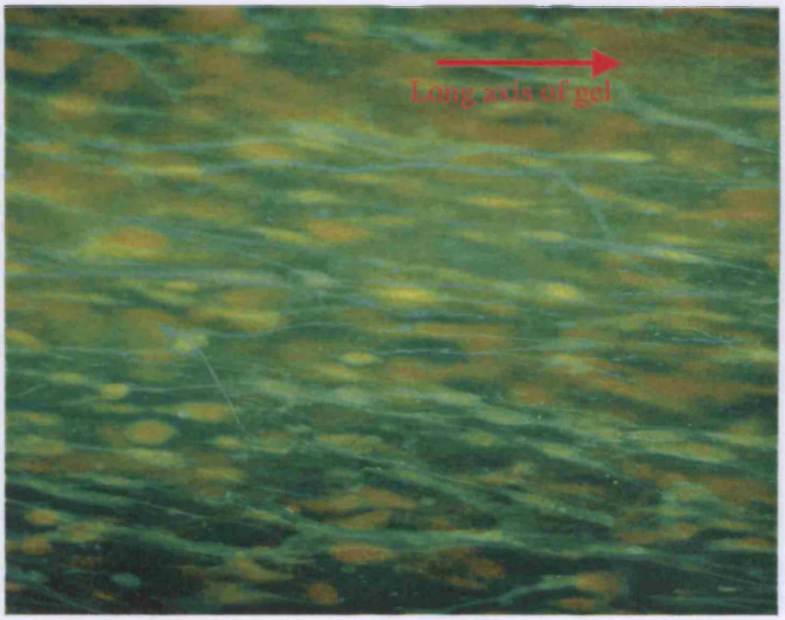


Figure 3.35: Micrograph of the aligned zone of a three dimensional collagen gel seeded with Dupuytren's nodule fibroblasts (x 200 magnification). At the conclusion of the experimental run on the culture force monitor the gel was fixed in 10% formal saline, and then stained immunohistochemically for alpha smooth muscle actin. Note alignment of cells along the long axis of the gel.

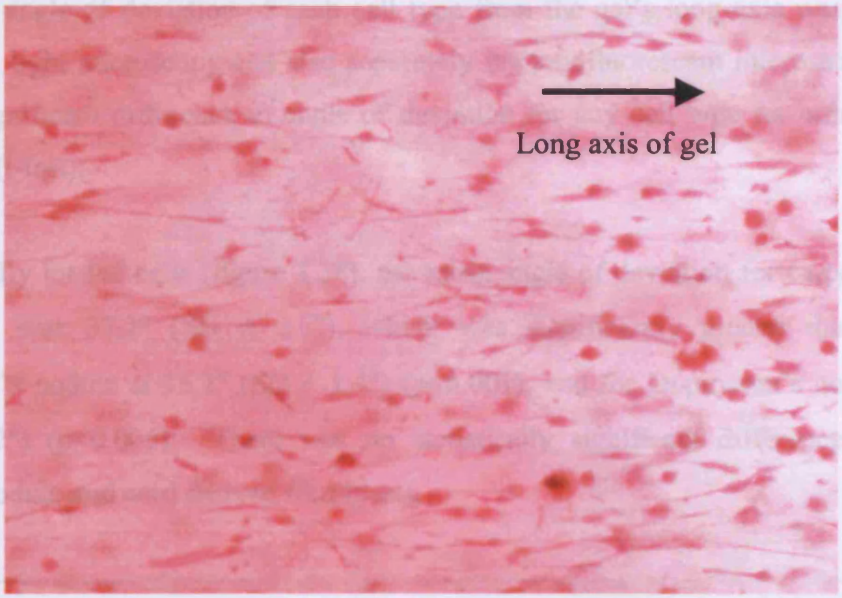


Figure 3.36: Micrograph of the aligned zone of a three dimensional collagen gel seeded with Dupuytren's cord fibroblasts (x 200 magnification). At the conclusion of the experimental run on the culture force monitor the gel was fixed in 10% formal saline, and then stained with 1% toluidine blue. Note alignment of cells along the long axis of the gel.

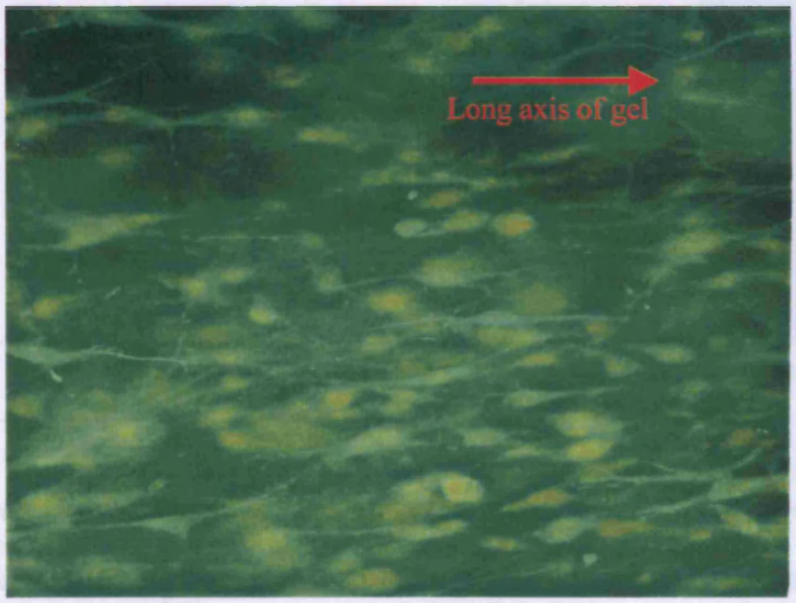


Figure 3.37: Micrograph of a the aligned zone of three dimensional collagen gel seeded with Dupuytren's cord fibroblasts (x 200 magnification). At the conclusion of the experimental run on the culture force monitor the gel was fixed in 10% formal saline, and then stained immunohistochemically for alpha smooth muscle actin. Note alignment of cells along the long axis of the gel.

The mean angle of deviation of each cell type from the gel's long axis was calculated using both light microscopy and also separately immunofluorescent microscopy. There was no significant difference in angle of deviation for any cell type for either staining technique (t-test).

For statically loaded cells (figure 3.38), the mean angle of deviation for carpal ligament fibroblasts was 37.2° (SD ± 2.0°), which was significantly greater than that for Dupuytren's nodule at 16.2° (SD ± 1.4°) ($p<0.001$), and for Dupuytren's cord at 22.8° (SD ± 2.2°) ($p<0.005$). There was no statistically significant difference in angles between nodule and cord derived fibroblasts.

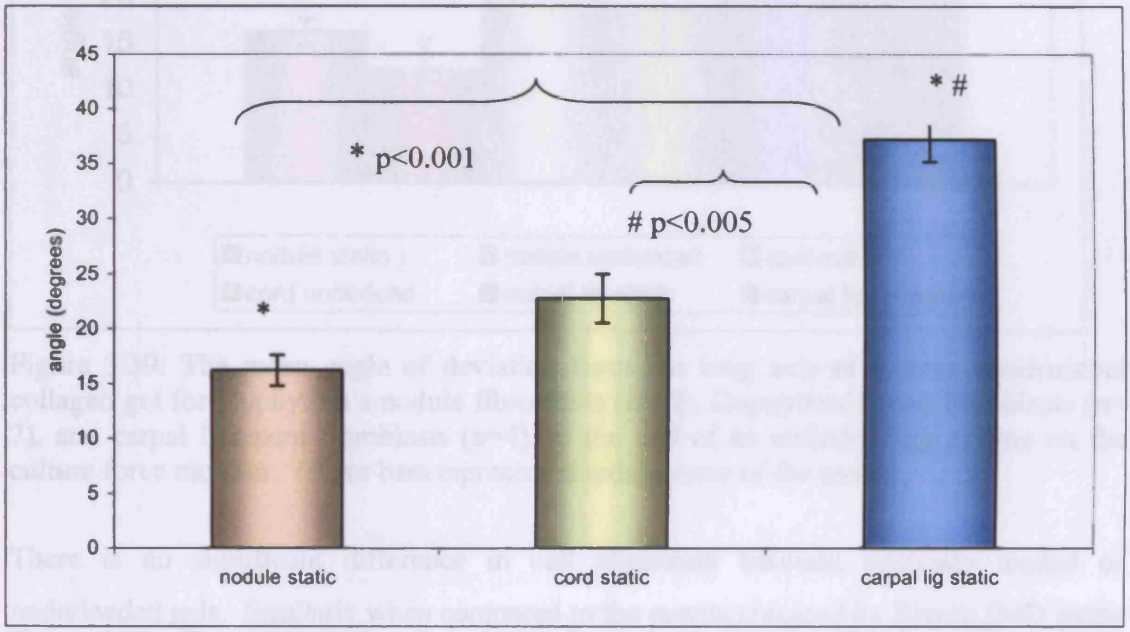


Figure 3.38: The mean angle of deviation from the long axis of a three dimensional collagen gel for Dupuytren's nodule fibroblasts (n= 4), Dupuytren's cord fibroblasts (n= 4), and carpal ligament fibroblasts (n= 4), at the end of a static loading regime on the culture force monitor. (Error bars represent standard error of the mean). * indicates a statistically significant difference ($p<0.001$) for the angle of deviation between nodule and cord, and for carpal ligament and nodule. # indicates a statistically significant difference ($p<0.005$) for the angle of deviation between carpal ligament and cord.

For underloaded gels, the mean angle of deviation of carpal ligament fibroblasts was 32.2° (SD ± 7.7°), and this was significantly greater than that of Dupuytren's nodule at 12.1° (SD ± 3.5°) ($p<0.026$), although not significantly different from that of Dupuytren's cords that displayed an angle of deviation of 18.9° (SD ± 4.9°). (figure 3.39)

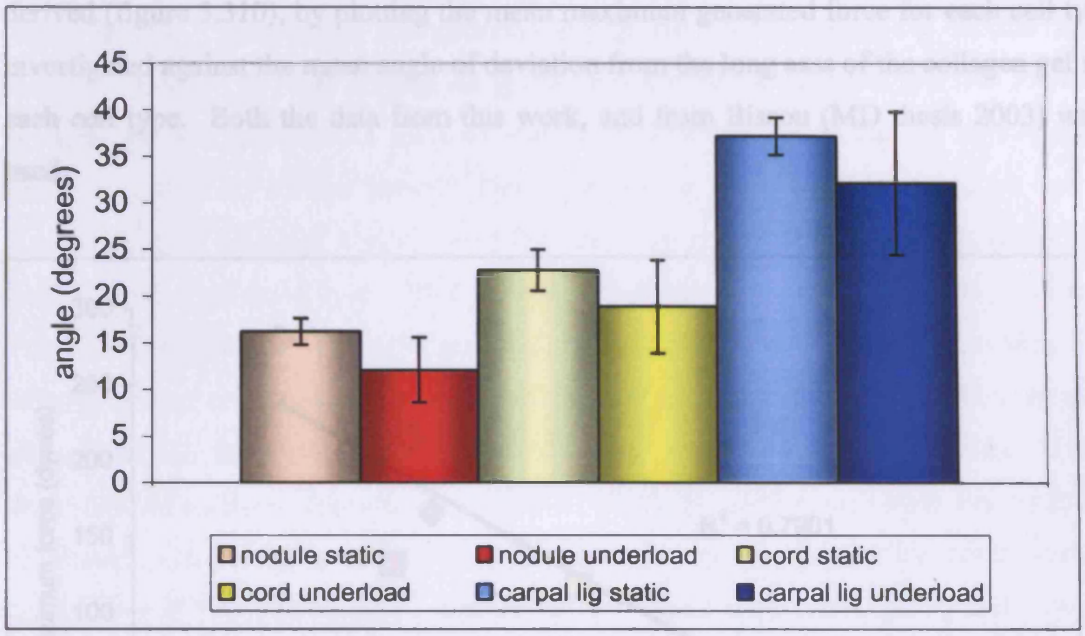


Figure 3.39: The mean angle of deviation from the long axis of a three dimensional collagen gel for Dupuytren's nodule fibroblasts (n= 7), Dupuytren's cord fibroblasts (n= 7), and carpal ligament fibroblasts (n=4), at the end of an underloading regime on the culture force monitor. (Error bars represent standard error of the mean)

There is no significant difference in cell alignment between statically loaded or underloaded gels. Similarly when compared to the results obtained by Bisson (MD thesis 2003), there is no significant difference in cell alignment between statically loaded, underloaded or overloaded gels.

It must be noted that when these cells were stimulated with TGF-β, the mean angle of deviation from the long axis decreased significantly in each cell type investigated compared to un-stimulated values (Bisson MD thesis 2003). For nodule fibroblasts this was 7.8° (SD ± 1.9°), for cord it was 14.7° (SD ± 6.1°), and for carpal ligament this

dropped to 15.8° (SD ± 6.5°). All of these cell types generated a significant increase in contractile force after stimulation with TGF-β1, in comparison to no stimulation (Bisson MD thesis 2003), which corresponds with the increasing cell alignment along the gel's lines of principle strain.

From this a graph of fibroblast alignment against maximum generated force has been derived (figure 3.310), by plotting the mean maximum generated force for each cell type investigated against the mean angle of deviation from the long axis of the collagen gel for each cell type. Both the data from this work, and from Bisson (MD thesis 2003) were used.

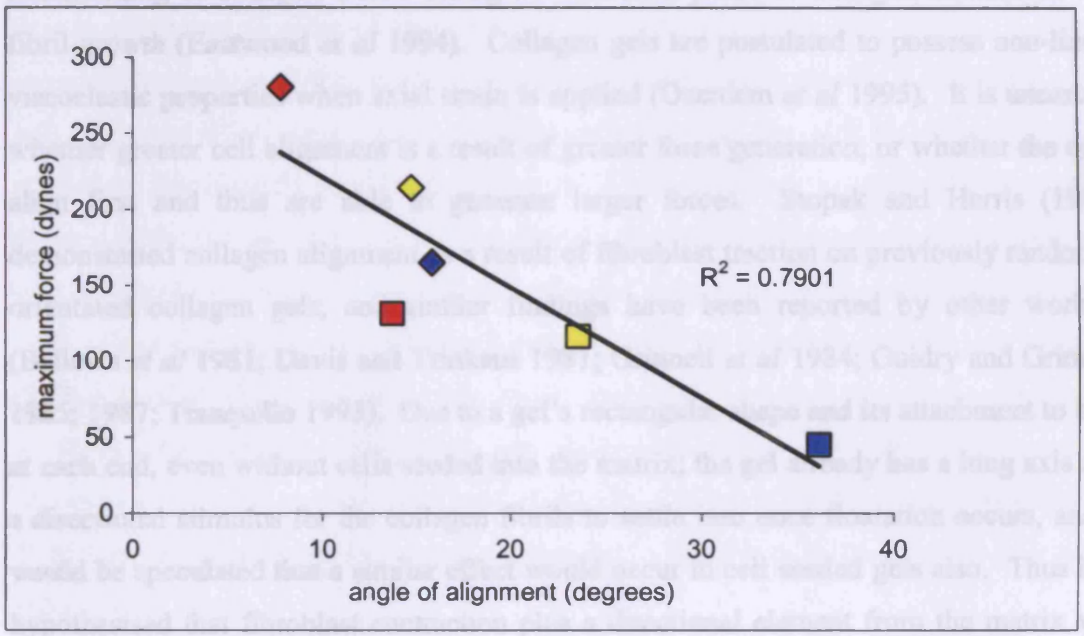


Figure 3.310: - Correlation curve of maximum generated force vs. fibroblast alignment with and without TGF-beta stimulation. (Key : ■ square box represents fibroblasts without stimulation; ♦ diamond represents fibroblasts stimulated with TGF beta. ● Dupuytren's nodule; ● Dupuytren's cord; ● carpal ligament

It may be observed from this graph that cell alignment correlates in a linear manner with the maximum force generated by a population of fibroblasts. As force increases, cell alignment increases also. It is postulated that when force is high, fibroblasts will align

parallel to the direction of force in order to minimise the load across the cell, whereas at lower forces alignment is less critical.

3.35 DISCUSSION

From this analysis fibroblast alignment corresponds with overall force generation in a linear fashion. Fibroblasts that generate the greatest force within the culture force monitor appear to be most aligned along the long axis of the collagen gel. An acellular collagen gel will generated a force of around 20 dynes if left within the culture force monitor system for several hours. This phenomenon was hypothesised to be due to intermeshing of collagen fibrils during the accretion phase of collagen maturation and fibril growth (Eastwood *et al* 1994). Collagen gels are postulated to possess non-linear viscoelastic properties when axial strain is applied (Ozerdem *et al* 1995). It is uncertain whether greater cell alignment is a result of greater force generation, or whether the cells align first and thus are able to generate larger forces. Stopak and Harris (1982) demonstrated collagen alignment as a result of fibroblast traction on previously randomly orientated collagen gels, and similar findings have been reported by other workers (Bellows *et al* 1981; Davis and Trinkaus 1981; Grinnell *et al* 1984; Guidry and Grinnell 1985; 1987; Tranquillo 1995). Due to a gel's rectangular shape and its attachment to bars at each end, even without cells seeded into the matrix, the gel already has a long axis and a directional stimulus for the collagen fibrils to settle into once floatation occurs, and it would be speculated that a similar effect would occur in cell seeded gels also. Thus it is hypothesised that fibroblast contraction plus a directional element from the matrix will cause fibroblast alignment.

There was a linear relationship between generated force and alignment, not only for Dupuytren's derived cells but also for carpal ligament cells stimulated with TGF-beta. Eastwood *et al* (1998) proposed that fibroblasts are able to perceive force within a matrix, and both orient themselves and adjust their own forces to minimise the load across the cell. This has been referred to as "stress-shielding" (Eastwood *et al* 1998). Fibroblasts have also been demonstrated to align by contact guidance of cells along an orientated

matrix (Guido and Tranquillo 1993; Curtis and Wilkinson 1997). Petroll *et al* (2004) used the technique of reflective light confocal imaging to visualise live cells and their surrounding fibrillar collagen matrix. Focal adhesions were orientated parallel to the direction of collagen fibril alignment in front of the cell. Mechanical stimulation rather than contact guidance was proposed to be the strongest guide for cells from recent work by Mudera *et al* (2000) who subjected fibroblasts to opposing dual cues.

It is hypothesised that those cells that are more contractile would be able to generate initially greater force, thus ensuring even better alignment of collagen fibrils, concomitant with bipolarity of cell shape. For carpal ligament derived cells generated force was only 45dynes, with average cellular alignment of 37° along the gel's long axis. It is possible that in view of these cells' low contractile ability that they are unable to orientate themselves or the surrounding collagen matrix along the longitudinal axis of the gel. However when these cells were stimulated with TGF-β1, both force generation and alignment significantly increased, suggesting a role for force in providing orientation. Several theories may explain why Dupuytren's fibroblasts align to a greater degree than control fibroblasts within a rectangular collagen gel. It has been postulated that these cells adhere or attach to the matrix to a greater extent than control cells e.g. via integrins (Riikonen *et al* 1995). Certainly it is known that TGF-β appears to increase the number of fibronexus adhesion complexes, and fibronectin fibrils for matrix attachment in Dupuytren's fibroblasts (Vaughan *et al* 2000). In addition it has been demonstrated that TGF-beta II receptors are increased in Dupuytren's fibroblasts in comparison to controls (Kloen *et al* 1995).

Mechanical stimulation of fibroblasts by underloading appeared to make no significant difference to cell orientation. There was a trend for alignment to increase after an underload all cell types investigated but this difference was not significant. As discussed previously (section 3.2), an underloading force applied across a cell seeded collagen gel resulted in an increase in cell contraction. Thus it may be hypothesised that an underloading force will also result in increasing cell alignment. The reduction in mechanical load by each underload was 30 dynes, and this may reflect the non-significant

changes seen in cell orientation. Bisson's work (MD thesis 2003) demonstrated no significant change in alignment between statically loaded cells and overloaded cells. It is hypothesised that this is because of continued contraction by Dupuytren's derived cells in response to an overloading stimulus.

It has been observed previously that a change in cellular morphology is required to alter gene expression (Benya and Shaffer 1982; Spiegelman *et al* 1982; 1983; Lee *et al* 1984; Zanetti and Solursh 1984). Unemori and Werb (1986) have shown that actin cytoskeletal changes within collagen gels are associated with an increase in procollagenase expression, probably at a pretranslational level. Similarly work by Mudera *et al* 2000 has shown an upregulation of matrix metalloproteinase activity in fibroblasts within a collagen gel that were exposed to conflicting mechanical cues and are thus unable to align. This is supported by a study on human tendon fibroblasts that demonstrated that fibroblast responses to mechanical stretching depended on cell orientation to the stretching direction (Wang *et al* 2004).

3.36 SUMMARY

- Fibroblast alignment correlates with maximum generated force in a linear fashion.
- Dupuytren's nodule derived fibroblasts exhibited greatest alignment along the gel's long axis, which was significantly greater than that of cord or carpal ligament derived fibroblasts
- Mechanical stimulation of fibroblasts by an underloading regime resulted in no significant changes in cell orientation

The next step in this research was to investigate the expression of enzymes important in the turnover of the extracellular matrix, the matrix metalloproteinases in response to mechanical stimulation. In addition gene expression of collagen deposition was also assessed.

CHAPTER 4

GENE EXPRESSION OF THE MATRIX METALLOPROTEINASES AND COLLAGEN BY DUPUYTREN'S FIBROBLASTS EXPOSED TO MECHANICAL STIMULATION

4.1 GENE EXPRESSION OF THE MATRIX METALLOPROTEINASES BY DUPUYTREN'S FIBROBLASTS EXPOSED TO MECHANICAL STIMULATION

4.11 INTRODUCTION

As mentioned previously cellular responses to alterations in applied mechanical load include changes in matrix synthesis, and the release of regulatory enzymes including the matrix metalloproteinases (Lambert *et al* 2001). Previous observations have shown that Dupuytren's fibroblasts display an abnormal contractile response to overloading mechanical stimuli (Bisson *et al* 2004). Application of underloading forces as described earlier has also shown an increase in contraction, so that it is postulated that any mechanical stimulus applied to Dupuytren's fibroblasts results in a contractile response. There is much information to be gained regarding the molecular events taking place during this time, in particular regarding expression of the matrix metalloproteinases and their tissue inhibitors. As described earlier (*Review of the Literature, sections 1.11, 1.12, and 1.17)*, the MMPs function in the degradation of the extracellular matrix as part of controlled matrix turnover. In normal physiological conditions expression and release of these enzymes is tightly regulated, but a loss of control may result in pathological fibrosis as seen in Dupuytren's disease. Alterations in the mechanical environment that cells reside in can also lead to changes in the production of these enzymes, both in normal and pathological conditions. Is Dupuytren's disease a result of an imbalance of these enzymes, and does mechanical load also play an exacerbating role?

MMP expression in Dupuytren's disease is an area that has been under investigated previously, the majority of work having focused on other tissues and cell types. Ulrich *et al* (2003) have looked at the levels of MMPs and their tissue inhibitors in the sera of patients with Dupuytren's disease, finding an imbalance in the ratio between the two, with an increase in TIMP-1 levels in Dupuytren's sufferers. However a limitation with

this work was that patient samples were not obtained in a controlled environment, so only an estimation of MMP levels could be deduced, in view of the fact that many external patient factors may affect MMP release, including anaesthesia and perioperative trauma. No research appears to have directly measured MMP expression in a series of Dupuytren's fibroblast cell cultures, so this is an area where there are some gaps in knowledge.

Research has investigated mechanical stimulation and MMP expression in Dupuytren's tissue (Bailey *et al* 1994; Tarlton *et al* 1998), finding that an increase in mechanical load induces increased expression of MMP-2 and MMP-9. It was hypothesized that this may be a cause of collagen breakdown with loss of mechanical strength of a contracture for example as seen in the continuous elongation technique (Messina and Messina 1991). The majority of researchers within the literature have employed cyclical loading regimes to measure responses to mechanical load (Yang *et al* 1998; Seliktar et al 2001; Sun and Yokota 2002; Asanuma *et al* 2003; Berry *et al* 2003), although Mudera *et al* (2000), and Prajapati *et al* (2000a and b), have both measured MMP expression or release using a culture force monitor model in dermal fibroblasts, both of these workers finding an upregulation of MMP-2 and MMP-9 in response to mechanical loading.

4.12 AIMS

- To investigate the baseline expression of MMPs and TIMPs by Dupuytren's fibroblasts and carpal ligament fibroblasts statically loaded on the culture force monitor
- To determine the modulation of these MMPs and TIMPs by underloading, and overloading regimes

4.13 HYPOTHESES

- Dupuytren's derived fibroblasts will display a greater baseline expression of MMPs and TIMPs in comparison to carpal ligament derived fibroblasts
- MMP and TIMP expression will decrease with mechanical underloading in Dupuytren's fibroblasts and in carpal ligament derived fibroblasts
- MMP and TIMP expression will increase with mechanical overloading in Dupuytren's fibroblasts but not in control carpal ligament derived cells.

4.14 MATERIALS AND METHODS

Fibroblast seeded collagen gels were set up on the culture force monitor and allowed to contract over a 20 hour period (*Material and Methods 2.3*). At this time point they were either allowed to contract for a further 4 hours (static load), or exposed to a series of uniaxial underloads, or uniaxial overloads (*Materials and Methods 2.3.3, 2.3.5-6*). After removal from the culture force monitor (*Materials and Methods 2.3.9*) the gels were snap frozen in liquid nitrogen and stored at -80°C in a freezer immediately until being used for RNA extraction as outlined previously in section 2.6-2.8 (*Materials and Methods)*. There were 3 groups composed of Dupuytren's nodule, Dupuytren's cord and carpal ligament derived gels. Each group was subdivided into statically loaded gels (n=4-5), overloaded gels (n=4-6), and underloaded gels (n=4-6). A change in MMP expression as a ratio compared to GAPDH expression was quantified using RT-PCR (section 2.8).

4.15 RESULTS

4.2 THE EXPRESSION OF MMPS BY DUPUYTREN'S FIBROBLASTS EXPOSED TO STATIC LOADS

Fibroblasts seeded within a longitudinally orientated collagen gel were left to contract within the culture force monitor. No external stimuli were applied across the gel, but by virtue of the gel's attachment to the fixed point and force transducer the gel can be said to have a static load applied to it (as discussed in section 1.14 *Introduction*). From the previous section it was demonstrated that Dupuytren's derived cells demonstrate an increased contractile ability, compared to control carpal ligament derived cells, with greater force generation and alignment over a 24-hour period.

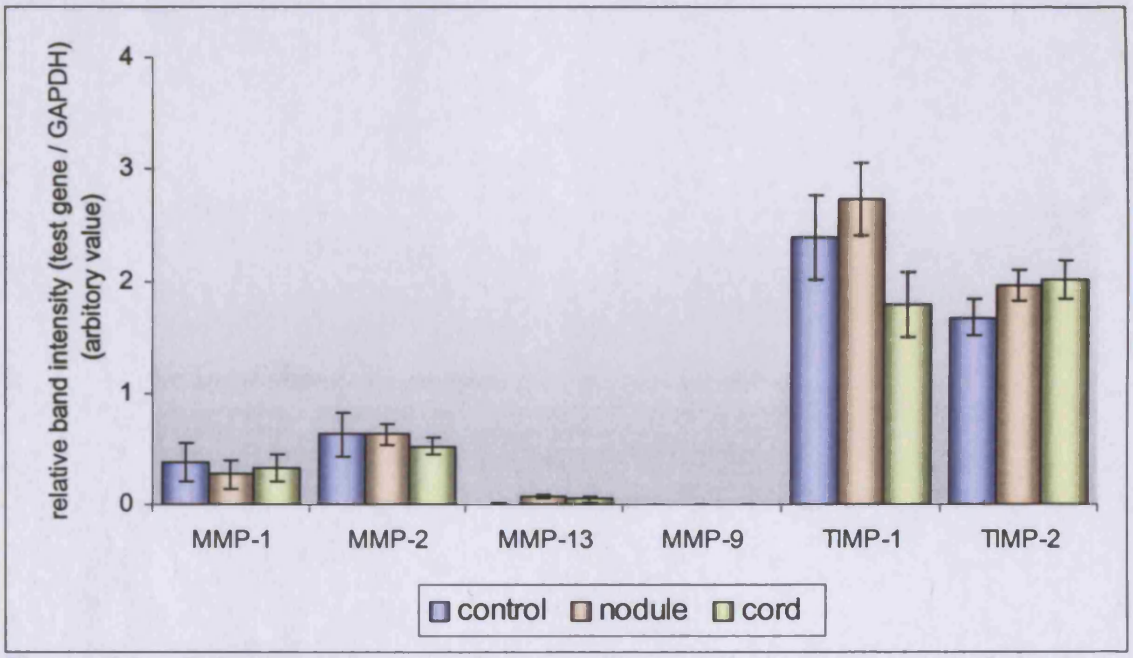


Figure 4.1:- Relative expression of the matrix metalloproteinases and their tissue inhibitors in n=5 control carpal ligament, Dupuytren's nodule and Dupuytren's cord fibroblasts exposed to static load only. Transcription of each gene is expressed as a ratio of the band intensities of the test gene / GAPDH. Each data point represents the mean band intensity. Error bars represent standard errors of the mean.

From the histogram (figure 4.1) it can be seen that expression of MMP-1 and MMP-2 was low in comparison to the housekeeping gene (*For densitometric pictoral demonstration of each PCR product analysed here please see Appendix X (a-h) for details).* (A ratio of 1 indicates that expression of the investigated gene is the same as that of the internal housekeeping gene GAPDH). For MMP-1 absorbance values were 0.375 (SEM ± 0.17) for carpal ligament, 0.274 (SEM ± 0.13) for nodule, and 0.322 (SEM ± 0.12) for cords. Values for MMP-2 ranged from 0.516 to 0.63 (SEM ± 0.1), with no significant difference in expression between cell lines. In contrast expression of the natural inhibitors of matrix metalloproteinases, TIMP-1, and TIMP-2 was significantly greater ($p<0.001$) than for the MMPs in each cell type. For TIMP-1, absorbance values were 2.39 (SEM ± 0.4) for carpal ligament, 2.73 (SEM ± 0.33) for nodule and 1.78 (SEM ± 0.28) for cord. Values for TIMP-2 were 1.67 (SEM ± 0.15) for carpal ligament, 1.95 (SEM ± 0.13) for nodule and 2.00 (SEM ± 0.17) for cords.

It can be seen that expression of MMP-13, and MMP-9 was minimal in all cell lines investigated with relative absorbance values of less than 0.1. However despite low levels of expression of MMP-13 in all cases there was a significantly greater baseline expression in nodule derived fibroblasts ($p<0.05$) compared to controls (seen in greater detail in figure 4.4 and 4.5). For nodule this absorbance value was 0.067 (SEM ± 0.016), compared to 0.008 (SEM ± 0.004) for controls and 0.049 (SEM ± 0.02) for Dupuytren's cord

Thus it appears that in general, in both diseased and normal palmar fascia fibroblasts, expression of TIMPs is higher than that of the matrix metalloproteinases, which may be extrapolated to reflect little matrix turnover.

4.21 DISCUSSION

Few workers have investigated gene expression of MMPs in Dupuytren's disease or in palmar fascia. Ulrich *et al* (2003) measured the serum concentrations of TIMP-1, and 2,

and MMPs- 1,2, and 9 in patients with either active stage disease or residual disease as defined by Luck (1959). These were compared with serum concentrations from patients undergoing routine carpal tunnel surgery. Thus patient populations in comparison to this study are very similar. Serum TIMP-1 concentrations were found to be significantly higher in Dupuytren's patients in comparison to controls, with significantly higher levels seen in those patients with active disease. There was no difference in serum TIMP-2 concentrations between groups. Similarly there was no difference in MMP levels between groups. Levels of TIMP-1 were greater than those of the MMPs in all cases, although TIMP-2 levels were similar to those of the MMPs. The same workers have demonstrated elevated TIMP-1 levels in pathological dermal scars also (Ulrich *et al* 2003). They hypothesized that an elevated systemic TIMP level may result in lack of degradation of the extracellular matrix components, and thus progressive fibrosis.

Gene expression was measured in a very controlled environment in this *in-vitro* collagen gel model, whereas for Ulrich *et al*'s work (2003), enzyme levels were taken directly from patients' sera with a large degree of external factors being involved such as operative stress, anaesthesia, and hormonal factors. It has been recorded (Herouy *et al* 2001; Belotti *et al* 1999; Parsons *et al* 1997; Ravanti and Kahari 2000), that a large number of stimuli including mechanical load and growth factors may affect MMP expression and production, and this is a limitation of Ulrich's (2003) study. In view of the different models used, the results reported here are not comparable with those of Ulrich *et al* (2003).

In both normal palmar fascia and Dupuytren's derived cells baseline expression of TIMP-1 and 2 were elevated in comparison to the MMPs with no significant difference between cell origin. We can postulate that in cells exposed to static loads where no external stimulation has been applied, a low level of expression of MMPs may indicate that matrix turnover is also low. When the extracellular matrix is in a steady state there is an equal balance between matrix degradation and production. Perhaps higher levels of TIMPs are required to maintain an equilibrium, and this would reflect their increased expression in this unstimulated state. In non-wounded skin there are very low measured

basal levels of MMP-1, MMP-9 and TIMP-1 suggesting minimal roles in normal skin maintenance (Soo *et al* 2000).

Thus it can be concluded that the differences seen in the Dupuytren's fibroblast cellular contraction profiles in comparison to control carpal ligament likely to be unrelated to MMP expression, given the similar levels of gene expression between Dupuytren's and control carpal ligament fibroblasts. The baseline enhanced levels of TIMPs in all cases investigated suggests that matrix turnover is unlikely to be occurring within this experimental model.

4.3 THE EXPRESSION OF MMPS AND TIMPS IN DUPUYTREN'S FIBROBLASTS EXPOSED TO MECHANICAL STIMULATION

4.31 UNDERLOADS

Following the application of a series of 4 uniaxial underloads to the collagen gels, the changes in gene expression were measured and documented below. In view of the very low levels of expression of MMP-13 and MMP-9 by all cell types these results will be displayed using separate histograms in order that a scale of zero to one on the y axis for relative band intensity may be used.

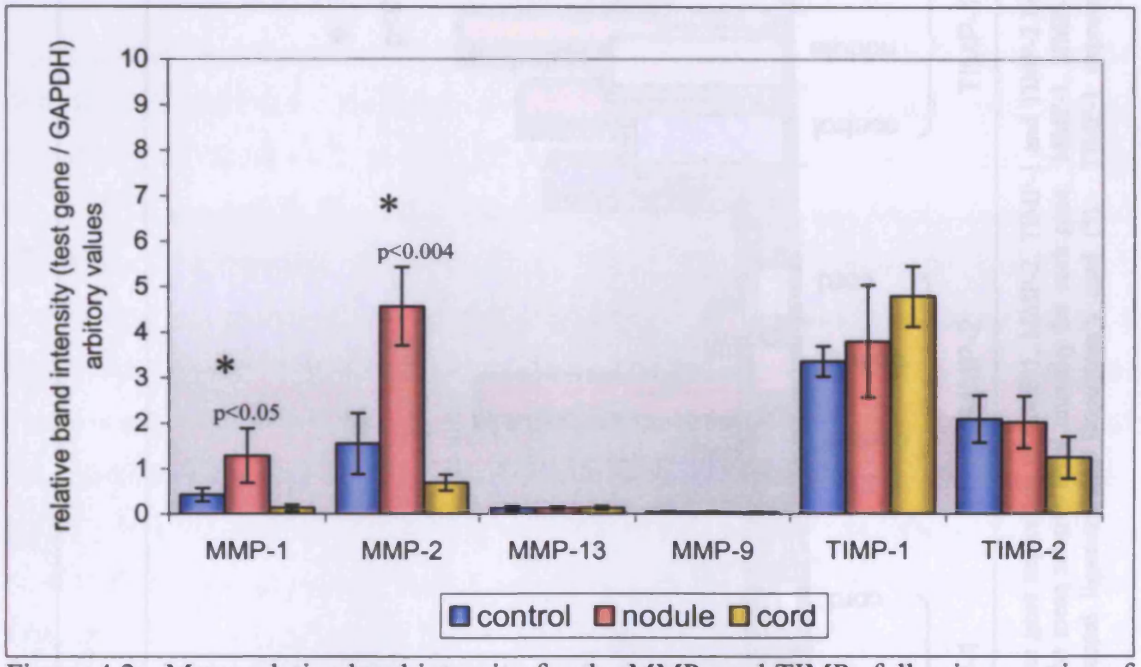


Figure 4.2:- Mean relative band intensity for the MMPs and TIMPs following a series of uniaxial underloads for n=4 carpal ligament, n=4 Dupuytren's nodule and n=6 Dupuytren's cord derived fibroblasts. Error bars represent standard errors of the mean. MMP-1 and MMP-2 expression is significantly greater in Dupuytren's nodule in comparison to control carpal ligament and Dupuytren's cord (*) (Figure 5 overleaf gives a diagrammatic representation of the comparison between static loads and underload using the same data)

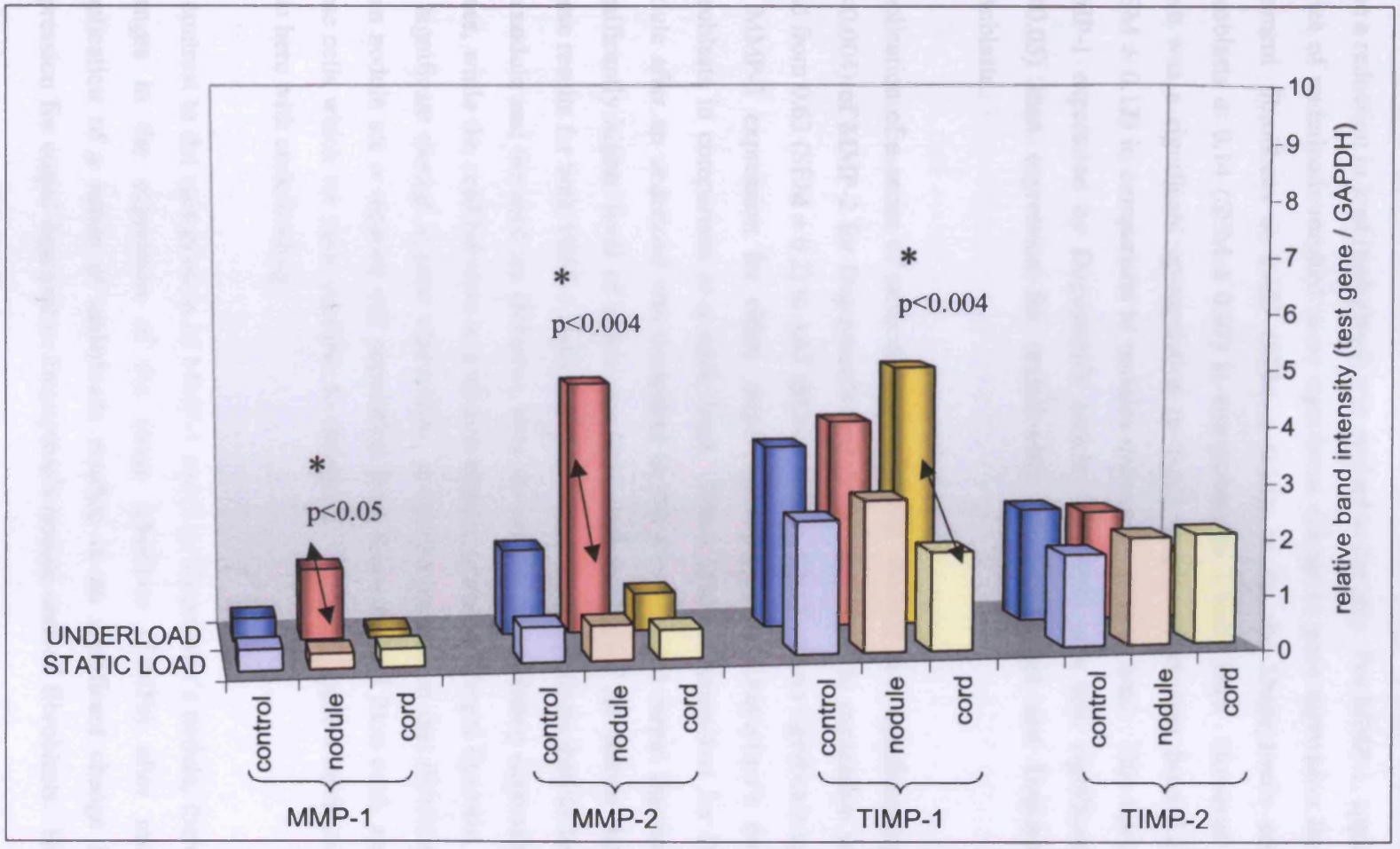


Figure 4.3:- Histogram to compare the gene expression of MMP-1, MMP-2, TIMP-1 and TIMP-2 between 2 different mechanical stimuli: static loading, and underloading. Each bar represents the mean relative band intensity for each gene. MMP-1, MMP-2 gene expression is significantly greater for Dupuytren's nodule in comparison to control carpal ligament and Dupuytren's cord (*). TIMP-1 expression is significantly greater for Dupuytren's cord (*).

From the previous 2 histograms (figure 4.2 and figure 4.3), it can be seen that an external mechanical load applied to the fibroblasts results in a change in gene expression. In this case a reduction in load (underload) was applied to the gels. For MMP-1, application of a series of underloads resulted in no significant change in gene expression for the carpal ligament fibroblasts at 0.423 (SEM ± 0.14), or for the Dupuytren's cord derived fibroblasts at 0.14 (SEM ± 0.07) in comparison to a static load. However for nodule there was a significant up-regulation ($p<0.05$) in gene expression from 0.275 to 1.29 (SEM ± 0.12) in comparison to nodules exposed to a static load. This upregulation in MMP-1 expression by Dupuytren's nodule fibroblasts was also significantly greater ($p<0.05$) than expression for underloaded carpal ligament and Dupuytren's cord fibroblasts.

Application of a series of uniaxial underload also resulted in a significant upregulation ($p<0.004$) of MMP-2 for Dupuytren's nodule in comparison to expression with a static load from 0.63 (SEM ± 0.2) to 4.67 (SEM ± 0.86). There was no significant upregulation of MMP-2 expression for either carpal ligament or for Dupuytren's cord derived fibroblasts in comparison to a static load. When MMP-2 expression for Dupuytren's nodule after an underload was compared to that for cord and carpal ligament, again a significantly higher level of expression ($p<0.004$) was noted for nodule derived cells. These results for both MMP-1 and MMP-2 gene expression indicate that the behaviour of the nodule and the cord are different, with the nodule up-regulating expression of these genes, while the cord behaves in a similar manner to control carpal ligament and shows no significant change in gene expression. It may be interpreted that fibroblasts derived from nodule are a separate cell population from those derived from cord, and that it is these cells which are most sensitive to changes in externally applied mechanical load as seen here with underloading.

In contrast to the upregulation of MMP-1 and 2 by Dupuytren's nodule, there were few changes in the expression of the tissue inhibitors (TIMPs) after underloading. Application of a series of underloads resulted in no significant change in TIMP-1 expression for carpal ligament or Dupuytren's nodule derived fibroblasts. However for

Dupuytren's cord there was a significant increase ($p<0.004$) from 1.78 (SEM ± 0.28) to 4.79 (SEM ± 0.66). With regard to the expression of TIMP-1 between cell types after underloading, no significant difference in expression of TIMP-1 was seen between carpal ligament or Dupuytren's derived cells. Similarly for TIMP-2 there was no significant change in gene expression between cell types. Expression levels remained unchanged from those of a static load with values of 2.09 (SEM ± 0.52) for control, 2.02 (SEM ± 0.57) for nodule and 1.25 (SEM ± 0.46) for cords. Thus within this experimental set-up, it can be interpreted that TIMP-2 gene expression is not affected by a reduction in externally applied uniaxial load. However TIMP-1 gene expression within Dupuytren's cord fibroblasts is up-regulated by reduced load. From this it can be extrapolated that in the cord matrix degradation is inhibited by the up-regulated TIMP-1 when load is reduced, and thus overall matrix turnover may also be reduced in this situation. This suggests that mechanical load may contribute to the net matrix production as seen in the Dupuytren's cord.

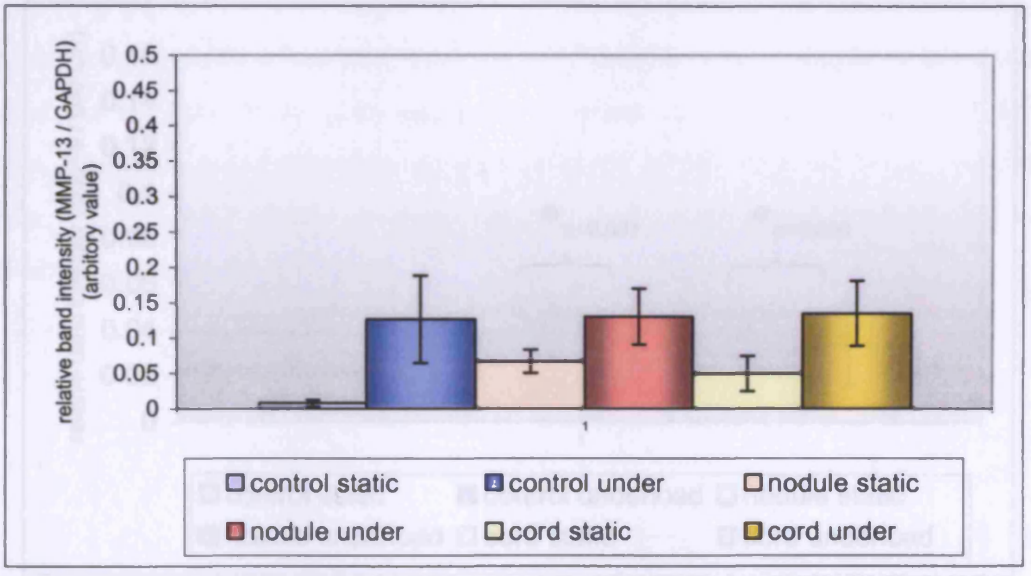


Figure 4.4:- Expression of MMP-13 after simple static loading or after an underloading regime for control carpal ligament (n=4), Dupuyten's nodule (n=4) and Dupuytren's cord (n=5). Error bars represent standard errors of the mean.

From the histogram above (figure 4.4) an underloading regime results in an upregulation of MMP-13 expression in both the control and Dupuytren's derived fibroblasts. Despite

the trend for upregulation these differences are not significant for any cell type. MMP-13 has been shown in osteoblasts to be up-regulated when mechanical load is applied (Yang *et al* 2004), but it was uncertain of the effects on reducing load. This data implies that both control palmar fascia fibroblasts derived from carpal ligament and Dupuytren's fibroblasts react in a similar manner to underloading with regards MMP-13 expression.

For MMP-9 expression levels are extremely low in all cases. However exposure to underloading results in an upregulation of MMP-9 expression in all cell types (figure 4.5). This up-regulation is significant ($p<0.003$) for Dupuytren's nodule with an increase from 0.002 (SEM ± 0.002) to 0.046 (SEM ± 0.009). Similar significant increases ($p<0.003$) occurred for Dupuytren's cord from 0.0016 (SEM ± 0.009) to 0.034 (SEM ± 0.018). For carpal ligament there was an increase in MMP-9 expression from 0.003 (SEM ± 0.002) to 0.045 (SEM ± 0.018), although this was not significant ($p>0.057$).

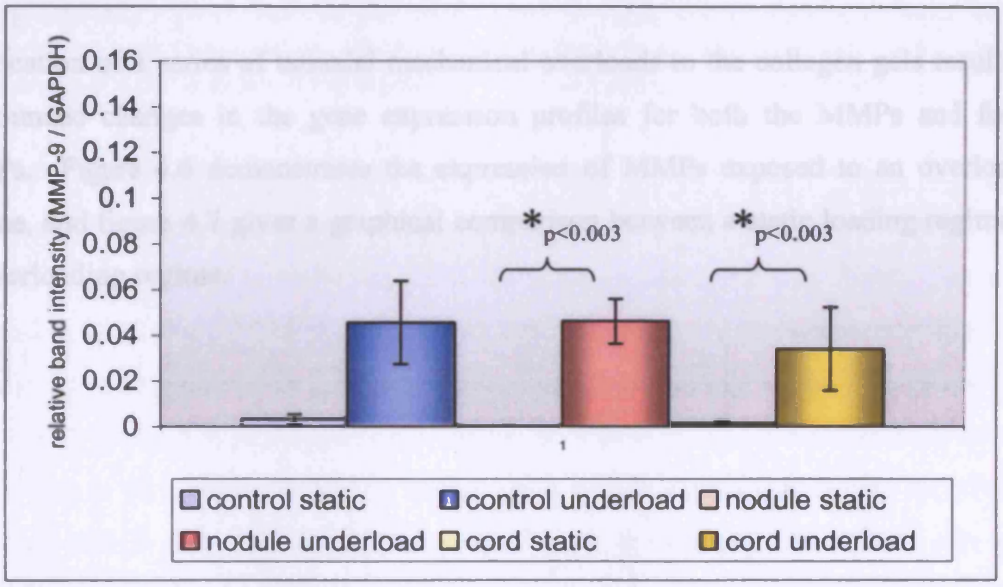


Figure 4.5:- Expression of MMP-9 after simple static loading or after an underloading regime for control carpal ligament (n=4), Dupuyten's nodule (n=4) and Dupuytren's cord (n=5). Error bars represent standard errors of the mean. Expression of MMP-9 increased significantly after underloading for Dupuytren's cell lines ($p<0.003$).

This data demonstrates that expression of MMP-9 in Dupuytren's fibroblasts is altered by a reduction in externally applied load. This change is also mimicked in control carpal ligament. Previous work has demonstrated that expression of this gene is up-regulated by changes in mechanical load (Mudera *et al* 2000; Von den Hoff 2003) in both dermal fibroblasts and in periodontal ligament fibroblasts. Given the low level of expression of this gene in all cases and the similarity in the change of expression in response to underloading, it may be postulated that in this experimental set-up, it is not an abnormality in MMP-9 gene expression that is responsible for the overall difference in contractility seen between Dupuytren's and control fibroblasts.

4.32 OVERLOADS

Application of a series of uniaxial mechanical overloads to the collagen gels resulted in pronounced changes in the gene expression profiles for both the MMPs and for the TIMPs. Figure 4.6 demonstrates the expression of MMPs exposed to an overloading regime, and figure 4.7 gives a graphical comparison between a static loading regime and an overloading regime.

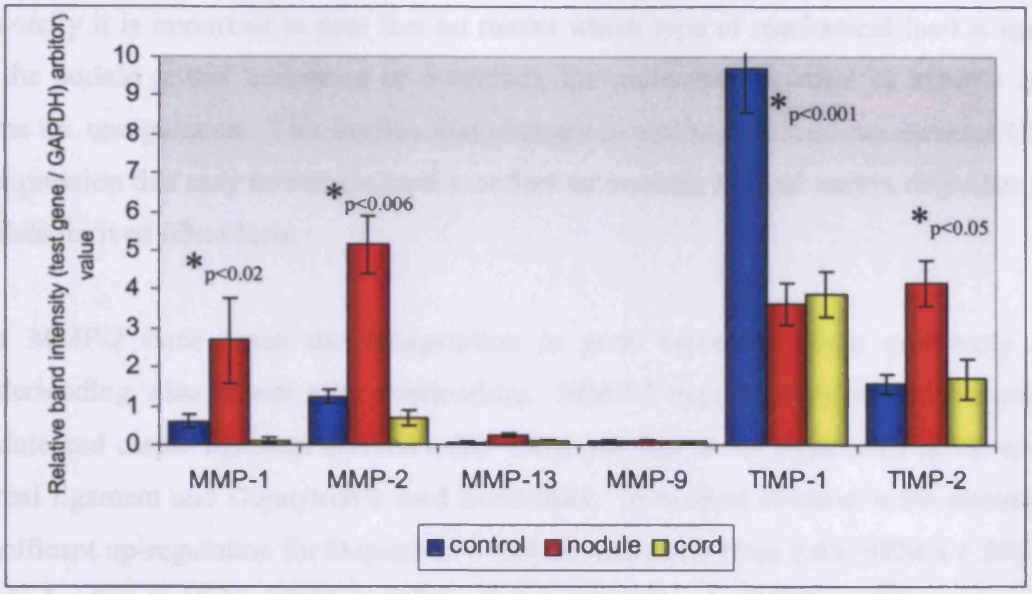


Figure 4.6:- Mean relative band intensity for the MMPs and TIMPs following a series of uniaxial overloads for n=4 control carpal ligament, n=5 Dupuytren's nodule and n=6 Dupuytren's cord derived fibroblasts. Error bars represent standard errors of the mean. The asterixes * indicate the significantly greater gene expression for MMP-1 , MMP-2 and TIMP-2 for Dupuytren's nodule in comparison to cord and carpal ligament, and also the significantly greater expression of TIMP-1 for the carpal ligament fibroblasts in comparison to cord and nodule. (Figure 4.7 overleaf gives a diagrammatic representation of the comparison between static loads and overloads using the same data)

After application of a series of overloads there is a significant increase in MMP-1 expression for Dupuytren's nodule. For control carpal ligament MMP-1 expression rises from 0.375 (SEM 0.17) to 0.60 (SEM ± 0.18) although this is not significant. For nodule expression rises from 0.274 (SEM ± 0.13) to 2.68 (SEM ± 1.1), which is a significant increase ($p<0.05$). However MMP-1 expression for Dupuytren's cord fibroblasts drops from 0.322 (SEM ± 0.12) in statically loaded gels to 0.12 (SEM ± 0.07) in overloaded gels, which is significant ($p<0.05$). Dupuytren's nodule fibroblasts display significantly increased MMP-1 expression after overloading in comparison to controls and cords ($p<0.02$). There is no difference in MMP-1 expression between an overload and an underload stimulus for nodule, cord or carpal ligament. Thus it appears that in this experimental set up it is the nodule derived fibroblasts that display greatest up-regulation of MMP-1 when both mechanically overloaded and also underloaded. This suggests first of all a functional difference between nodule and cord with respect to this gene.

Secondly it is important to note that no matter which type of mechanical load is applied to the nodule (either underload or overload), the molecular response of MMP-1 is the same i.e. upregulation. This implies that changes in mechanical load can increase MMP-1 expression that may be extrapolated to reflect an increase in local matrix degradation by nodule derived fibroblasts.

For MMP-2 once again the upregulation in gene expression seen previously after underloading, also occurs after overloading. MMP-2 expression increased for control, nodule and carpal ligament derived cells. However this is not significant in the case of carpal ligament and Dupuytren's cord fibroblasts. In marked contrast is the statistically significant up-regulation for Dupuytren's nodule with a rise from 0.63 (SEM ± 0.09) after static loading to 5.16 (SEM ± 0.74) after overloading ($p<0.003$). When comparing overloading gene expression to underloading gene expression there is no difference in MMP-2 expression for all cell lines. The up-regulation of MMP-2 in Dupuytren's nodule is significantly greater than that for both cord and carpal ligament ($p<0.006$). This data provides additional weight to the extrapolation given above. An up-regulation of both MMP-1 and MMP-2 for Dupuytren's nodule is indicative of an increased ability to degrade the extracellular matrix in comparison to cord or carpal ligament. It also demonstrates the similarity of molecular behaviour between carpal ligament and cord with regards to MMP gene expression, with the nodule acting in a separate manner.

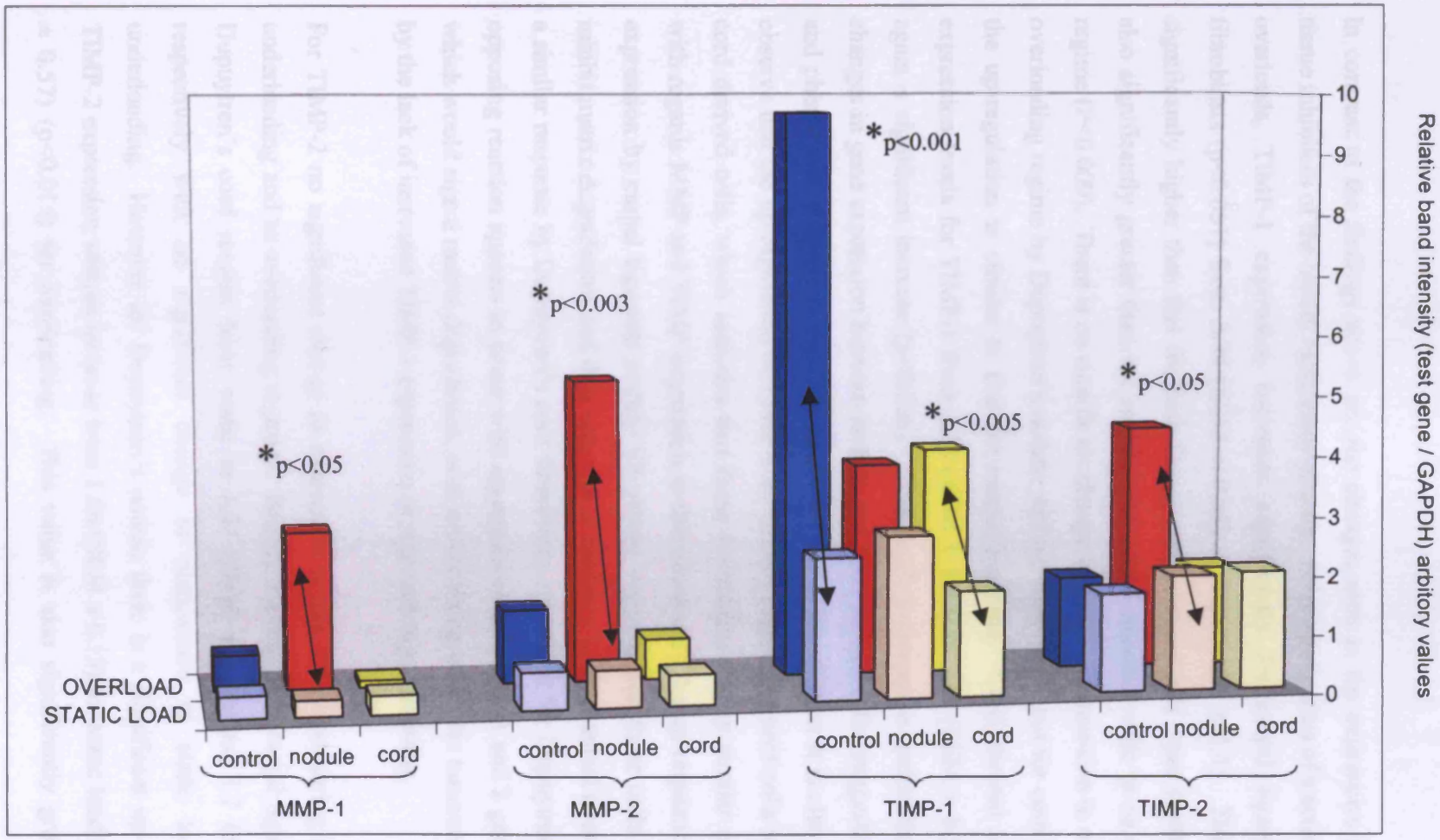


Figure 4.7: Histogram to compare the gene expression of MMP-1, MMP-2, TIMP-1 and TIMP-2 between 2 different mechanical stimuli: static loading, and overloading. Each bar represents the mean relative band intensity for each gene. Note the significantly greater expression of MMP-1, MMP-2, and TIMP-2 for Dupuytren's nodule in comparison to static load (*). Note also the significantly greater TIMP-1 expression for control carpal ligament and cord.

In contrast to the findings above are the changes seen in the expression levels of the tissue inhibitors of the matrix metalloproteinases. After application of a series of uniaxial overloads, TIMP-1 expression increases significantly for carpal ligament derived fibroblasts ($p<0.001$) from 2.39 (SEM ± 0.38) to 9.68 (SEM ± 1.1). This increase is significantly higher than that for both Dupuytren's derived cell types ($p<0.001$), and is also significantly greater than the response of carpal ligament cells to an underloading regime ($P<0.009$). There is no significant change in TIMP-1 expression in response to an overloading regime by Dupuytren's nodule derived fibroblasts, but for cord derived cells the up-regulation is similar to that for carpal ligament. Cord derived cells increase expression levels for TIMP-1 from 1.78 (SEM ± 0.28) to 3.86 (SEM ± 0.59), which is again a significant increase ($p<0.006$). This result indicates as earlier the contrasting changes in gene expression between nodule and cord derived cells, suggesting functional and phenotypic differences between these cell types. In addition it is also important to observe that the up-regulation of TIMP-1 by control carpal ligament cells is also seen in cord derived cells, which indicates that these fibroblasts display similar characteristics with regards MMP and TIMP expression in this situation. The up-regulation in TIMP-1 expression by carpal ligament control fibroblasts suggests that these cells are acting to inhibit matrix degradation, and thus turnover in response to mechanical overloading, with a similar response by Dupuytren's cord fibroblasts. However for Dupuytren's nodule an opposing reaction appears to occur with up-regulation of MMP-1 and 2 gene expression which would signal matrix degradation, with no inhibition of matrix turnover as indicated by the lack of increased TIMP-1 expression in response to overloading.

For TIMP-2 no significant change in expression occurs when comparing static load to underloading and to overloading regimes. Expression levels for carpal ligament and for Dupuytren's cord remain fairly static at 1.57 (SEM ± 0.24) and 1.7 (SEM ± 0.51) respectively with no significant change in comparison to static loading or to underloading. However for Dupuytren's nodule there is a significant up-regulation of TIMP-2 expression with an increase from 1.96 (SEM ± 0.13) for static load to 4.16 (SEM ± 0.57) ($p<0.015$) for overloading. This value is also significantly greater than the

response to an underloading regime ($p<0.02$). Up-regulation by nodules is also significantly greater when comparing these values to those of carpal ligament and Dupuytren's cord ($p<0.015$). Up-regulation of TIMP-2 has also been observed after mechanical loading in periodontal fibroblasts (He *et al* 2004) and fetal lung fibroblasts (Yokota *et al* 2002). As introduced earlier the role of the TIMPs is not only in the inhibition of MMP activity, but they are also involved in causing enhanced cellular proliferation, which may explain the up-regulation of TIMP-2 in the Dupuytren's nodule only.

For MMP-13 up-regulation of gene expression was seen for Dupuytren's nodule only which occurred to a significantly greater extent than with an underload (figure 4.8). Band intensity increased from 0.067 (SEM ± 0.016) for statically loaded gels to 0.24 (SEM ± 0.03) for overloaded gels, which was significant ($p<0.002$). For carpal ligament and for cord derived cells there was no significant change in MMP-13 expression after overloading in comparison to static loading or underloading. The upregulation seen in nodule derived cells was significantly greater than the values seen for carpal ligament controls and for Dupuytren's cord ($p<0.05$). Once again this result supports the postulation that the nodule appears to play a key role in matrix degradation in response to applied mechanical load.

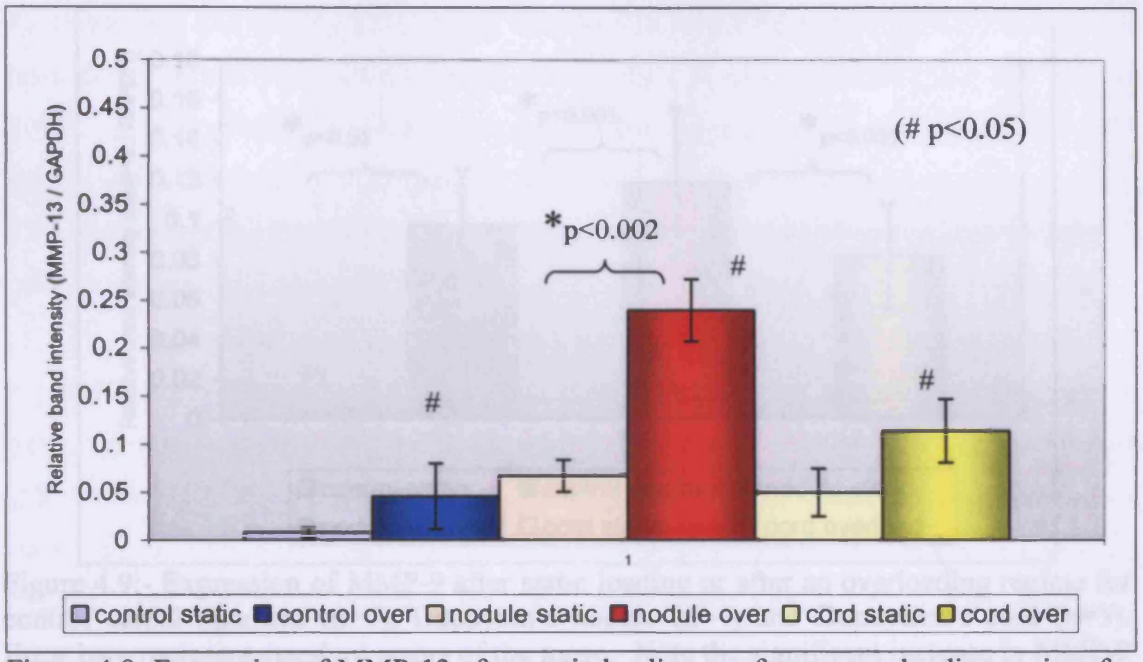


Figure 4.8: Expression of MMP-13 after static loading or after an overloading regime for control carpal ligament (n=4), Dupuyten's nodule (n=4) and Dupuytren's cord (n=5). Error bars represent standard errors of the mean. Note the significant increase in gene expression for Dupuytren's nodule after overloading (* $p<0.002$). Note also that MMP-13 gene expression for nodule was significantly greater than that for cord or control carpal ligament (# $p<0.05$).

The up-regulation in expression of MMP-9 that was seen after underloading in all cases was also seen after overloading (figure 4.9). From minimal MMP-9 expression in all investigated statically loaded fibroblasts, there was an increase to 0.09 (SEM ± 0.03) for control carpal ligament, to 0.12 (SEM ± 0.03) for nodule and to 0.08 (SEM ± 0.02) for cord derived cells. Up-regulation was significant for each cell type investigated ($p<0.05$ for carpal ligament; $p<0.005$ for Dupuytren's fibroblasts). There was no significant difference between overloading and underloading regimes.

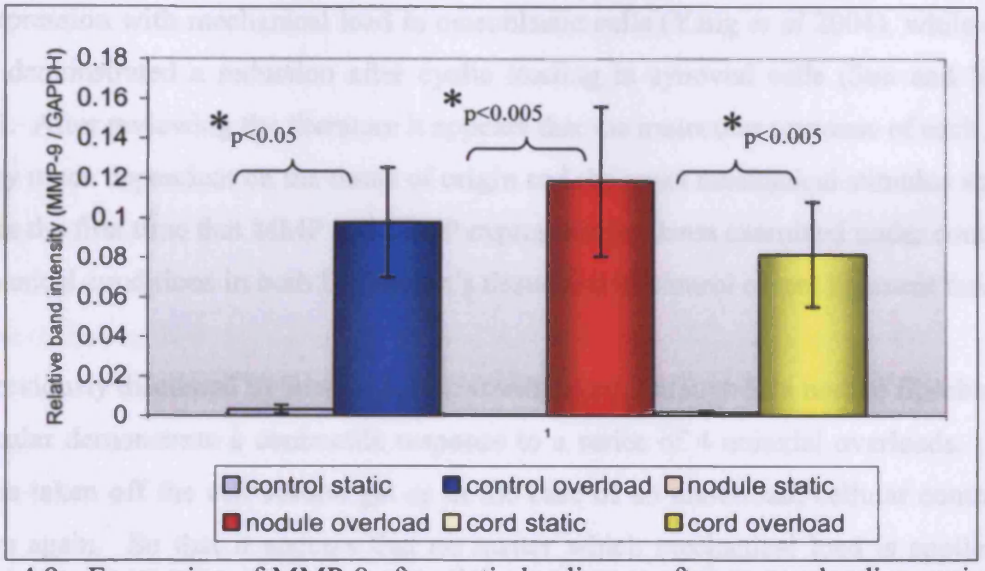


Figure 4.9:- Expression of MMP-9 after static loading or after an overloading regime for control carpal ligament (n=4), Dupuyten's nodule (n=4) and Dupuytren's cord (n=5). Error bars represent standard errors of the mean. Note the significant increase in MMP-9 expression after an overload compared to static loading (*) in all cases.

It may be postulated that in this experimental set-up, that the up-regulation of MMP-9 is a normal and usual response to a change in mechanical load, given the similarity in response by all three cell types to both overloading and underloading. This is also supported by previous work on both human dermal fibroblasts and human periodontal fibroblasts (Mudera *et al* 2000; Von den Hoff 2003).

4.33 DISCUSSION

Mechanical load has been shown in many cell types to stimulate production of MMP-1, MMP-2 and MMP-9 (Li *et al* 1999; Seliktar *et al* 2001; WuDunn 2001; Asanuma *et al* 2003; Von den Hoff 2003; He *et al* 2004). These have included cardiac fibroblasts, vascular smooth muscle cells, trabecular meshwork cells, and periodontal ligament fibroblasts in both cell monolayer and collagen gel models. Other work has demonstrated no change in MMP-1 and 2 expression after application of a mechanical load e.g. in scleral fibroblasts (Yamaoka *et al* 2001). Some studies have shown an increase in MMP-

13 expression with mechanical load in osteoblastic cells (Yang *et al* 2004), while others have demonstrated a reduction after cyclic loading in synovial cells (Sun and Yokota 2002). After reviewing the literature it appears that the molecular response of each MMP is very much dependant on the tissue of origin and the exact mechanical stimulus applied. This is the first time that MMP and TIMP expression has been examined under controlled mechanical conditions in both Dupuytren's tissue and in control carpal ligament fascia.

As previously discussed in Section 1.18 (*Introduction)*, Dupuytren's nodule fibroblasts in particular demonstrate a contractile response to a series of 4 uniaxial overloads. When load is taken off the cell seeded gel as in the case of an underload, cellular contraction occurs again. So that it appears that no matter which mechanical load is applied, the response of Dupuytren's nodule derived cells is by contraction. The molecular expression of MMPs has been observed here within a single snap-shot in time, with the gene expression being measured thirty minutes after a series of 4 underloads or overloads. Time and laboratory limitations did not permit the examination of these molecular changes at separate time points as this would give us a more in depth picture of the intracellular events as they occur. It is for these reasons that a static load has been utilized as the standard with which to compare the other loading regimes.

From this work it may be postulated that it is the Dupuytren's nodule which is most active with regards to MMP expression. There is a distinct significant up-regulation of all of the MMPs under investigation here (MMP-1,2,9, and 13), after both underloading, and to a greater degree after overloading. In contrast is the behaviour of the Dupuytren's cord which parallels that of the control carpal ligament derived cells. These displayed no up-regulation of MMP-1, 2, or 13 after a mechanical stimulus, excepting MMP-9. From this we may conclude that there is some fundamental difference in the make-up of these cells. It is postulated that the Dupuytren's cord derived fibroblasts come from a different cell population to those of the nodule.

When seeded into a collagen gel – the behaviour of the nodule and cord is remarkably similar as shown by their force generated on the culture force monitor. Both generate

significantly greater force than control carpal ligament fibroblasts, and both continue to contract with no evidence of tensional homeostasis at 48 hours. In addition when subjected to an overload, both display a contractile response (Bisson *et al* 2004), although this is more significant in the nodule. Previous work investigating the differences in cellular behaviour between nodule and cord has been inconclusive. Moyer *et al* (2002) analysed gap-junction intercellular communication and free floating circular collagen gel contraction in nodule and cord derived cell lines over a period of 8 passages, finding that as passage number increased the behaviour of the nodule derived fibroblasts became similar to that of cord, with reduced contractility, and increased coupling index (a measure of increased gap junction communication). They concluded that this provided weight to the theory favoured by Hueston (1985) that the Dupuytren's nodule represented the early stage of the disease, and that with time nodules will change their phenotype and progress into cords. The histological appearance of cells within nodule and cord are different (Luck 1959), and since the discovery of the myofibroblast by Gabbiani and Majno (1972) a lot of work has investigated the location of this cell type within the Dupuytren's disease nodule/cord complex. Some workers (Badalamente *et al* 1983; Vande Berg *et al* 1984; Qureshi *et al* 2001) have demonstrated that the cord generally lacked myofibroblasts, whereas Bisson *et al* (2003) demonstrated their presence in cord albeit in low proportions (2.7% myofibroblast content). When stimulated with TGF-beta however they found a significant increase in myofibroblasts within cord cultures to levels similar to that for nodule (25% myofibroblast content).

The data presented here provides new evidence for a difference between nodule and cord fibroblasts with regards MMP gene expression. It does not however help the debate as to which, if either, of these Dupuytren's lesions arises first. As seen from the static loading results, the baseline expression of MMP-1,2,9 and 13 in all cell types is very low, with a greater expression of TIMP-1 and 2. As soon as a change in mechanical load is applied, the nodule and the cord behave in converse ways with MMP, and TIMP-2 upregulation for nodule, and TIMP-1 up-regulation for cord. It is therefore postulated that these are two separate cell populations, each with a different role to play in the disease. On a molecular level it is the nodule that is the most sensitive to mechanical load and active

when it comes to protease gene expression. No matter which mechanical load is applied, the result is increased gene expression of MMP-1,2,9 and 13. For the cord, there is very little change in MMP expression, and behaviour is remarkably similar to that for carpal ligament. It may be extrapolated from this that the cord is relatively quiescent, and does not play a major role in matrix remodeling activity. By virtue of the cords close relationship to the nodule it is entirely possible that these separate entities are part of the same unit, but with different roles to fill in the process of forming a flexion contracture.

An up-regulation in the expression of the MMPs in response to mechanical stimulation has been previously reported in Dupuytren's disease (Tarlton *et al* 1998), and other cell types (Seliktar *et al* 2001; Asanuma *et al* 2003; Von den Hoff 2003; He *et al* 2004). Seliktar *et al* (2001) exposed human vascular smooth muscle cells in a 3-D construct to either static loads or cyclical loads and found a significant up-regulation of MMP-2 production using gelatin zymography when mechanically stimulated. They also measured an increase in strength of the tested construct after mechanical stimulation. The same cell types have been shown to increase their expressed levels of MMP-1, MMP-2 and MMP-9 when exposed to a static load, whereas cyclic loading resulted in a downregulation (Yang *et al* 1998; Asanuma *et al* 2003). It was proposed that an increase in static load seen in arteries with atheroma may act as a stimulus for matrix breakdown and possible plaque failure, and thus distal vascular occlusion. Transgenic mice with overexpression of MMP-3, or MMP-1 have developed increased reactive stroma and an increased collagen content within the ECM, and for mice with increased expression of MMP-1, left ventricular hypertrophy occurs (Sympson *et al* 1994; D'Armiento *et al* 1997). Similarly MMP-1, 2, and 9 levels were enhanced in preloaded dermal fibroblasts subjected to cyclic strain (Berry *et al* 2003), and it was suggested that this results in a shift to a more active catabolic state. In the case of the smooth muscle cells static load occurs as a result of a pathological disease state such as atheroma, and an increase in MMP activity is an action performed by these cells in order to improve flow. The difference with Dupuytren's disease is that these changes as far as we know are not a normal response to an abnormal mechanical situation within the hand, but an abnormal response to a normal mechanical situation.

Arnoszky *et al* (2004) found a strong inverse correlation between MMP-1 expression and static load applied to rat tendon fibroblasts. An increase in load resulted in a significant fall in expressed MMP-1. Load deprivation in tendon cells has been shown to result in degradation of the ECM, and proteolytic action of the MMPs has been thought to have been a causative factor (Goomer *et al* 1999; Majima *et al* 2000). The action of cytochalasin-D to disrupt the actin cytoskeleton resulted in a similar level of MMP-1 to load-deprived fibroblasts, which supported the hypothesis that a cytoskeletally based mechanosensory tensegrity system was involved in the control of MMP gene expression. Work on periodontal ligament cells yielded similar findings with mechanical load in collagen gels preventing matrix degradation, and free floating gels showing enhanced MMP-2 and 9 activity (Von den Hoff 2003). In this work it appears that a mechanical overloading results in an increase in expressed MMP-1 in Dupuytren's nodule. However regarding static load alone the levels of expressed MMP-1 are low in Dupuytren's and in carpal ligament cells.

Kessler *et al* (2001) concluded from their work, that mechanically loaded fibroblasts are activated to a synthetic phenotype characterized by connective tissue synthesis in concert with the induction of protease inhibitors, while downregulating proteases and inflammatory mediators. They observed induction of ECM synthesis of collagen I and tenascin C, with downregulation of MMP-1 in a mechanically loaded gel in comparison to a free floating gel. In parallel the protease inhibitors TIMP-1 and TIMP-3 were induced by a mechanical load. It was thought that an increased level of these inhibitors could disturb the balance between matrix synthesis and degradation leading to accumulation of extracellular matrix. Our work contrasts with this study. We see no downregulation of MMP activity after mechanical load in the normal carpal ligament control cells, and an up-regulation in the Dupuytren's nodule cells. One can postulate that in order for a tissue to remodel, first an element of matrix degradation and "softening" must take place in order that cell motility can occur within the matrix, followed by cellular contraction and then finally knitting together of the "softened" matrix with deposition of new extracellular matrix materials. Thus the initial event may

well be an up-regulation of MMP activity in order for this to occur. A repeated continuation of this process may lead to further tissue contraction, and eventually long term net matrix deposition leading to contracture

Compressive forces applied to periodontal ligament fibroblasts results in decreases of type I collagen and fibronectin protein, downregulation of collagen I gene expression, and upregulation of MMP-2, with no change in TIMP-2 expression (He *et al* 2004). In contrast a mechanical load increased expression of MMP-2 and TIMP-2. This indicated that these cells have the ability to perceive different types of mechanical stimuli, and respond in a different manner to each. It can be seen here that Dupuytren's nodule derived cells display the same responses to mechanical load as the cells described above, and thus can be termed mechanosensitive, in contrast to the carpal ligament and Dupuytren's cord derived cells. An upregulation of MMPs may be a normal response for periodontal ligament derived fibroblasts where a key role in the maintenance of a stable tooth is the detection of mechanical load. This lays weight to the findings that normal palmar fascia cells have a low baseline contractility, and do not upregulate MMPs when exposed to a mechanical load. It can be postulated that the palmar fascia is not mechanosensitive. It is possible that an insult to the palmar fascia fibroblast be it chemical, traumatic, or genetic may suddenly trigger these cells to become particularly sensitive to load, thus resulting in the characteristic fibrotic changes seen clinically in Dupuytren's disease.

Levels of TIMP-1 and TIMP-2 remain unchanged after mechanical loading in trabecular meshwork cells (WuDunn 2001). Similar findings have been documented for vascular smooth muscle cells (Asanuma *et al* 2003). However work on synovial cells, chondrocytes and fetal lung cells has demonstrated an increase in expression of TIMP-1 and 2 after cyclical loading (Honda *et al* 2000; Xu *et al* 1999; Sun and Yokota 2002), whereas a downregulation of TIMP-1 occurs in human scleral fibroblasts (Yamaoka *et al* 2001). With regards to pathological conditions, chronic leg wound fibroblasts have been shown to have a decreased expression of MMP-1 and 2, with increases in expression of TIMP-1 and TIMP-2. It was suggested that the impaired ability of chronic wound

fibroblasts to reorganize the extracellular matrix in vitro is related to this change in protease activity (Cook *et al* 2000). Similar patterns of TIMP expression have also been seen in the dermis of scleroderma patients (Takeda *et al* 1994) and in fibroblasts derived from hypertrophic and keloid scars (Arakawa *et al* 1996; Neely *et al* 1999). When we analyse the results seen from mechanical underloading of fibroblasts in our own model, there is no significant change in the expression of TIMP-2 for normal and Dupuytren's derived fibroblasts. A trend for up-regulation of TIMP-1 is seen after underloading for all cell types, although this is only significant for cord derived cells. Once mechanical overloading is applied there is a significant upregulation of TIMP-1 for carpal ligament in particular, but also for cord. There appears to be no reciprocal up-regulation of TIMP-1 gene expression to "mop-up" protease expression in the Dupuytren's nodule. This may go some way to explaining the continued matrix remodeling seen in the disease process, whereas in normal palmar fascia the release of MMPs might be very closely controlled by this up-regulation in TIMP-1 resulting in little matrix turnover. Thus we can postulate that in normal palmar fascia, cells are non-mechanosensitive, and are in a stable state with regards matrix turnover. If stimulated by a sudden increase in mechanical load, these fibroblasts will react with an up-regulation of TIMP-1 in order to block any of the matrix degrading activity of the MMPs, and thus maintain the tissue in a steady state. In contrast for Dupuytren's nodule an opposite reaction is postulated to occur. After stimulation up-regulation of MMP activity begins with no reciprocal "blocking" activity of TIMP-1, and this may lead to matrix degradation along with new matrix remodeling.

The use of MMP inhibition as a therapeutic strategy to oppose matrix remodeling in Dupuytren's disease is a concept that may be suitable for further investigation. Certainly in the failing human heart it appears that a similar process to that occurring in the Dupuytren's nodule occurs with increased MMP activity, while TIMP-1 activity is decreased (Li *et al* 2000). Animal models have displayed improved cardiac pump function after application of a synthetic MMP inhibitor (Spinale *et al* 1999).

The main limitation of this work was that only RNA expression was measured. It would have been useful to have investigated MMP and TIMP activity via additional techniques

such as gelatin zymography and Western blotting as this would have given more information on protein levels of these enzymes, and also information on whether enzymes were inactive or active.

4.34 SUMMARY

- Baseline TIMP-1 and 2 expression in all statically loaded fibroblasts is significantly greater than that of MMP-1,2,9 and 13. There is no significant difference in gene expression of MMPs and TIMPs between carpal ligament and Dupuytren's fibroblasts after static loading.

- A change in mechanical load results in significant up-regulation of MMP gene expression by Dupuytren's nodule fibroblasts. This is most significant after overloading. There is no up-regulation of MMPs by cord or carpal ligament derived fibroblasts.

- There is a reciprocal significant up-regulation of TIMP-1 expression by carpal ligament derived cells after mechanical loading, with a similar significant response by cord derived cells. This response is absent in nodule derived fibroblasts.

- It is postulated that there is a complex interplay of events when mechanical load changes – this begins with localised matrix breakdown, which can then allow structural alterations in the ECM, further tissue contraction, and eventually long term net matrix deposition leading to contracture.

Having established the molecular response of these fibroblasts to mechanical stimulation, the molecular behaviour of Dupuytren's derived fibroblasts to stimulation with TGF-β1 was then investigated.

4.4 GENE EXPRESSION OF THE MATRIX METALLOPROTEINASES BY DUPUYTREN'S FIBROBLASTS PRE-INCUBATED WITH TGF-BETA AND EXPOSED TO MECHANICAL STIMULATION

4.41 INTRODUCTION

TGF-β1 is a ubiquitous polypeptide growth factor that is thought to play a central role in fibrotic conditions, having been shown to increase collagen deposition (Alioto *et al* 1994; Reed *et al* 1994) and enhance contraction of fibroblast populated collagen lattices (Montesano and Orci 1988; Riikonen *et al* 1995; Vaughan *et al* 2000; Brown *et al* 2002). It is also documented that TGF-β1 promotes fibroblast to myofibroblast differentiation in cell cultures, and although the exact relationship between increased lattice contraction and myofibroblast upregulation is controversial several authors have proposed a direct correlation (Desmouliere *et al* 1993; Yokozeki *et al* 1997). It is therefore conceivable that TGF-β1 acts at several levels to promote cellular contraction, as a primary mechanoregulatory growth factor as suggested by Brown *et al* (2002), by increasing integrin expression (Riikonen *et al* 1995), and also by stimulating cytoskeletal component upregulation as in the transformation of myofibroblasts.

TGF-β_1 has been implicated in the causation or progression of Dupuytren's disease for some time, traditionally being described as a profibrotic growth factor (Alioto *et al* 1994; Kloen *et al* 1995; Badalamente *et al* 1996). When TGF-β_1 is applied to the collagen gel model used here and previously by Bisson (MD thesis 2004), it is seen to stimulate a higher generation of force by both Dupuytren's and carpal ligament fibroblasts, with some of that effect occurring early in the contraction profile. After exposure to a series of uniaxial overloads, there was an enhancement of the contractile response seen previously only for Dupuytren's nodule without TGF-β1 stimulation (Bisson *et al* 2004). This contractile response to overloading was additionally seen in cord and carpal

ligament derived fibroblasts, whereas without stimulation by TGF-β1, these cells had previously responded by a reduction in contraction, and a decrease in measured force. The mechanism by which TGF-β1 stimulation was affecting this response was not addressed in this previous work, but it was speculated that TGF-β1 was possibly increasing the sensitivity to mechanical stimulation within the investigated population of fibroblasts.

No previous work has investigated the effects of TGF-β1 on MMP and TIMP expression in studies on Dupuytren's disease or carpal ligament derived fibroblasts, and given the findings documented earlier, it was felt that this work would provide greater insight into the molecular processes occurring in Dupuytren's fibroblasts exposed to this growth factor.

4.42 AIM

- To determine the MMP and TIMP gene expression responses of Dupuytren's nodule, cord and carpal ligament fibroblasts to mechanical loading after stimulation with TGF-β1.

4.43 HYPOTHESIS

- TGF-β1 will downregulate MMP expression and upregulate TIMP expression as a reflection of its profibrotic activity in control carpal ligament, Dupuytren's nodule and Dupuytren's cord fibroblasts.

4.44 RESULTS

After pre-incubation with TGF-beta1, fibroblast seeded three dimensional collagen gels were left to contract on the culture force monitor, and at 20 hours were subjected to a

series of uniaxial overloads, in a manner identical to that documented previously (*Materials and Methods section 2.33-2.36*). The changes in gene expression are seen in the histogram below (figure 4.10), and overleaf in a pictoral comparison between overloads with or without TGF-beta1 (figure 4.11).

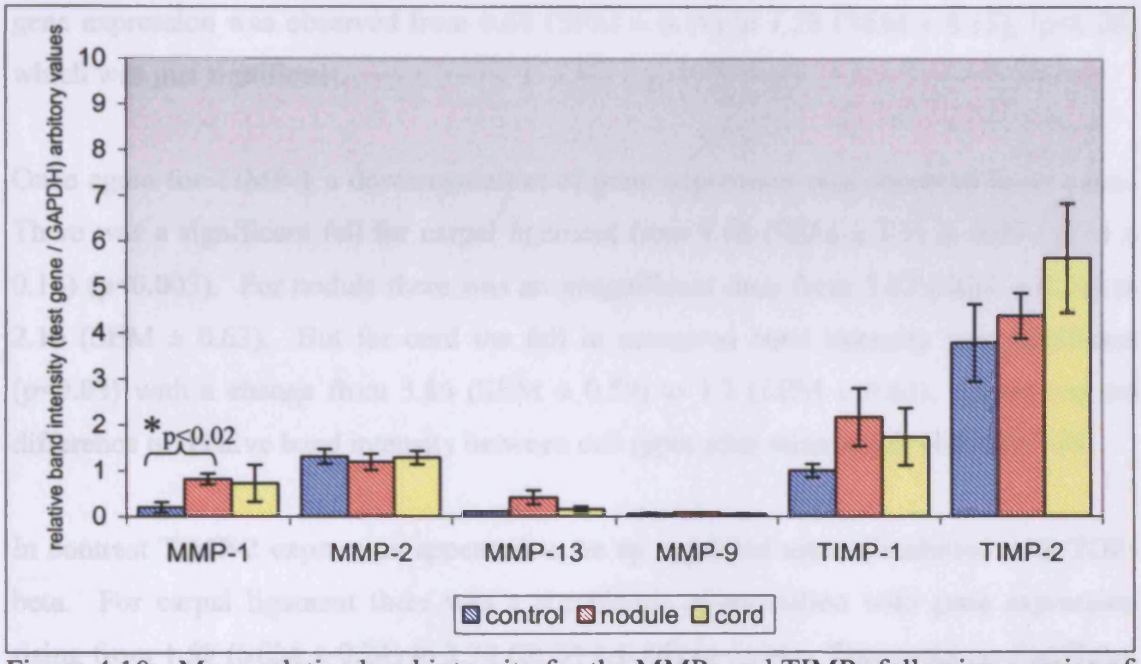


Figure 4.10:- Mean relative band intensity for the MMPs and TIMPs following a series of uniaxial overloads after pre-incubation with TGF-β1 for n=4 control carpal ligament, n=5 Dupuytren's nodule and n=5 Dupuytren's cord derived fibroblasts. Error bars represent standard errors of the mean. Note the significantly greater MMP-1 expression for Dupuytren's nodule in comparison to control carpal ligament (*$p<0.02$). (Figure 4.11 overleaf gives a diagrammatic representation of the comparison between overloads and overloads with TGF-β1 using the same data)

Stimulation with TGF- β1 resulted in a downregulation of MMP-1 gene expression for carpal ligament, and Dupuytren's nodule. For control carpal ligament MMP-1 expression fell from 0.6 (SEM ± 0.18) to 0.19 (SEM ± 0.11), although this was not significant. Similarly for nodule a fall from 2.68 (SEM ± 1.1) to 0.81(SEM ± 0.12) was observed which was significant ($p<0.03$). For cord there was no significant change in MMP-1 expression after stimulation. Of note is that after stimulation with TGF-β1, expression of MMP-1 remained significantly higher for Dupuytren's nodule in comparison to control ($p<0.02$), with no difference noted between nodule and cord.

For MMP-2 a similar trend was observed with a downregulation of gene expression after stimulation with TGF-β1 for Dupuytren's nodule. For carpal ligament no significant change was observed. For nodule a fall from 5.16 (SEM ± 0.74) to 1.19 (SEM ± 0.18) was seen, which was significant ($p<0.006$). However for Dupuytren's cord an increase in gene expression was observed from 0.69 (SEM ± 0.19) to 1.28 (SEM ± 0.15), ($p<0.05$) which was just significant.

Once again for TIMP-1 a downregulation of gene expression was observed in all cases. There was a significant fall for carpal ligament from 9.68 (SEM ± 1.1) to 0.99 (SEM ± 0.14) ($p<0.003$). For nodule there was an insignificant drop from 3.62 (SEM ± 0.53) to 2.16 (SEM ± 0.63). But for cord the fall in measured band intensity was significant ($p<0.03$) with a change from 3.86 (SEM ± 0.59) to 1.7 (SEM ± 0.62). There was no difference in relative band intensity between cell types after stimulation with TGF- β1.

In contrast TIMP-2 expression appeared to be up regulated after stimulation with TGF-beta. For carpal ligament there was a significant up-regulation with gene expression rising from 1.57 (SEM ± 0.24) to 3.78 (SEM ± 0.84) ($p<0.04$). There was no significant difference in TIMP-2 expression for nodule derived cells with or without TGF-β1. However for cord, expression of TIMP-2 rose from 1.7 (SEM ± 0.51) to 5.64 (SEM ± 1.19), which was significant ($p<0.008$).

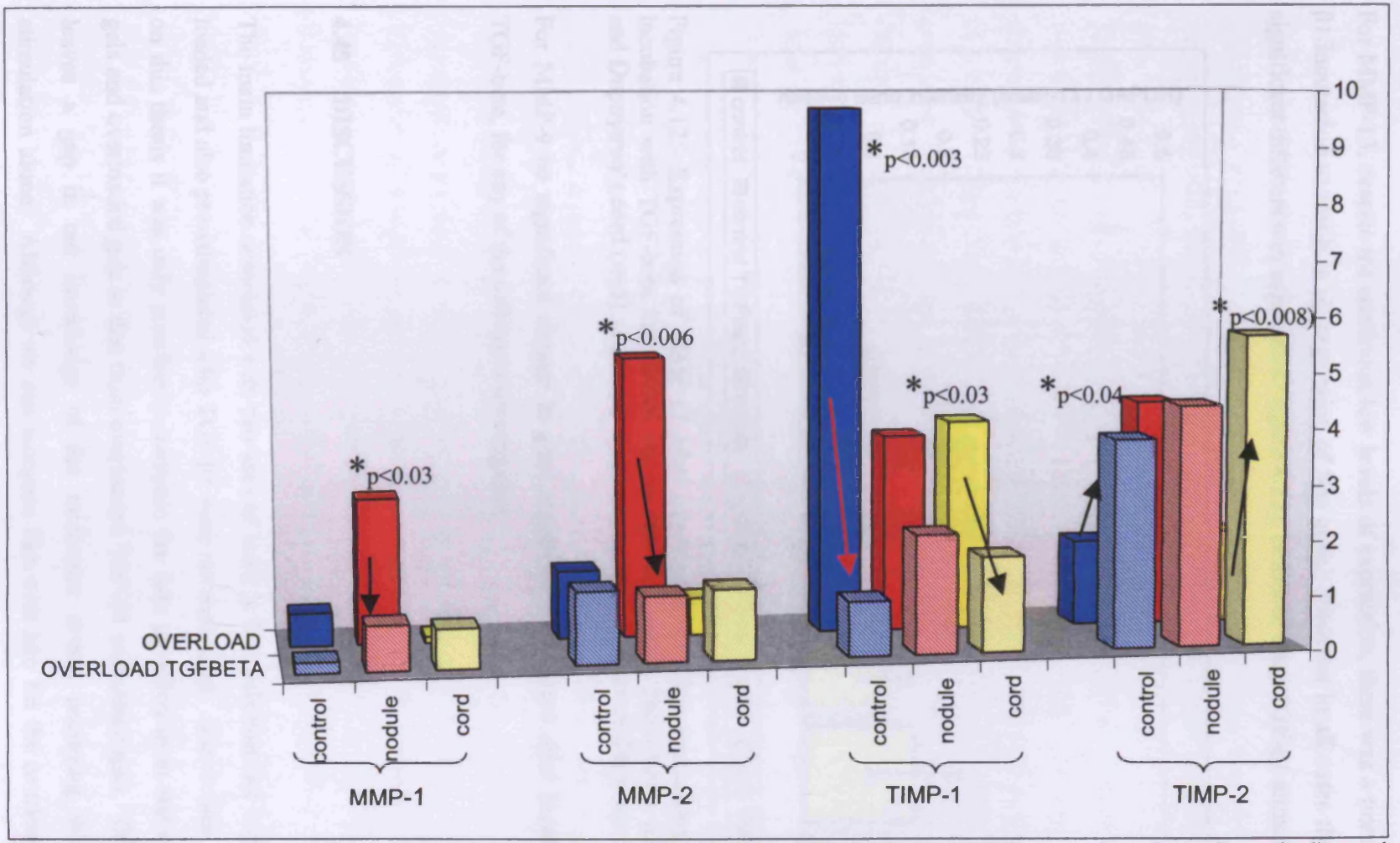


Figure 4.11: Histogram to compare the gene expression of MMP-1, MMP-2, TIMP-1 and TIMP-2 between 2 different mechanical stimuli: overloading, and overloading with TGF-beta. Each bar represents the mean relative band intensity for each gene. Note the significant downregulation of MMP-1, MMP-2 for nodule, and TIMP-1 for cord and carpal ligament*. Note the significant up-regulation of TIMP-2 for cord and carpal ligament*

For MMP-13, despite the continued low levels of expression, there was a trend for TGF-β1 incubation to result in up-regulation of this gene. However in all cases there was no significant difference in expression (figure 4.12), before or after TGF-β1 stimulation.

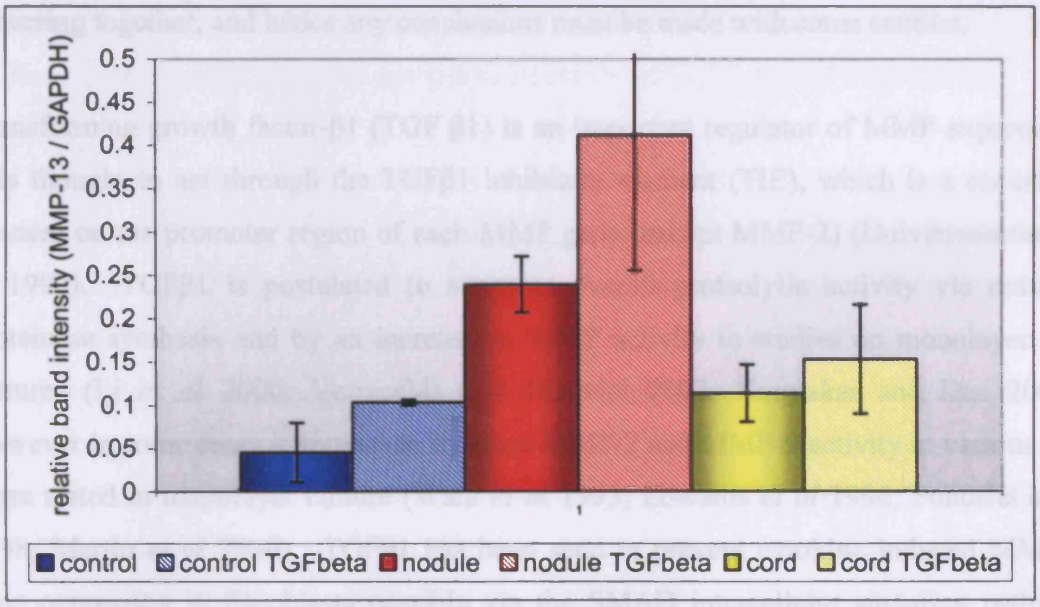


Figure 4.12:- Expression of MMP-13 after overloading or after overloading with pre-incubation with TGF-beta for control carpal ligament (n=4), Dupuyten's nodule (n=4) and Dupuytren's cord (n=5). Error bars represent standard errors of the mean

For MMP-9 no significant change in gene expression was noted after incubation with TGF-beta, for any of the cell types investigated.

4.45 DISCUSSION

The main limitation associated with this area of work is that gels that had been statically loaded and also pre-stimulated with TGF-β1 were not examined. Due to time constraints on this thesis it was only possible to compare the data provided from statically loaded gels and overloaded gels to that from overloaded TGF-β1 stimulated gels. This therefore leaves a gap in our knowledge of the molecular events occurring with TGF-β1 stimulation alone. Although we can compare like with like for the overloaded gels, it

would have been ideal to compare like with like for gels that had not been mechanically stimulated. In these results we are interpreting the molecular response to two cues – the first being a mechanical strain, and the second being the influence of a growth factor. The genetic expression of MMPs to each of these cues alone may differ to them occurring together, and hence any conclusions must be made with some caution.

Transforming growth factor-β1 (TGF β1) is an important regulator of MMP expression. It is thought to act through the TGFβ1 inhibitory element (TIE), which is a *cis*-acting element on the promoter region of each MMP gene (except MMP-2) (Duivenvoorden *et al* 1999). TGFβ1 is postulated to suppress overall proteolytic activity via reduced proteinase synthesis and by an increase in TIMP activity in studies on monolayer cell cultures (Li *et al* 2000; Verrecchia and Mauviel 2002; Karmakar and Das 2002). However in some cases it appears to increase MMP-2 and MMP-9 activity in various cell types tested in monolayer culture (Wahl *et al* 1993; Edwards *et al* 1996; Poncelet *et al* 1998; Martin *et al* 2000). TGFβ1 has been seen to prevent cytokine induced MMP-1 gene expression in fibroblasts possibly via the SMAD intracellular signaling pathway (Yuan and Varga 2001; Hall *et al* 2003). Other workers have implicated other intracellular signaling pathways in the induction of TIMP-1 including c-fos, c-jun and JunD, with no dependence on the SMAD pathway (Hall *et al* 2003). Similarly treatment of monolayer peritoneal mesothelial cells with TGFβ1 has resulted in a decrease in MMP-1 expression, while increasing expression of TIMP-1, whereas TIMP-2 expression was unaffected (Ma *et al* 1999). Similar work on cell monolayers yielded similar results in myometrial smooth muscle cells (Ma and Chegini 1999). These workers found that baseline expression of TIMPs were higher than those of MMPs in a similar manner to this study. However in the presence of TGF-β1, there was a reduction in MMP-1 and 3 expression, and an increase in TIMP-1 and 2 expression. It was hypothesized that high TIMP expression in vivo may allow only limited proteolysis leading to deposition rather than degradation of the ECM. In TGF beta1 overexpressing transgenic mice, it has been seen that interstitial collagenase activity was reduced in the myocardium in comparison to controls, but expression of MMP-2 and 9 was not affected. TIMP-1 and 2 expression were significantly increased (Seeland *et al* 2002). These changes were thought to

promote myocardial fibrosis in vivo. Vascular smooth muscle cells as monolayers were exposed to either static load or cyclical mechanical load on a flexorcell apparatus, and levels of MMPs were measured (O'Callaghan and Williams 2000). It was found that after 5 days of cyclical load collagen concentrations increased, and MMP-2 activity increased. In addition TGF-beta expression increased. It was concluded that strain induced ECM accumulation was not due to inhibition of ECM protein degradation.

Our work has yielded slightly different results in comparison to the literature above. Expression of MMP-1 and 2 in overloaded gels certainly fell in all cell types investigated, and this was significant in the case of Dupuytren's nodules. This correlates with most previous reported work. Expression of MMP-9 and 13 did not change significantly before or after TGF-β1 stimulation. For TIMPs, an upregulation in expression of TIMP-2 was seen to occur in all cell types, in particular for Dupuytren's cord, and carpal ligament. However TIMP-1 expression appeared to be down regulated by the addition of TGF-β1 in this experimental series. This trend was again seen to occur in all three cell types investigated. Certainly the reduction in MMP expression, and increase in TIMP-2 expression observed is consistent with the pro-fibrotic nature of TGF-beta. However the reduction in TIMP-1 expression is an unusual finding. There are several explanations for these findings.

It appears that TGF-β1 acts to "damp-down" the activity of the MMP-1 and 2 in Dupuytren's nodule, while having little effect on cord and carpal ligament. MMP-9 and 13 activity was unaffected by TGF-β1 in all cases. Based on our earlier findings with overloading it seems that there is a complex interplay of events occurring at a genetic level here. It can be postulated that in the first instance exposure to mechanical force will cause an increase in MMP activity, which will allow cellular migration, and local matrix degradation to occur, with the resultant further action of cellular contraction and matrix shortening. It is possible that the addition of TGF-β1 will allow the cells to downregulate their MMP activity, and switch to a state of matrix deposition and net production. As in any physiological or pathological condition there will by many processes occurring in parallel at any one time. This work in vitro is, of course, a gross oversimplification of

events occurring in vivo, but it does provide an idea of activity at a cellular level. TIMP-2 expression is up regulated by TGF-β1 in cord and carpal ligament, with no change in expression for nodule. It can be postulated that nodule derived cells are already acting to block any excess MMP activity once stimulated by overloading, and that TGF-β1 cannot enhance this effect. It can be extrapolated that in cord and carpal ligament MMP inhibition is beginning to occur, and cause a switch to matrix deposition from matrix equilibrium.

As mentioned earlier the findings regarding TIMP-1 expression are different to those of previous workers investigating other cell types. When we compare the expression of TIMP-1 here to that of the statically loaded gels, there is no significant change in TIMP-1 expression. It was hypothesised that TIMP-1 would be up regulated after stimulation with TGF-β1, and that this up-regulation would be significant given the enhanced response of this gene to overloading. Given that stimulation with TGF-β1 has resulted in a down-regulation of the MMPs, it can be postulated that it is also beginning to down-regulate TIMP-1 in addition, as matrix degradation stops and net production begins, with the up-regulation of other genes involved in new matrix synthesis. However, we are looking at a single time point, and gene expression may change over several minutes. By investigating expression of TIMP-1 over a longer time course it may be possible to deduce the exact events occurring, although this was beyond the scope of this project. It is theorised that an up-regulation of TIMP-1 expression after stimulation with TGF-β1, may then lead to a feedback inhibition of TIMP-1 expression after an additional stimulus such as an overload, which may lead to a reduction in this gene's expression as seen here. As discussed earlier, it would also have been beneficial to have information regarding TIMP-1 expression in unstimulated cells, as this would have provided valuable information to explain these findings.

4.46 SUMMARY

- TGF-β1 down-regulates MMP-1 and 2 expression, with no effect on expression of MMP-9 and 13 in all of the cell types investigated. This is consistent with thte results of previous workers.

- TGF-β1 causes an up-regulation of TIMP-2 activity in the case of carpal ligament and Dupuytren's cord derived cells.

- The finding of a reduction in TIMP-1 expression in all cases may be explained by a feedback inhibition response resulting from stimulation by both mechanical and growth factor derived cues.

- It is postulated that TGF-β1 acts to dampen down the matrix degrading activity in order to allow matrix deposition to begin.

Given the above findings, investigation of the genes responsible for matrix deposition (collagen I and collagen III) was the next step in the elucidation of matrix deposition in Dupuytren's disease.

4.5 MESSENGER RNA SIGNALLING FOR COLLAGEN I AND COLLAGEN III BY DUPUYTREN'S FIBROBLASTS EXPOSED TO MECHANICAL STIMULI AND TO TGF-B1

4.51 INTRODUCTION

Mechanical forces have been known to be important regulators of extracellular matrix homeostasis. The composition of the ECM is adapted according to the load that is applied through it (Flint and Poole 1990). Previously we looked at the molecular signaling and regulatory mechanisms involved in matrix breakdown. In this section we aimed to establish the effects of mechanical strain on the genes responsible for collagen production.

Collagen type I is the predominant form of collagen in normal palmar fascia, with both type I and type III seen in Dupuytren's disease lesions (Bailey *et al* 1977; Brickley-Parsons *et al* 1981). As mentioned previously the stimulus for type III collagen production remains unexplained, compared to its disappearance in a healing wound. In addition to this it is unknown exactly which factors are involved in the continued deposition of collagen I and III in the Dupuytren's cord. By examining collagen expression in fibroblasts given set mechanical cues, the stimulus to this activity may be elucidated.

TGF-β induces the synthesis of many ECM proteins such as collagen, fibronectin and tenascin, and it is thought to inhibit the matrix degrading enzymes, thus playing an important role in fibrosis. TGF-β stimulates the COL1A2 promoter activity via the SMAD pathway, resulting in enhanced type I collagen gene expression and synthesis (Ghosh 2002). The effects of this growth factor on collagen expression under this experimental regime were investigated in this section.

4.52 AIMS

- To investigate the baseline expression of types I and III collagen by Dupuytren's fibroblasts and carpal ligament fibroblasts exposed to a static load.
- To determine the effects of underloading and overloading regimes on Collagen I and III gene expression.
- To determine the effects of TGF-β1 on collagen I and III expression.

4.53 HYPOTHESES

- Baseline expression of collagen I will be low in all cases exposed to static loads
- Baseline expression of Collagen III will be greater in the case of Dupuytren's fibroblasts
- A mechanical loading regime will up-regulate collagen III expression
- TGF-β1 expression will cause an up-regulation of both collagen I and collagen III expression

4.54 METHODS

As outlined earlier in section 2.6-2.8 (*Materials and Methods*), and in section 4.14 (*Results).*

4.55 RESULTS

The baseline expression of collagen I and of collagen III is fairly constant for all cell lines investigated (figure 4.13a and b), with little difference in expression between the collagen types. For collagen I relative band intensity was 0.65 (SEM ± 0.12) for control carpal ligament, 0.76 (SEM ± 0.06) for nodule, and 0.87 (SEM ± 0.08) for cord. There was no significant difference in levels of expression between cell types. For collagen III values were not significantly different to those of collagen I with a relative expression of 0.43 (SEM ± 0.11) for control carpal ligament, 0.75 (SEM ± 0.05) for nodule and 0.87 (SEM ± 0.18) for cord. It is however noted that collagen III expression was significantly greater for Dupuytren's nodule and cord in comparison to controls ($p<0.017$). This correlates with findings by previous workers investigating collagen III content in Dupuytren's tissue in comparison to normal fascia (Bailey *et al* 1977; Bazin *et al* 1981). It can be interpreted that this may represent an increase in matrix remodeling and new collagen deposition by these fibroblasts in Dupuytren's disease.

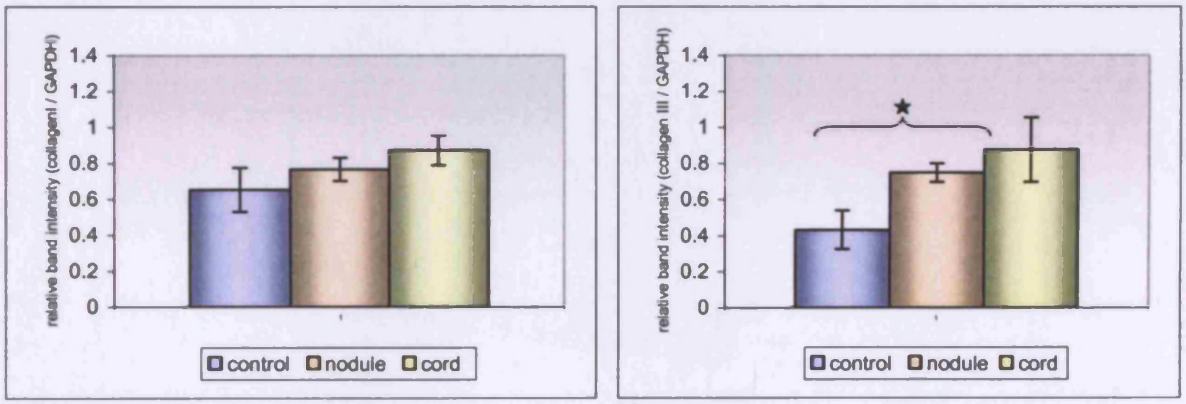


Figure 4.13a and b:- Expression of collagen I (a) and collagen III (b) by Dupuytren's nodule (n=5), cord (n=5) and carpal ligament fibroblasts (n=4) exposed to a static load only. Error bars represent standard errors of the mean ★ The star in fig 15b demonstrates a significantly higher level of expression of collagen III for Dupuytren's derived cells ($p<0.017$).

Following a series of uniaxial underloads, and overloads the expression of collagen I and III was examined again. For collagen I (figure 4.14), a mechanical underload leads to a decrease in collagen gene expression, although this trend was only significant in the case of Dupuytren's cord ($p<0.005$), with a fall in collagen I expression from 0.87 (SEM ± 0.08) to 0.43 (SEM ± 0.07). Mechanical overloading however led to no alteration in gene expression when compared to a static applied load, with no significant change in gene expression for any cell type investigated.

In contrast for collagen III (figure 4.15), mechanical load makes no significant difference to this gene's expression in any of the cell lines investigated.

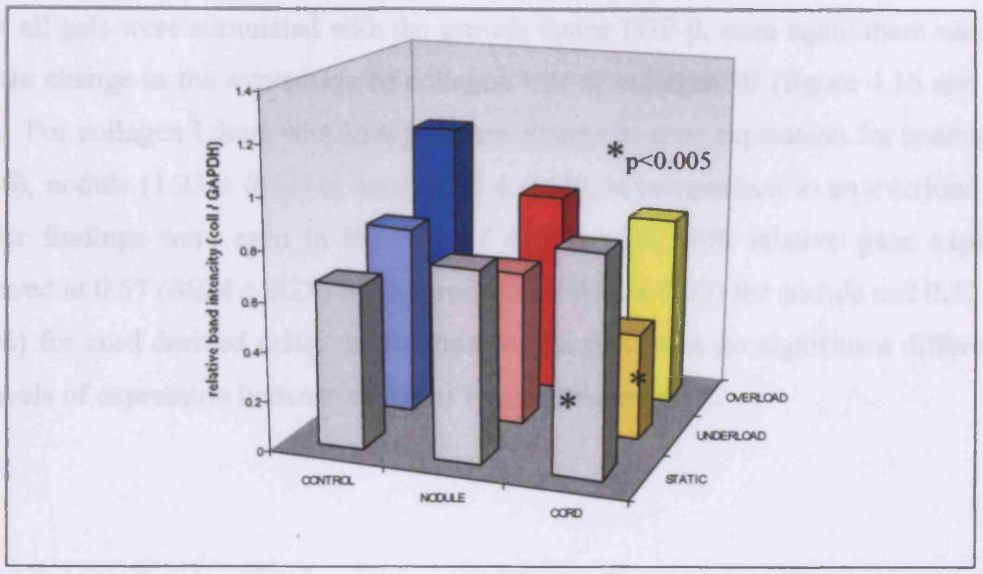


Figure 4.14:- Diagrammatic representation of collagen I expression by mechanically stimulated fibroblasts derived from Dupuytren's nodule, cord and carpal ligament. A comparison of static vs underloading and overloading reveals no significant difference in collagen I expression for control and nodule, but a significant fall for cord (*) after underloading

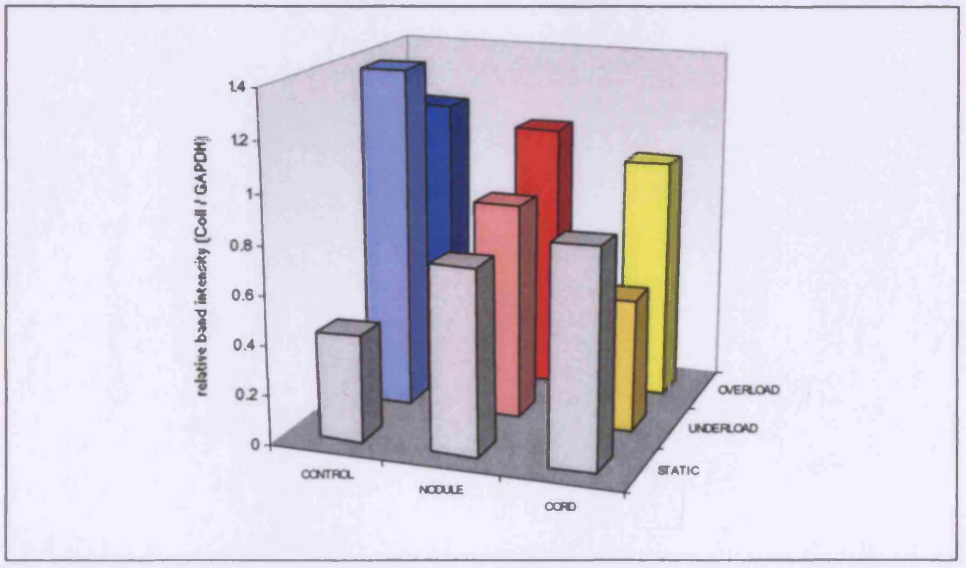


Figure 4.15:- Diagrammatic representation of collagen III expression by mechanically stimulated fibroblasts derived from Dupuytren's nodule, cord and carpal ligament. A comparison of static vs. underloading and overloading reveals no significant difference in collagen III expression

When all gels were stimulated with the growth factor TGF-β, once again there seemed to be little change in the expression of collagen I or of collagen III (figure 4.16 and figure 4.17). For collagen I there was no significant change in gene expression for control (0.95 ± 0.46), nodule (1.23 ± 0.42) or cord (0.76 ± 0.18), in comparison to an overload alone. Similar findings were seen in the case of collagen III, with relative gene expression measured at 0.67 (SEM ± 0.25) for control, 0.81 (SEM ± 0.23) for nodule and 0.32 (SEM ± 0.06) for cord derived cells. In addition to this there was no significant difference in the levels of expression between collagen I and collagen III.

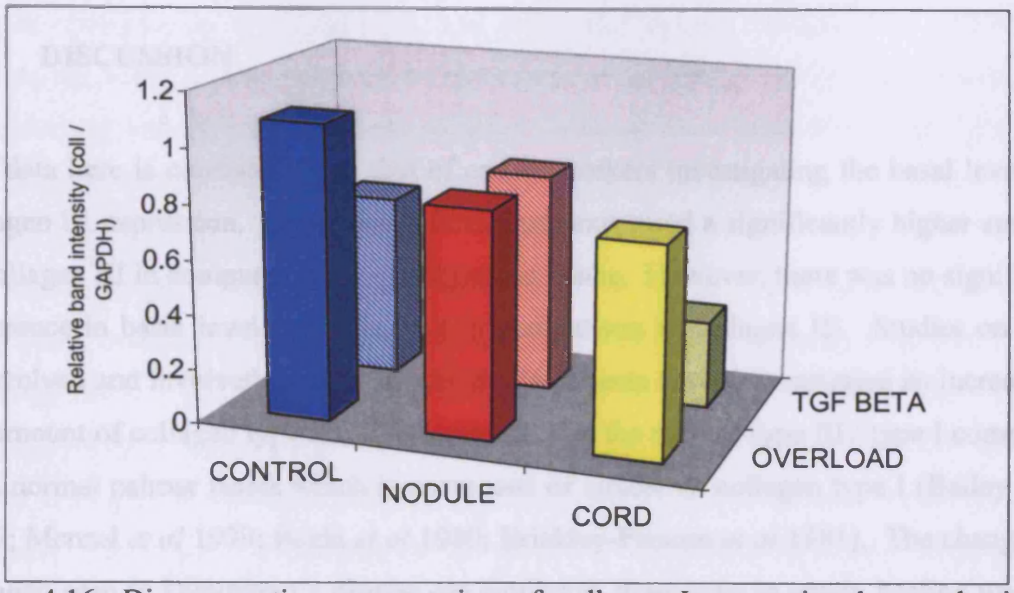


Figure 4.16: Diagrammatic representation of collagen I expression by mechanically stimulated fibroblasts derived from Dupuytren's nodule, cord and carpal ligament. A comparison of overloading and overloading with TGF-β1 stimulation reveals no significant difference in collagen I expression

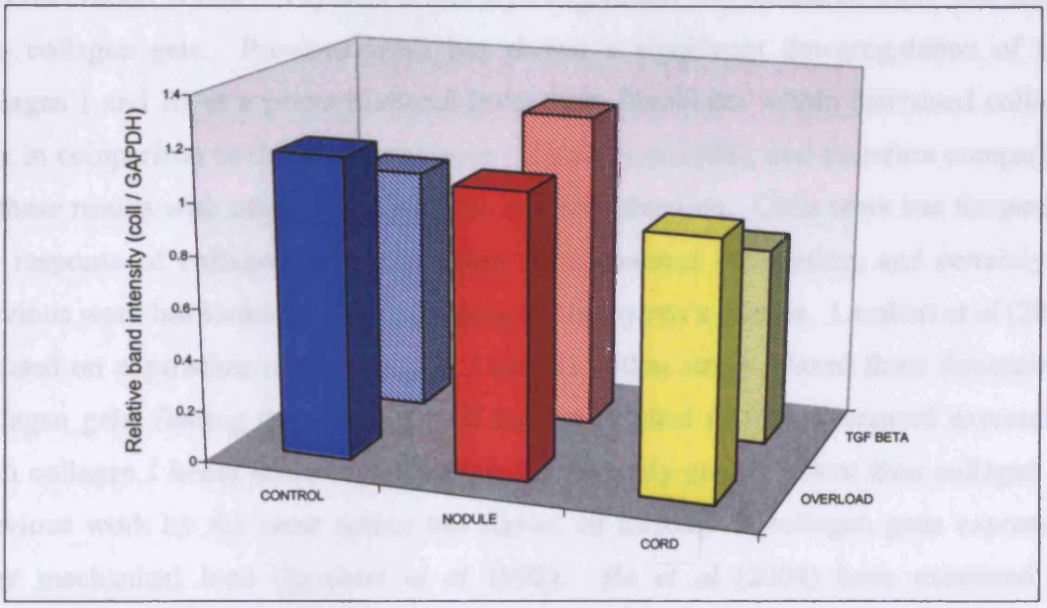


Figure 4.17: Diagrammatic representation of collagen III expression by mechanically stimulated fibroblasts derived from Dupuytren's nodule, cord and carpal ligament. A comparison of overloading and overloading with TGF-β1 stimulation reveals no significant difference in collagen III expression

4.56 DISCUSSION

The data here is consistent with that of earlier workers investigating the basal levels of collagen III expression. Dupuytren's fibroblasts expressed a significantly higher amount of collagen III in comparison to normal palmar fascia. However, there was no significant difference in basal levels of collagen I in comparison to collagen III. Studies on both uninvolved and involved fascia of Dupuytren's patients have demonstrated an increase in the amount of collagen type III, with an increase in the ratio of type III / type I compared with normal palmar fascia which is composed of almost all collagen type I (Bailey *et al* 1977; Menzel *et al* 1979; Bazin *et al* 1980; Brickley-Parsons *et al* 1981). The changes in collagen seen in Dupuytren's disease are similar to those seen in newly healing wounds and in embryogenesis.

An observation in this test system is that gene expression was measured from cells seeded into collagen gels. Previous work has shown a significant downregulation of both collagen I and III at a pretranslational level from fibroblasts within restrained collagen gels in comparison to those in monolayer (Mauch *et al* 1988), and therefore comparison of these results with others must take this into consideration. Little work has focused on the response of collagen gene expression to mechanical stimulation, and certainly no previous work has looked at this in the case of Dupuytren's disease. Lambert *et al* (2001) focused on expression of procollagens I and III within stress relaxed three dimensional collagen gels, finding that relaxation of tension resulted in their decreased expression, with collagen I being down-regulated to a significantly greater extent than collagen III. Previous work by the same author has shown an increase in collagen gene expression after mechanical load (Lambert *et al* 1992). He *et al* (2004) have examined the expression of collagen I in periodontal ligament fibroblasts in response to both compression and mechanical loading in a cell monolayer system, finding that a 10% compression resulted in a decrease in expression whereas 10% mechanical loading increased expression. Similarly Xu *et al* (1999) displayed similar up-regulation of collagen I in response to intermittent load in fibroblasts, while Swartz *et al* (2001)

showed upregulation of collagen III, and to a lesser degree collagen I in response to mechanical load in lung fibroblasts. Kessler *et al* (2001) aptly summarized that mechanically loaded fibroblasts appear to be activated to a "synthetic" phenotype with the induction of collagen gene expression.

The limitation with many studies investigating changes in ECM gene expression in response to mechanical load is that in some cases changes in expression have only been observed after several days (Chiquet *et al* 2003). In the case of this work, we have investigated only one time point i.e. at 24 hours. It can therefore be postulated in the case of the collagen genes that up-regulation may have occurred if the experiment had continued over a more prolonged period of time. Within the short time scale of this experiment, important regulatory changes may not have occurred. It was beyond the scope of this project to look at this at later time points, but certainly this is an area that may merit further work in the future.

With regards to mechanical stimulation and collagen I gene expression, the results here correspond to those in the literature for a reduction in mechanical load, with down-regulation of collagen I expression. However in contrast there was no up-regulation when an overloading regime was applied. This may have occurred for several reasons. It is postulated that in the control samples, static loading of cells is sufficient to cause a change in collagen I gene expression already without the added stimulus of an overload, and thus no change in gene expression would be noted. In order to confirm this one would need to measure gene expression in gels with no mechanical load applied at all. As described by Chiquet et al (2003), in principal there are three different mechanisms by which a gene may be regulated by mechanical signals. Firstly a cellular mechanotransduction pathway may activate a transcription factor that binds to a "mechano-responsive" regulatory element in the gene promoter. In a secondary response, a mechanical signal would first induce the transcription and synthesis of a nuclear factor, which then transactivates a specific gene. The third mechanism is that mechanical stress might induce the synthesis or secretion of a growth factor that indirectly regulates gene expression via an autocrine or paracrine feedback loop. It is

possible that it is this third mechanism that controls collagen gene expression, which may explain the absence of a response to the overloading regime. If gene expression was observed over a longer time point a change may well be noticed. Certainly it has been shown that collagen I gene induction does depend upon autocrine release of TGF-β (Lindahl *et al* 2002).

TGF-β has been thought to stimulate fibroblasts to synthesise increased quantities of type I collagen (Montesano and Orci 1988; Alioto *et al* 1994). Certainly work by Reed et al (1994) has shown that TGF-β can stimulate an increase in mRNA expression of collagen I in dermal fibroblasts seeded into three dimensional collagen gels (after 44 hours of exposure). Alioto *et al* (1994) showed an increase in collagen synthesis in Dupuytren's fibroblasts in comparison to normal palmar fascia after exposure to TGF-β in a study of cells in monolayer. Interestingly there was a cut off in the dose of TGF beta used, whereby for normal fascia there was no further increase in collagen synthesis at doses greater than 0.3ng/ml, whereas this did not occur for Dupuytren's derived cells. Similarly work on human kidney mesangial cells has demonstrated an up-regulation of both collagen I and III after TGF-β stimulation in cell monolayer (Poncelet and Schnaper 1998). The results displayed here are in contrast to the studies above. It was shown here that TGF-β1 did not cause any significant change in collagen I or in collagen III gene expression when simultaneously mechanically overloaded. It is possible that because the cells were prestimulated with TGF-β1 for three days prior to contraction in a collagen gel, that a feedback down regulation of collagen expression may have occurred during that time. In addition it may be that a combination of both the mechanical stimulus plus TGF-β results in no change to gene expression as the two stimuli may act in opposing ways. Further work is required to answer the questions raised by these results.

MMPs not only play a role in matrix degradation but they also modulate collagen synthesis. The end result is often an increase in MMPs accompanied by increased fibrosis such as has been demonstrated most often in the failing heart (Li *et al* 1999; Li *et al* 2000). Collagen expression was increased after intermittent mechanical strain of fetal rat lung cells, without affecting the expression of MMPs or TIMPs (Xu *et al* 1999). It

was suggested that mechanical load appears to result in an increase in ECM synthesis and that this is not due to decreasing activity of degradative enzymes.

In summary there is a very complex interplay of events occurring at a molecular level, when fibroblasts are mechanically stimulated. Although this work has provided information on the molecular events occurring at one moment in time, there is still a vast amount of information that is as yet unknown. More progress is yet to be made regarding how a mechanical signal is transferred via signaling pathways to activate certain genes, and how activation of these genes results in structural changes in the extracellular matrix.

4.57 SUMMARY

- Dupuytren's fibroblasts demonstrate a greater baseline expression of collagen III in comparison to normal palmar fascia

- Mechanical underloading results in a trend for downregulation of type I collagen expression in all cell types (although this remains significant only for Dupuytren's cord)

- There is no up-regulation of collagen expression after an overload in any cell type investigated. This is postulated to be related to a possible feedback loop effect.

- TGF-β1 does not appear to affect collagen gene expression within this experimental set up.

CHAPTER 5

THE QUANTIFICATION OF PERMANENT MATRIX REMODELLING BY DUPUYTREN'S FIBROBLASTS

5.1 INTRODUCTION

It has been postulated that contracture is a result of two separate processes occurring in parallel, firstly cell mediated contraction of the matrix, and secondly continuous matrix remodelling, leading to permanent contracture (Harris *et al* 1981; Flint and Poole 1990; Tomasek *et al* 2002). Much research has focused on the first process, but very little has focused directly on the second. Some studies have looked at collagen fibril orientation and alignment (Bell *et al* 1979), whereas others have studied collagen non-covalent bonding, and biochemical assays of collagen turnover (Guidry and Grinnell 1985) in order to understand the process of matrix remodelling. Other work has examined contraction in stress-relaxed circular collagen gels with and without the disruption of the cellular actin cytoskeleton by the drug cytochalasin-D (see *Introduction 1.21*). Guidry and Grinnell (1985) found complete reversibility of contraction in fibroblast seeded circular collagen gels after the addition of cytochalasin-D indicating that no permanent physical change had occurred to the gel i.e. the diameter of the gel was the same before contraction at the start of the experiment, as it was after cellular contraction had been eliminated. A later study (Grinnell and Ho 2002) demonstrated a permanent shortening of the matrix even when cellular contractile action was eliminated. These studies were limited because they did not involve any method of quantifying the strength or extent of the matrix shortening that had occurred.

Recently a tissue engineered model of collagen remodelling under tension based on a three dimensional collagen matrix has been developed that is capable of quantifying the extent of spatial remodelling (Marenzana *et al* 2004). They used the culture force monitor model to quantify the force generated within a fibroblast populated collagen lattice in a rat tendon model. They distinguished cell dependant force from cell independent force by the addition of cytochalasin-D, which eliminated the cell-dependant element of contraction (figure 5.1). The physical shortening of the matrix persisted even after cellular contraction was eliminated. This physical shortening has been termed the residual matrix tension (RMT). Matrix remodeling was identified as early as 18 hours and increased with time so that by 65 hours 45% of the maximal force generated was

retained within the gel after inactivation of the actin cytoskeleton. When these cells were incubated with TGF-β1, the amount of remodeling increased by over 100% after the 18-hour time point.

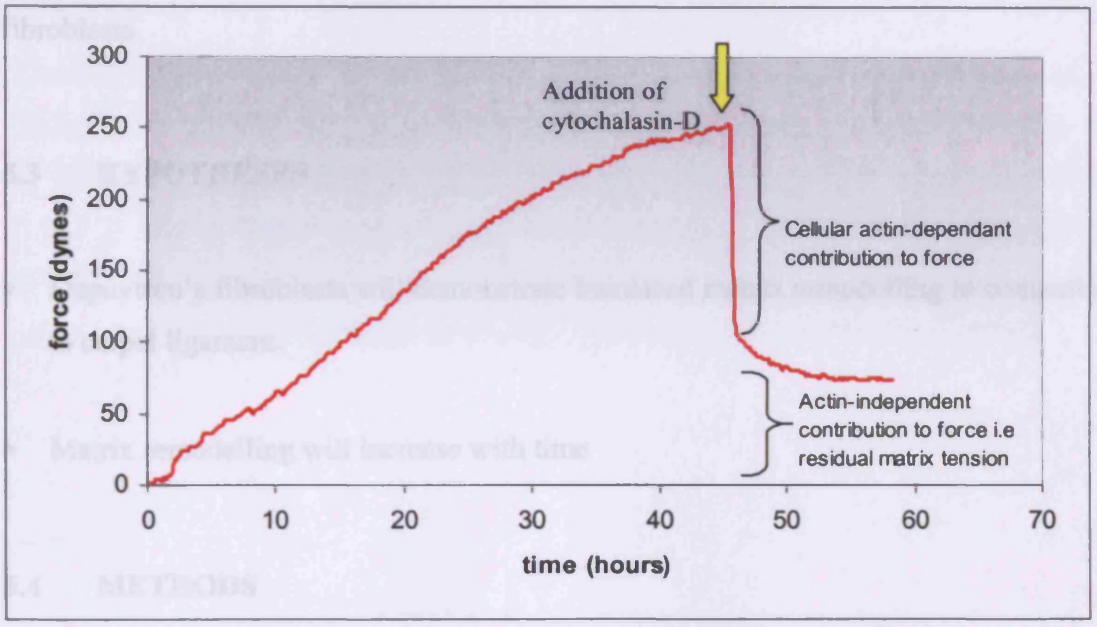


Figure 5.1:- A typical contraction profile of a human fibroblast cell line demonstrating the cellular and remodelled components of force generation. Note that force is rapidly generated by the fibroblasts and this increases with time reaching a maximum force of 250 dynes at 48 hours. At this time point cytochalasin-D is added and it can be seen that force rapidly decreases, and then gradually plateaus leaving a residual measured force of 70 dynes.
The sudden drop is known as the active cellular actin dependant component of contraction. The residual measured force is therefore the actin independent "remodelled" residual matrix tension. This demonstrates an accurate way to measure the amount of matrix remodelling a cell type can produce (Tomasek et al 2002; Marenzana et al 2004 in press).

These studies were the first to directly measure matrix remodelling in a collagen gel system, rather than using indirect measurements of this process, and the model supported the theory that only limited new collagen is required for matrix shortening or remodeling. The work outlined below is the first to directly measure matrix remodelling in Dupuytren's tissue.

5.2 AIMS

To investigate the contribution to tissue contracture made by matrix remodelling in Dupuytren's fibroblasts in comparison to that of normal palmar fascia and that of dermal fibroblasts

5.3 HYPOTHESES

- Dupuytren's fibroblasts will demonstrate increased matrix remodelling in comparison to carpal ligament.

- Matrix remodelling will increase with time

5.4 METHODS

Fibroblast populated collagen lattices were set up as described previously. The lattices were allowed to set for 30 minutes at 37°C in 5% CO_2 prior to floatation in 20ml of normal growth media and insertion into the culture force monitor. In half of the test cases of Dupuytren's fibroblasts the media was supplemented by the addition of ascorbic acid to give an overall concentration of 50μg/ml. Three separate experiments were conducted.

In the first the collagen lattices were left to contract over a period of 8 hours. At this point the maximum generated force was noted, and then a single dose of cytochalasin-D (Sigma, Poole, Dorset, UK), 20μl of 60mM in 0.5ml of normal growth media was added to the normal growth media filled chamber to give an overall concentration of 60 μM within the chamber in order to abolish cell mediated force generation. This was added rapidly in order to minimise disruption to the incubator temperature and CO_2 levels. Throughout this period force measurements were continuously recorded by the culture

force monitor in real time, and these were continued for at least 4 hours after addition of cytochalasin-D. The above process was followed in separate experiments occurring at 24 hours and at 48 hours in order to investigate the effects of incubation time on matrix remodelling.

Residual matrix tension (RMT) was determined by the measurement of force on the culture force monitor two hours following the addition of cytochalasin-D to the system by which time the drop in measured force had plateaued (*Review of the Literature section 1.20-1.2; Materials and Methods Section 2.35).* Collagen gels were fixed under tension in 4% paraformaldehyde before processing for light and electron microscopy as outlined previously (*Materials and Methods Section 2.36-2.5).*

5.5 RESULTS

5.51 Calculation of appropriate dose of cytochalasin-D

Cytochalasin-D was added to the experiments resulting in a rapid reduction in force as the actin cytoskeleton was disrupted. In order to calculate the correct dose that ensures complete disruption of the actin cytoskeleton a set of experiments was run adding a single dose of 20μM cytochalasin-D at 24 hours. There was an initial drop in force, which plateaued (Fig. 5.2). After 1 hour a second 20μM dose of cytochalasin-D was added and there was a further drop in force, which again plateaued. This was repeated again and a further drop noted. After this further doses of cytochalasin-D were added to the system with no further drops. It was at this point that it was felt that the actin cytoskeleton had been completely disrupted. Thus the concentration required to completely inactivate the actin cytoskeleton for Dupuytren's fibroblasts was calculated to be at 60μM. Confirmation of disruption of the actin cytoskeleton was visualised both via light microscopy and electron microscopy (as shown overleaf) (figure 5.3-5.6).

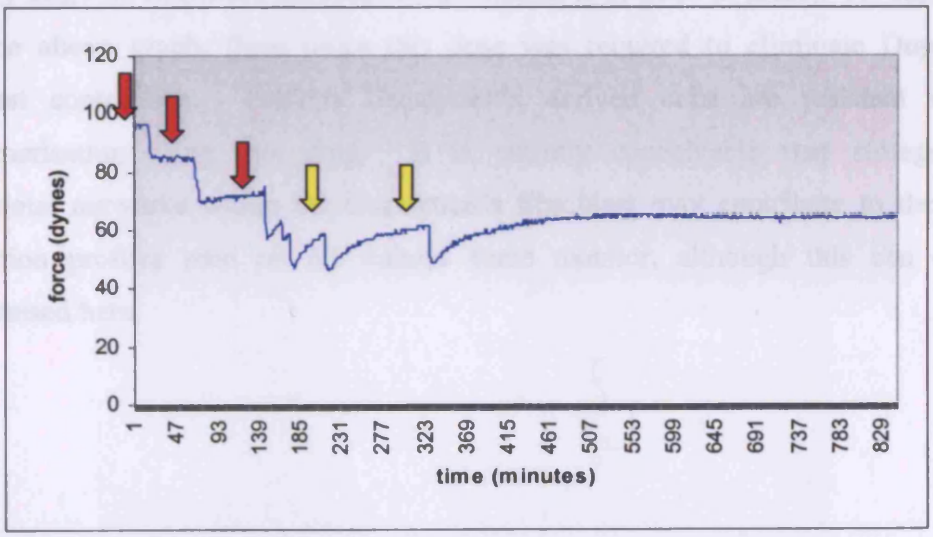


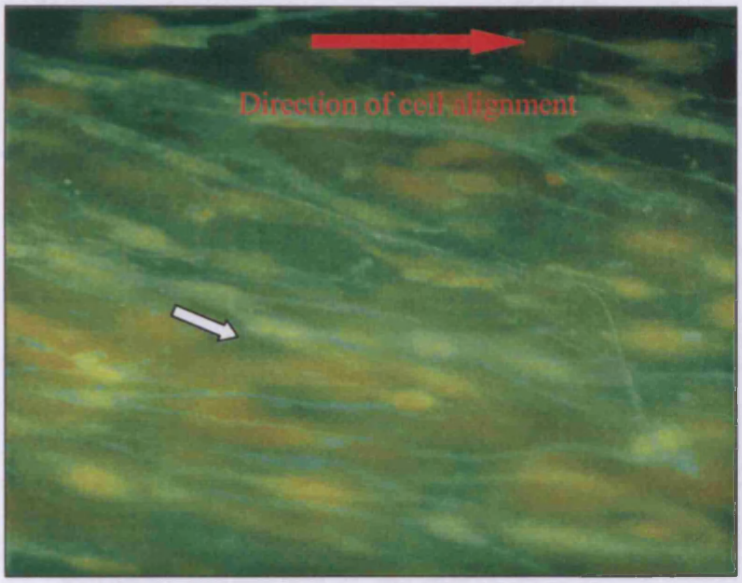
Figure 5.2:- Contraction profile of a Dupuytren's cord cell line after 24 hours demonstrating the effect of addition of 20μM increments of cytochalasin-D to the media. Red arrows indicate that there is a drop in measured force after each addition. However it can be seen that after further doses of cytochalasin-D (yellow arrows) that the measured force eventually plateaus.

These results were confirmed by performing a second set of experiments where the cytochalasin-D was added to the gel within the culture force monitor, and this was followed by the replacement of media with the addition of distilled water in order to perform hypotonic lysis of the cells. There was no further drop in force after the addition of distilled water.

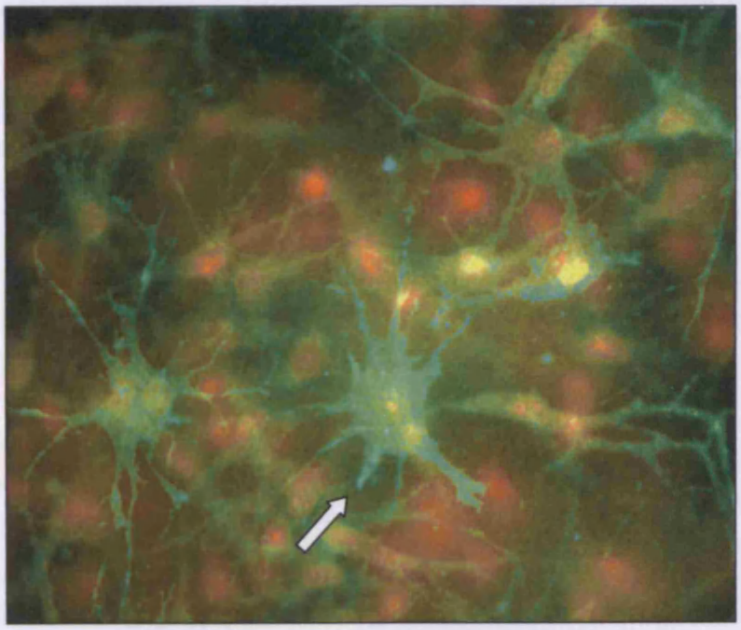
To finally confirm, another set of experiments were run, where instead of addition of cytochalasin-D, a 0.5 ml aliquot of simple normal growth media was added to the chamber. This confirmed that there was very minimal disruption to the system by opening the incubator door and adding fluid to the chamber.

The saturating dose of cytochalasin-D used here is much greater than that used in other experimental procedures on dermal, and rat tendon fibroblasts (Wakatsuki *et al* 2000; Grinnell and Ho 2002, Marenzana *et al* 2004 in press). Previously a dose of 20μg / ml was found to be sufficient to eliminate cell mediated contraction in rat tendon fibroblasts, and only 2μg / ml in dermal fibroblasts (Marenzana *et al* 2004 in press). As can be seen from the above graph, three times this dose was required to eliminate Dupuytren's fibroblast contraction. Perhaps Dupuytren's derived cells are resistant to actin depolymerisation using this drug. It is entirely conceivable that stronger actin cytoskeletal networks within the Dupuytren's fibroblast may contribute to the greater contraction profiles seen on the culture force monitor, although this can only be hypothesised here.

Figure 5.3 a and b. Immunohistochemical staining of fibroblast seeded collagen gels for α-smooth muscle actin (green fluorescence) before and after the addition of cytochalasin-D.

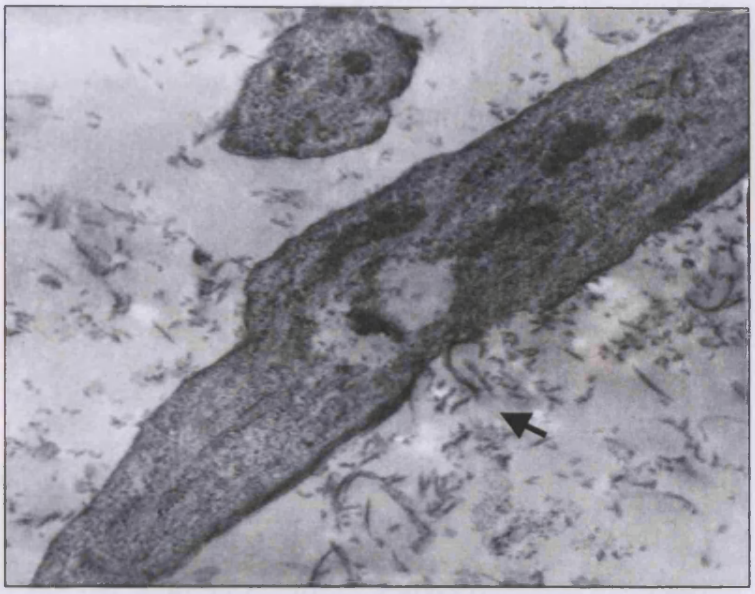


a) Dupuytren's fibroblasts aligned longitudinally along the collagen gel before disruption of the actin cytoskeleton (white arrow).

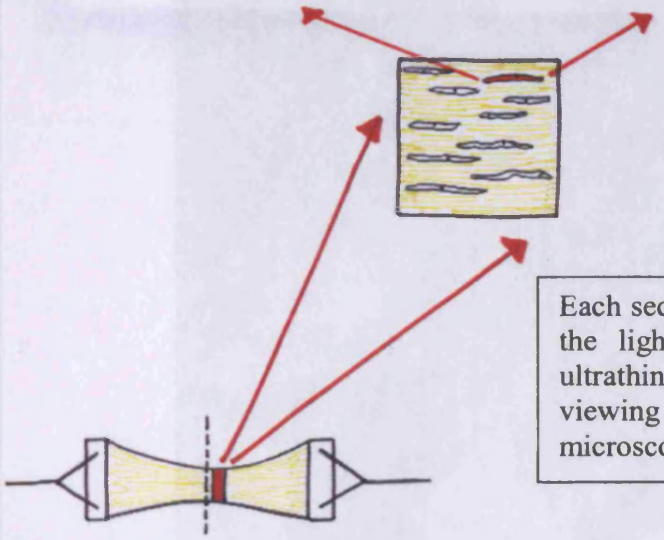


b) Dupuytren's fibroblasts after the addition of cytochalasin-D to the media. Note the cells have become rounded, and the actin filaments have become disrupted (white arrow).

Figure 5.4: Transmission Electron Micrograph of a Dupuytren's fibroblast allowed to contract within a collagen gel, without the addition of cytochalasin-D (x 8000)



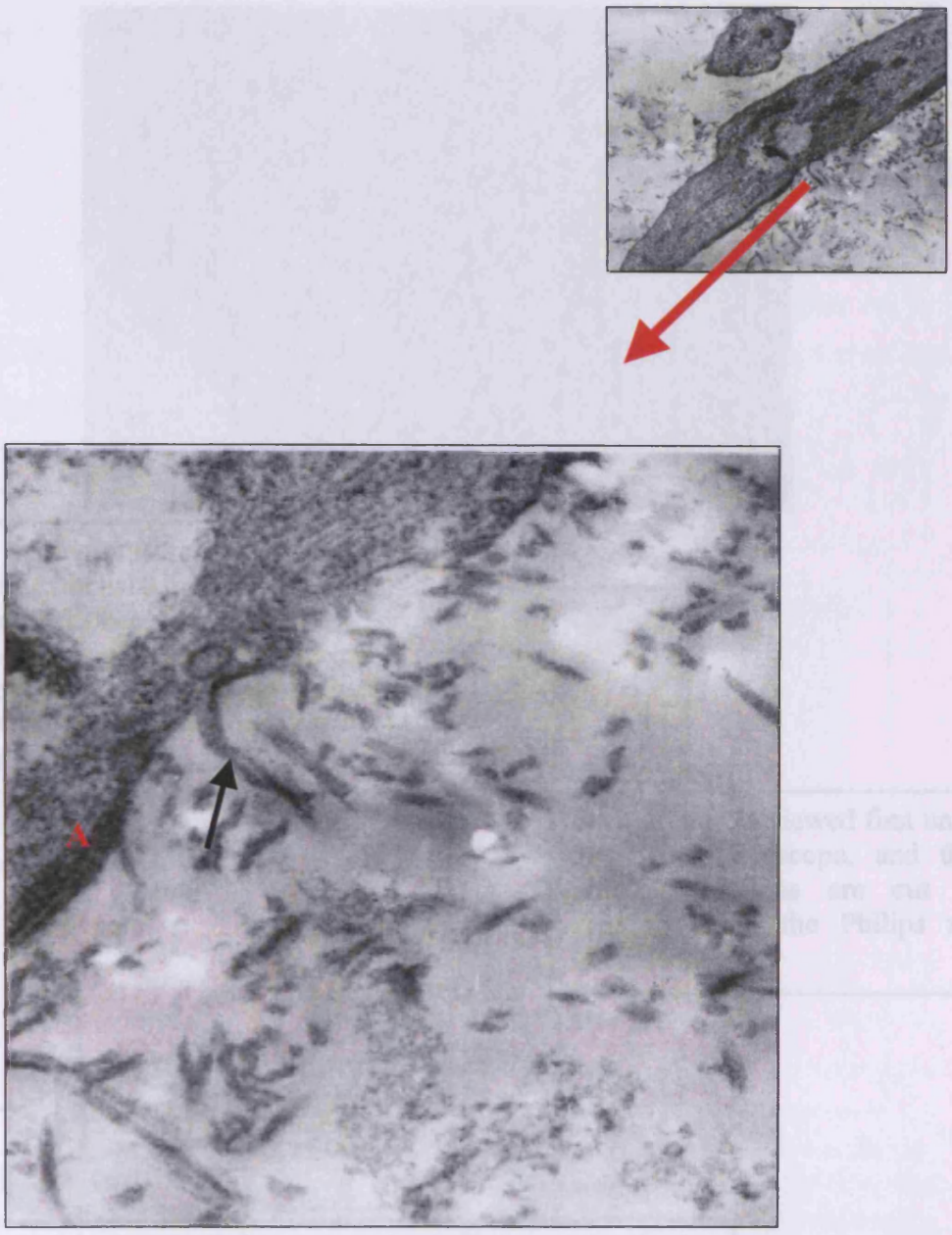
TEM of a Dupuytren's fibroblast before the addition of cytochalasin-D. Note the elongated appearance of the cell. In addition note the interaction of the cell (arrow) with the surrounding collagen fibrils



Each section is viewed first under the light microscope, and then ultrathin sections are cut for viewing under the Philips x12 microscope

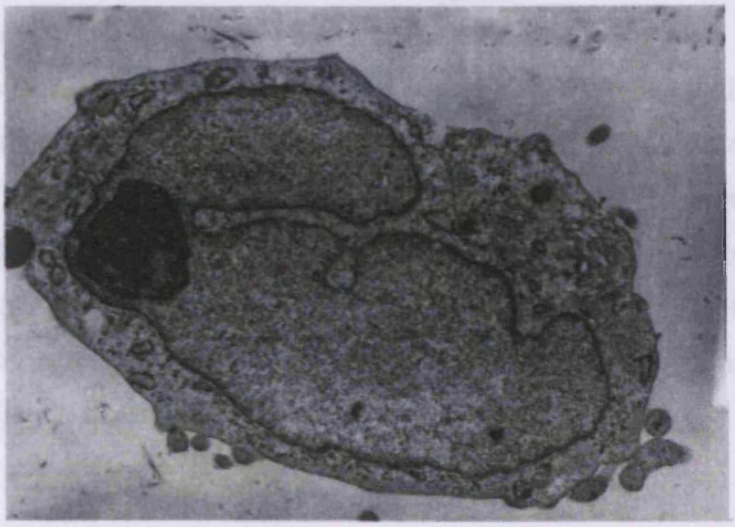
Collagen gel is removed from the culture force monitor and a small segment (seen in red) is excised and processed for TEM.

Figure 5.5: TEM magnified view of a Dupuytren's fibroblast demonstrating the interaction of the cell with the surrounding extracellular matrix at the fibronexus

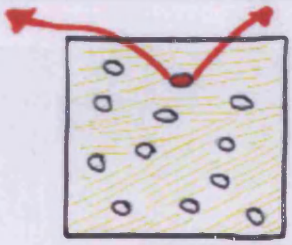


TEM shows the surface of the fibroblast interacting with the surrounding type I collagen matrix. Note the bundle of filamentous extracellular material extending from the fibroblast into the matrix (arrow), otherwise known as the fibronexus. This is closely associated with the intracellular actin microfilaments (A) (x 16,000) (*as first described by Tomasek et al 1987 in embedded tissue specimens; and later in 1991)*

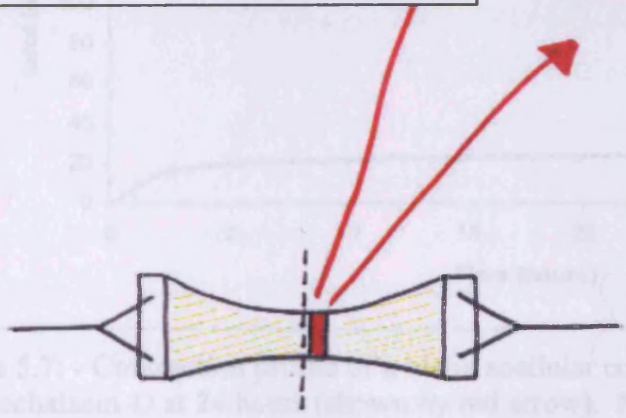
Figure 5.6: Transmission Electron Micrograph of a Dupuytren's fibroblast after addition of cytochalasin-D at 48 hours. (X 8000)



TEM of a single fibrobroblast after addition of cytochalasin-D to the gel. Note the cell has become rounded, and the lack of stress fibres. There are no pseudopodia, and the cell is no longer able to exert a tractional force on its surrounding matrix



Each section is viewed first under the light microscope, and then ultrathin sections are cut for viewing under the Philips x12 microscope



Collagen gel is removed from the culture force monitor and a small segment (seen in red) is excised and processed for TEM.

5.52 EFFECT OF CYTOCHALASIN-D ON BLANK ACELLULAR GELS

A typical contraction profile of an acellular blank gel is shown in the figure below with addition of cytochalasin-D at 24 hours (Fig. 5.7). It can be seen that as the cytochalasin-D is added there is a reduction in measured force, which quickly recovers, and the force returns to the measured value recorded prior to interruption of the system. This measured flux in force is due to the opening of the incubator door and addition of fluid to the system that is recorded by the sensitivity of the force transducer. This result indicates that the cytochalasin-D has very little effect on the force generation by a simple collagen gel and that this chemical does not alter the stiffness of the collagen matrix.

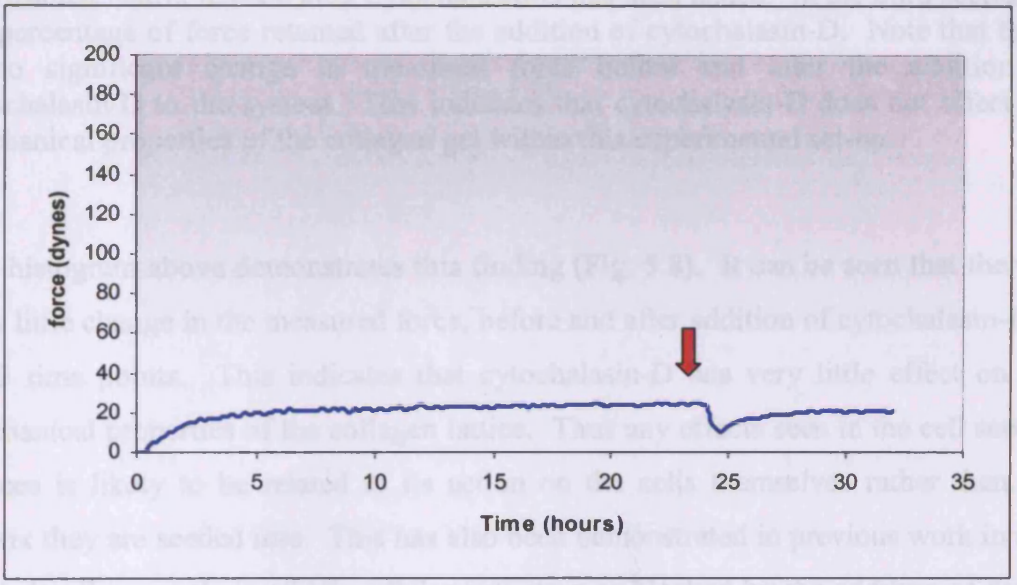


Figure 5.7: - Contraction profile of a blank acellular collagen lattice with the addition of cytochalasin-D at 24 hours (shown by red arrow). Note the slow recovery from the initial drop in force as the chemical is added. The drop in force seen was postulated to be due to the sensitivity of the force transducer to the addition of fluid to the culture force monitor mould.

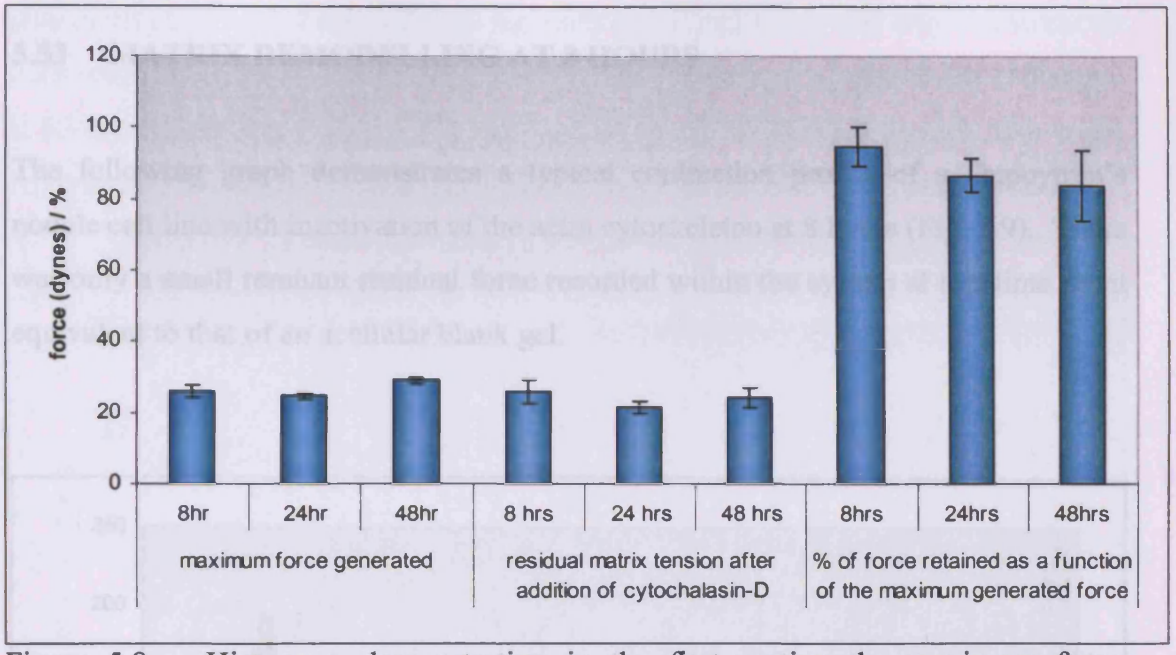


Figure 5.8: - Histogram demonstrating in the first section the maximum force generated at 8, 24 and 48 hours for n=3 acellular blank gels. In the second section is the residual matrix tension after cytochalasin-D has been added. In the third section is the percentage of force retained after the addition of cytochalasin-D. Note that there is no significant change in measured force before and after the addition of cytochalasin-D to the system. This indicates that cytochalasin-D does not affect the mechanical properties of the collagen gel within this experimental set-up.

The histogram above demonstrates this finding (Fig. 5.8). It can be seen that there is very little change in the measured force, before and after addition of cytochalasin-D at all 3 time points. This indicates that cytochalasin-D has very little effect on the mechanical properties of the collagen lattice. Thus any effects seen in the cell seeded lattices is likely to be related to its action on the cells themselves rather than the matrix they are seeded into. This has also been demonstrated in previous work in cell seeded collagen gels in which cellular activity was blocked by the addition of hypo-osmotic media sufficient to cause cell lysis (Marenzana *et al* 2004 in press). The addition of cytochalasin-D to this system had no additional effect on residual matrix tension.

5.53 MATRIX REMODELLING AT 8 HOURS

The following graph demonstrates a typical contraction profile of a Dupuytren's nodule cell line with inactivation of the actin cytoskeleton at 8 hours (Fig. 5.9). There was only a small remnant residual force recorded within the system at this time point equivalent to that of an acellular blank gel.

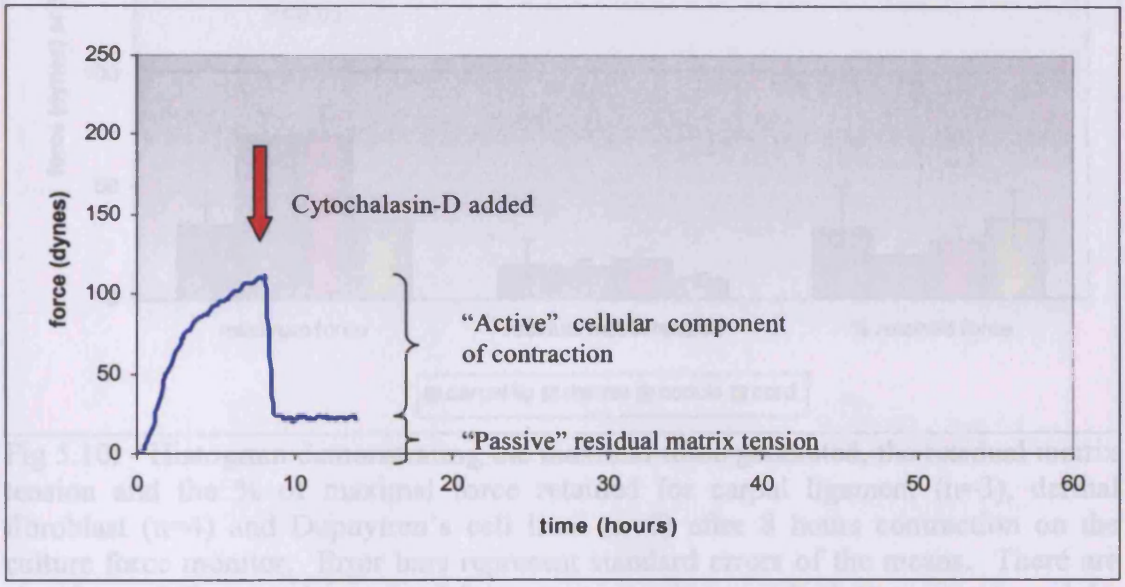


Figure 5.9: - Contraction profile for a Dupuytren's fibroblast seeded collagen gel with cytochalasin-D added (red arrow) at 8 hours. There is a fall in generated force to 20 dynes, the equivalent of a profile for a blank acellular gel. Note the two phases of force generation – the active cellular component which is eliminated by the cytochalasin-D, and the passive residual matrix tension which is the force recorded by the culture force monitor once the cellular component is eliminated.

The following histogram demonstrates the effects of inactivation of the actin cytoskeleton on various cell lines after they are allowed to contract for 8 hours (Fig. 5.10). The maximal force generated at this time point is significantly greater for Dupuytren's nodule and dermal fibroblast cell lines (force = 72.6 dynes; SEM ± 13 and 11) in comparison to that of Dupuytren's cord (35 dynes SEM ± 7) and carpal ligament (33 dynes SEM ± 8).

However the measured force i.e. the residual matrix tension (RMT) within the system after addition of cytochalasin-D was minimal for all cell types, and was equivalent to the baseline blank acellular gel (Fig 5.7; fig 5.9). There was no significant difference in RMT between any cell type. Thus it may be concluded that at this early time point there was no evidence of permanent matrix remodelling within the collagen matrix.

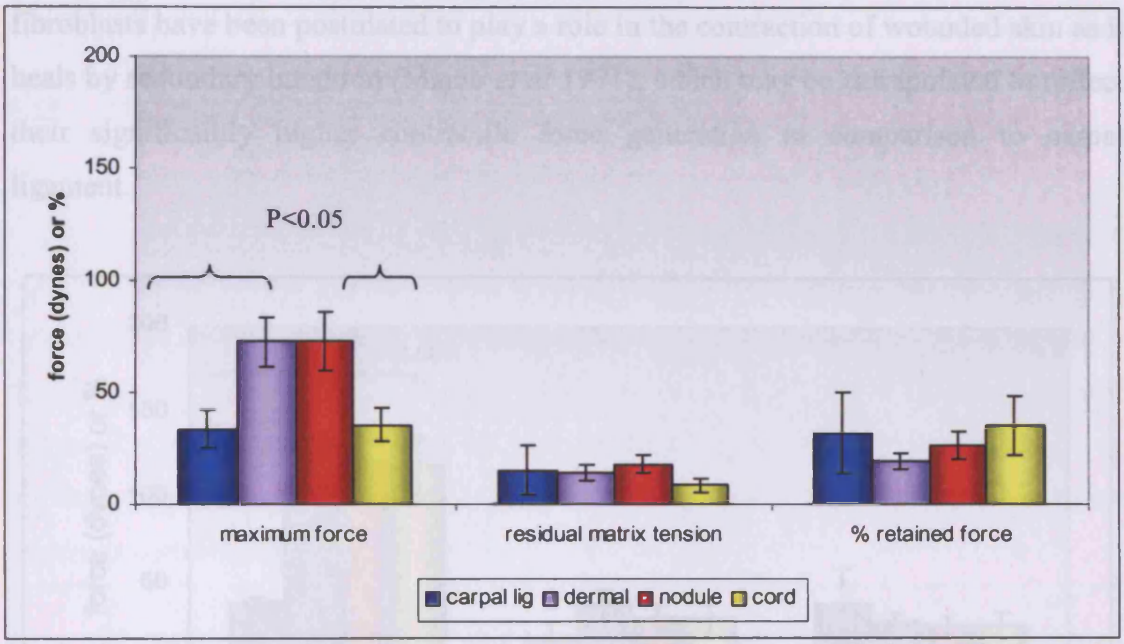


Fig 5.10: - Histogram demonstrating the maximal force generated, the residual matrix tension and the % of maximal force retained for carpal ligament (n=3), dermal fibroblast (n=4) and Dupuytren's cell lines (n=7) after 8 hours contraction on the culture force monitor. Error bars represent standard errors of the means. There are significant differences in maximal force generation between both Dupuytren's nodule, carpal ligament and Dupuytren's cord groups. Similarly these differences are also significant for the dermal fibroblast cell lines ($p<0.05$)

5.54 MATRIX REMODELLING AT 24 HOURS

By 24 hours the maximal force generated for carpal ligament cell lines has reached a plateau at 39 dynes (SEM ± 2), as has that for the dermal fibroblasts at 130 dynes (SEM ± 20). However both Dupuytren's nodule and cord cell lines continue to contract, reaching maximal forces of 120 dynes (SEM ± 13) and 118 dynes (SEM ± 17) respectively. There is a significant difference in maximum force between Dupuytren's and dermal cell lines in comparison to carpal ligament ($P<0.001$)(Fig.

5.11). The significantly lower level of contraction of carpal ligament fibroblasts has been documented previously (Bisson *et al* 2004), and was proposed to be due to these cells location in a stress-shielded area of matrix in-vivo. These values for dermal fibroblasts correspond with previous work using the culture force monitor model (Eastwood *et al* 1996; Brown *et al* 1998), and demonstrates the similar contractile ability of dermal fibroblasts in comparison to Dupuytren's fibroblasts. Dermal fibroblasts have been postulated to play a role in the contraction of wounded skin as it heals by secondary intention (Majno *et al* 1971), which may be extrapolated to reflect their significantly higher contractile force generation in comparison to carpal ligament.

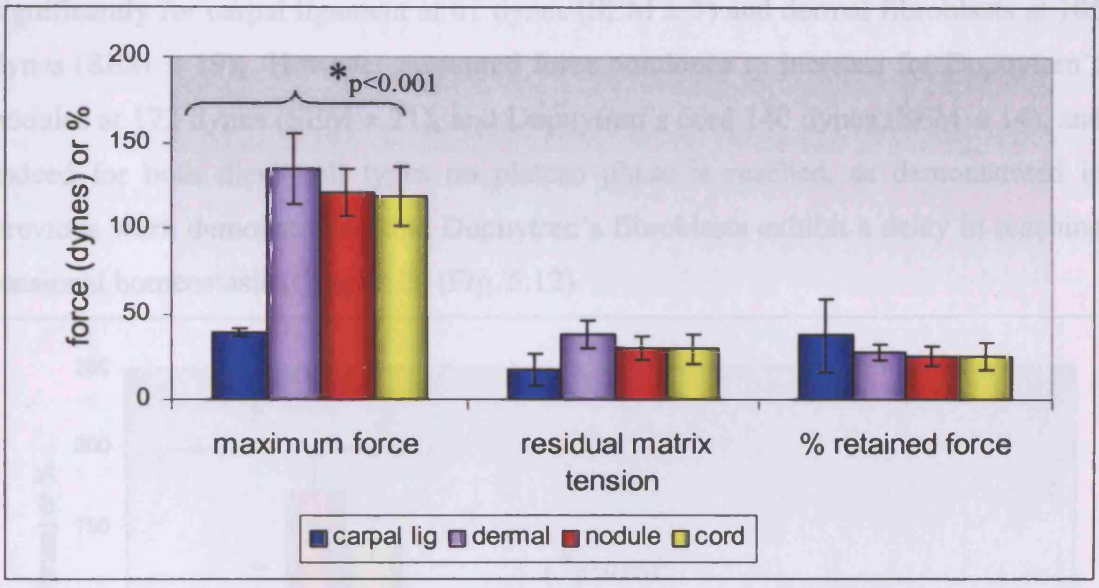


Figure 5.11: - Histogram demonstrating the maximal force generated, the residual matrix tension and the % of maximal force retained for carpal ligament (n=3), dermal fibroblast (n=4) and Dupuytren's cell lines (n=7) at 24 hours. Error bars represent standard errors of the means. There are significant differences in maximal force generation between both Dupuytren's nodule, carpal ligament and Dupuytren's cord groups. Similarly these differences are also significant for the dermal fibroblast cell lines ($p<0.001$). There is no significant difference in residual matrix tension between cell lines.

The residual matrix tension for all cell types at 24 hours is significantly higher that that at 8 hours ($p<0.05$), although there is no significant difference between cell types. For carpal ligament cell lines there was a residual matrix tension of 16.9 dynes (SEM ± 9 dynes) that varied very little from the baseline force measured for a blank acellular gel. However for Dupuytren's nodule and cord cell lines the residual matrix

tension was 29.8 (SEM ± 6) and 29.6 (± 8) dynes respectively. The value for dermal fibroblasts was not significantly greater at 37 dynes (SEM ± 7). If these values are taken as a percentage of the maximal force generated at his time point there is a mean "remodelled" force of around 25 % for dermal fibroblasts and Dupuytren's fibroblasts.

5.55 MATRIX REMODELLING AT 48 HOURS

At the 48-hour time point it is noted that maximum generated force has not changed significantly for carpal ligament at 61 dynes (SEM ± 3) and dermal fibroblasts at 105 dynes (SEM ± 19). However measured force continues to increase for Dupuytren's nodules at 172 dynes (SEM ± 21), and Dupuytren's cord 140 dynes (SEM ± 14), and indeed for both these cell types no plateau phase is reached, as demonstrated in previous work demonstrating that Dupuytren's fibroblasts exhibit a delay in reaching tensional homeostasis (Chapter 3) (Fig. 5.12).

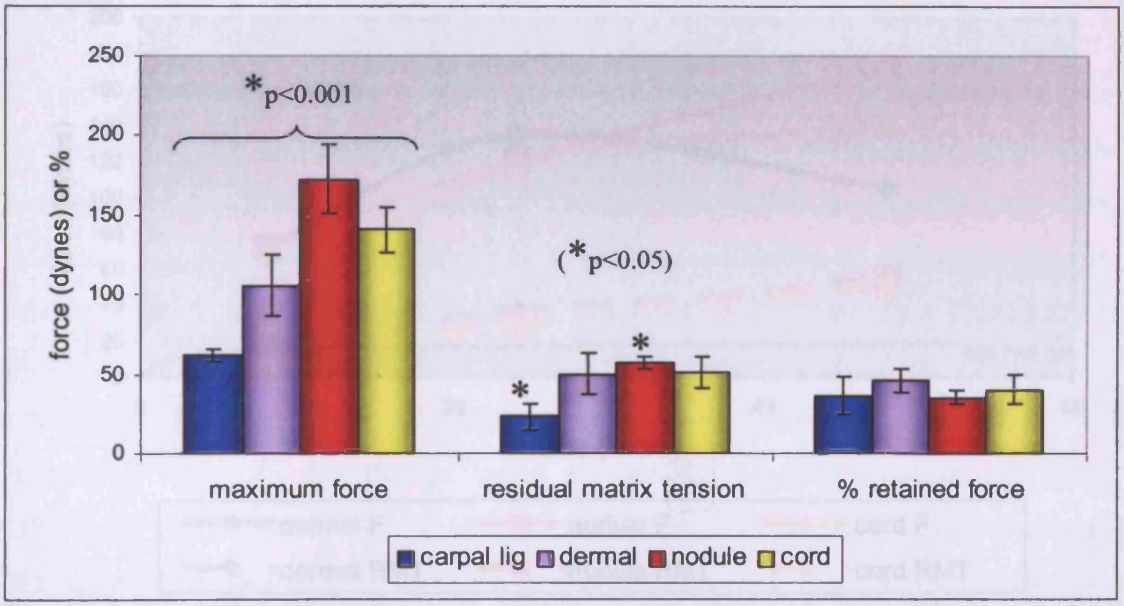


Figure 5.12: - Histogram demonstrating the maximal force generated, the residual matrix tension and the % of maximal force retained for carpal ligament (n=3), dermal fibroblast (n=4) and Dupuytren's cell lines (n=7) at 48 hours. Error bars represent standard errors of the means. There are significant differences in maximal force generation between both Dupuytren's nodule, carpal ligament and Dupuytren's cord groups. Similarly these differences are also significant for the dermal fibroblast cell lines ($p<0.001$). Dupuytren's nodules demonstrated a significant increase in residual matrix tension in comparison to carpal ligament ($p<0.05$).

The residual matrix tension at 48 hours for carpal ligament again remained minimal at 22 dynes (SEM ± 7.7), and no different from that of a blank acellular gel. It may be interpreted from this that these cells may be unable to permanently remodel a collagen matrix. It can be postulated that this is related to their low contractile force generation. For dermal fibroblasts RMT had increased to 49 dynes (SEM ± 12), for Dupuytren's nodules it was 56 dynes (SEM ± 3.5) and for cord it was 51 dynes (SEM ± 10). There was no significant difference in residual matrix tension between Dupuytren's fibroblasts and dermal fibroblasts, but Dupuytren's nodule fibroblasts remodelled the matrix significantly greater ($p<0.05$) than carpal ligament fibroblasts, and for cords this remodelling was approaching significance. For Dupuytren's cells this remodelling was 35% of the total force generated. For Dupuytren's derived cells it was noted that there was no significant difference in force generation or residual matrix tension at any time point if the cells were incubated with supplemented ascorbic acid.

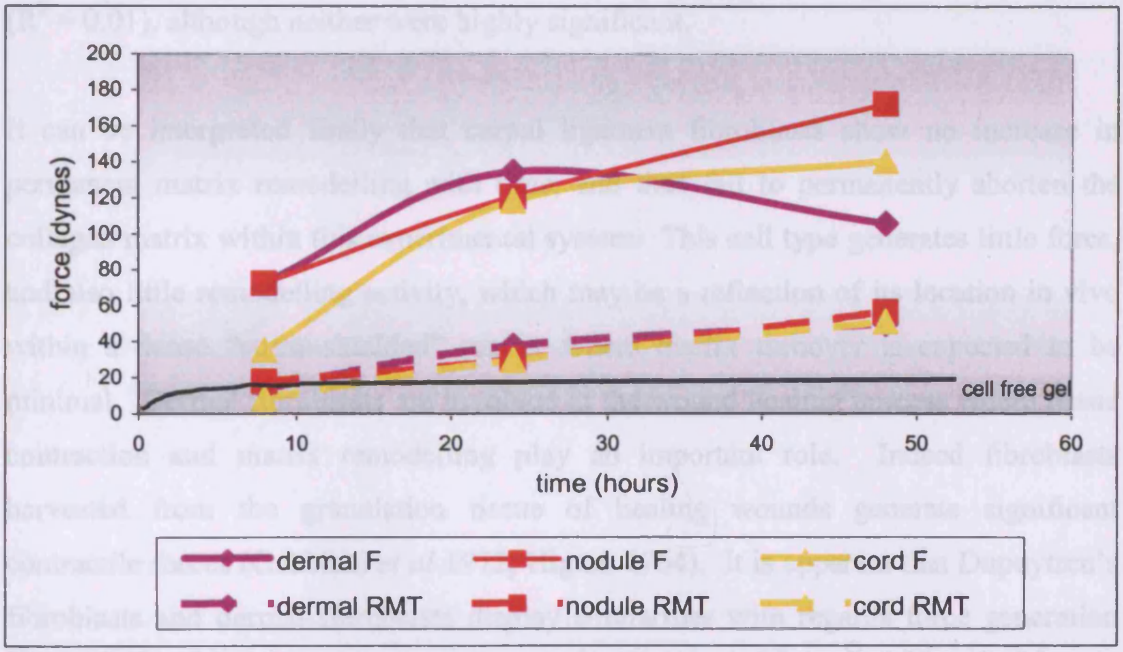


Figure 5.13: - Line chart demonstrating maximal generated force at 8,24 and 48 hours, and RMT at the same time points for dermal fibroblasts (n=4), Dupuytren's nodule (n=7) and Dupuytren's cord fibroblasts (n=7). The black line indicates the force generated by an acellular blank gel over the same time period. Note the near linear increase in RMT for Dupuytren's derived cells in comparison to dermal fibroblasts.

When these results are analysed together (figure 5.13) over 8 to 48 hours it appears that with time for Dupuytren's derived fibroblasts, both generated force, and the residual matrix tension increase. What is important in this graph is the overall shape of the curve. Values for carpal ligament fibroblast were omitted here as carpal ligament fibroblasts throughout this experimental series generated little force, and RMT was minimal, at the level of an acellular gel, at all time points investigated.

For normal dermal fibroblasts RMT is seen to increase significantly over the first 24 hours ($p<0.034$), with only a small increase in RMT over the full 48-hour period. This parallels the fibroblasts contractile behaviour in that the cells reach a plateau of force generation by 24 hours. In contrast is the RMT for nodule and cord derived cells. RMT for these increased in a near linear fashion with a significant increase between 8 to 24 to 48 hours each ($p<0.01$ (nod), $p<0.034$ (cord)). Once again the RMT parallels the cells' contractile behaviour with increasing force being generated throughout the 48-hour period. The correlation between force and RMT which was much more marked for Dupuytren's cells ($R^2 = 0.46$) in comparison to control cells ($R^2 = 0.01$), although neither were highly significant.

It can be interpreted firstly that carpal ligament fibroblasts show no increase in permanent matrix remodelling with time, and thus fail to permanently shorten the collagen matrix within this experimental system. This cell type generates little force, and also little remodelling activity, which may be a reflection of its location in vivo within a dense "stress-shielded" matrix where matrix turnover is expected to be minimal. Dermal fibroblasts are involved in the wound healing process where tissue contraction and matrix remodelling play an important role. Indeed fibroblasts harvested from the granulation tissue of healing wounds generate significant contractile forces (Gabbiani *et al* 1972; Higton 1964). It is apparent that Dupuytren's fibroblasts and dermal fibroblasts display similarities with regards force generation and permanent remodelling ability. This would be anticipated for dermal fibroblasts given their role in vivo. This similarity in behaviour between these cell types is an important finding.

5.56 MATRIX REMODELLING – THE "CREEP" ZONE

If the graph of force generation against time is examined in closer detail after the addition of cytochalasin-D it may be noted that there are 3 separate components of the curve (figure 5.14). There is an initial rapid drop in measured force over 10 minutes after the drug is added, then there is a slow decline in force over a 20 minute period before the curve plateaus at the level of the residual matrix tension.

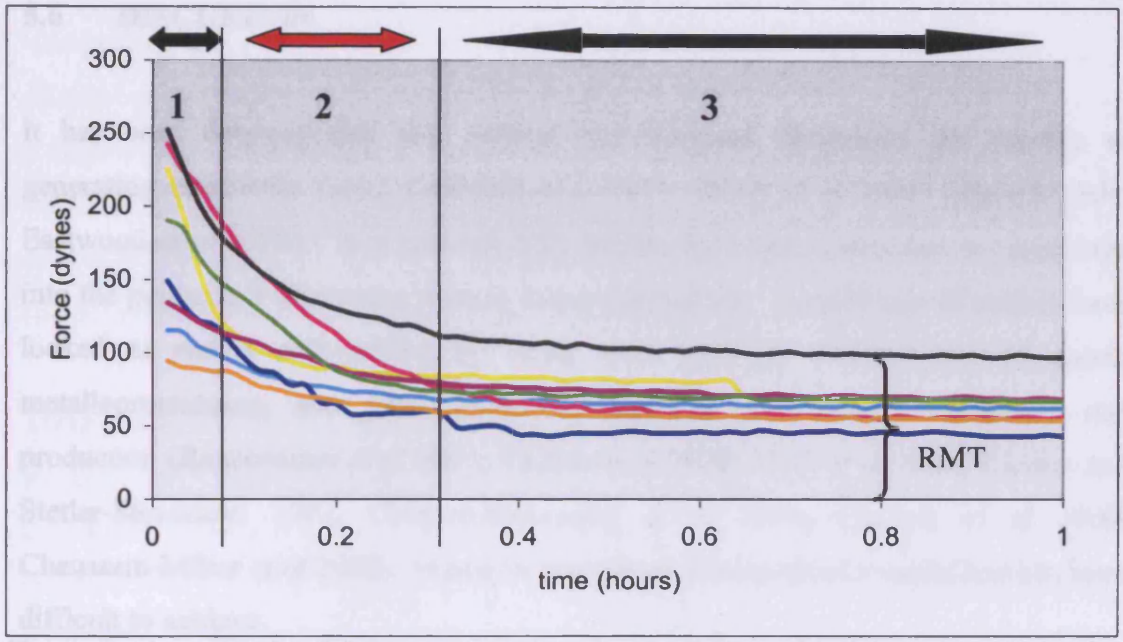


Figure 5.14: - Graph of force against time demonstrating the 3 components to the change in force after the addition of cytochalasin-D for n=8 Dupuytren's nodule cell lines at 48 hours. The first component shows a sharp initial drop in force (1), followed by a region of slow decline or "creep" (2), and finally a plateau phase (3) termed the residual matrix tension (RMT).

These changes have been observed in previous initial work by Marenzana *et al* (2004, unpublished data) on rat tendon fibroblasts. The changes within the creep zone were proposed to be due to the exposure of the collagen gel to an opposing force exerted by the force transducer. The initial drop in force was thought to be due to the loss of action of the cellular element by the cytochalasin-D. Prior to addition of this drug, the cells have contracted leading to a physical shortening of the gel, and hence a deformation of the strain gauge in an inwards direction. As force falls in the first phase, it was thought that the spring like action of the strain gauge being released and

attempting to return to its original resting position, leads to a slowing of the decline in force being measured as the gel begins to hold in its new "remodelled" position. Marenzana *et al* (2004, unpublished data) proposed that this area of creep is the result of breaking of the newest weakest bonds that had formed between collagen fibrils. This hypothesis was backed up by evidence of a linear relationship between "creep" and the maximal generated force for each experiment. Thus this data shows that RMT is the fixed tension that survives the application of recoil stress, and therefore RMT is an underestimate of the full degree of matrix remodelling.

5.6 DISCUSSION

It has been demonstrated that normal and diseased fibroblasts are capable of generating contractile force (Gabbiani *et al* 1971; Harris *et al* 1981; Grinnell 1994; Eastwood *et al* 1998). It is still not fully known how this contraction is transferred into the permanent shortening seen in tissue contracture. A multitude of studies have looked at matrix remodelling by using tools such as measurement of matrix metalloproteinases, and other molecular markers of collagen breakdown and production (Ragoowansi *et al* 2001; Tarlton *et al* 1998; Werb *et al* 1986; Kleiner and Stetler-Stevenson 1993; Chiquet-Ehrismann *et al* 1994; Chiquet *et al* 1996; Chaussain-Miller *et al* 2002). However precise measurement of remodelling has been difficult to achieve.

In order for a cell to contract and generate force its actin cytoskeleton must remain intact (Bell *et al* 1979). This was demonstrated by Kolodney and Wysolmerski in 1992, who disrupted the cytoskeleton of chick embryo fibroblasts using cytochalasin-D and found that generated force rapidly disappeared in a floating force transducer model. Similar findings have also been shown by Eastwood *et al* in 1996. It was therefore postulated from these models that cells may hold force via their attachment to the collagen matrix and subsequent cellular contraction. If cellular contraction is eliminated no residual force is left behind within the tissue matrix (Kolodney and Wysolmerski 1992; Rayan and Tomasek 1994).

Few workers have managed to quantify the ability of cells to permanently alter a collagen matrix. The earliest measurements were undertaken by Guidry and Grinnell (1985) who measured the diameter of circular free floating collagen lattices before and after the addition of cytochalasin-D or simple detergent to eliminate any cellular contribution to force generation. This study concluded that matrix remodelling increased with time with almost full gel re-expansion occurring within a few hours of cell seeding, and less occurring after over 20 hours. Grinnell and Ho (2002) using a stress-relaxed model demonstrated that collagen matrix contraction was completely actin dependant at day zero using a population of human dermal fibroblasts preincubated with TGF-β, but that by day 6 over 50% of generated force was retained within the collagen gel after the cellular dependant actin contribution to force was eliminated. This was thought to be due to reorganisation or remodelling of the collagen matrix by the cells. The present study shows that at 8 hours there was only a residual matrix tension of 14 dynes in the dermal fibroblast cell lines, a value similar to that of an acellular collagen gel, but by 48 hours residual matrix tension had increased to 49 dynes, which was over 45% of overall generated force.

There are difficulties in comparing the work here with that of Grinnell and Ho (2002), in view of the fact that they were using a circular stress relaxed system, and the duration of the experiments was longer. However, overall our findings are similar. It can be stated that matrix remodelling or stiffening of the collagen matrix will increase with time. It also appears that the cells that produce greater forces of contraction will also achieve a greater residual matrix tension. Many proposals regarding the exact mechanism of action of this matrix stiffening have been proposed. Some feel that remodelling is mainly a physical rearrangement of the collagen fibrils with the formation of new noncovalent bonds between fibrils (Guidry and Grinnell 1985; Eastwood *et al* 1998). Others have hypothesised that cellular contraction leads to matrix deformation, followed by secretion of new collagen and matrix deposition, before the process begins again (the slip and ratchet theory) (Tomasek *et al* 2002). Recent work has revealed that this may be due to a combination of both these processes (Parsons *et al* 1999; Grinnell and Ho 2002).

Wakatsuki *et al.* (2000) stated that the mechanical properties of any tissue are determined by the cell, the matrix and their interactions. They constructed a model by

which the contribution of each may be determined, using chick embryo fibroblasts embedded in a collagen matrix, via the application of uniaxial stretch measurements. The stiffness of a tissue is the sum of the active and the passive elements. The passive contribution is that of the extracellular matrix, whereas the active is dominated by cytoskeletal and some matrix contributions. These researchers found that the cellular contribution to force generation is made up of a contractile component (CC) due to actin-myosin interactions, and in series with that a cellular component dominated by the actin cytoskeleton, but not related to myosin interactions (SC), plus a matrix component at the point where the cells connect to the extracellular matrix (SM) via their actin network. In parallel with this cellular contribution to force is the residual cellular force (PC) remaining from the cells once the actin cytoskeleton is completely disrupted. This residual force (PC) is thought to be negligible as demonstrated by work disrupting intermediate filaments and microtubules in a similar system. The remainder of force within a cell filled collagen system is that provided by the tissue matrix itself (PM), and this is likely to be unaffected by any inactivation of the actin cytoskeleton.

The exact events that occur in order for the matrix to shorten are not elucidated for certain here. Certainly what this model clearly shows is that there are two elements of force generated by a cell seeded gel: - the cell dependent force, and the cell-independent force which increases with time as more force becomes permanently "held" or "enmeshed" within the collagen gel. The cell is appearing to transfer its force into the surrounding matrix by stabilising or changing, or adding to the structure of the collagen, hence residual matrix tension. It is proposed that once this action has occurred, the cell may then be free to contract again in order to deform and shorten the matrix to an even greater degree.

Collagen type I has been used as an extracellular matrix in this case, and there are some limitations that have been encountered in view of its make-up. Firstly collagen type I gels are made of homogenised collagen, with a significant water content. As cells contract this deforms the matrix and this may lead to the extrusion of water from the gel and a change in the gel's mechanical characteristics. Previous unpublished work from this lab (Marenzana, personal communication) has shown that if an acellular gel is mechanically overloaded; once the overload is released the gel will not

return to its previous resting position. This phenomenon is expected in viscoelastic materials such as collagen, and it is important to note that the changes expected from the material alone are distinct from the changes encountered in cell seeded gels exposed to cytochalasin-D (Marenzana *et al*, personal communication). Cell seeded gels will display some viscoelastic behaviour, but the overall increase in stiffness of the matrix is likely to be due to spatial changes in the collagen itself, rather than elasticity alone. Further mechanical work needs to be undertaken in this area to make any firm conclusions, and this was beyond the scope of this thesis.

Recent work on rat tendon fibroblasts using the culture force monitor model (Marenzana *et al* 2004) has shown that at 4 hours there was no residual measured force in the system after addition of cytochalasin-D; by 18 hours this had increased to a residual matrix tension of 43 dynes or 35 % of the maximum force generated. By 24 hours residual matrix tension had increased to 86 dynes or 36.7% of the maximum force generated, and by 65 hours a 295-dyne value for residual matrix tension was recorded or 45% of the maximal force. Similarly for all of the cell lines investigated in this study it was seen that residual matrix tension increased with time of incubation. Indeed at 8 hours for all cases there was no evidence of permanent matrix remodelling, with the value for residual matrix tension below that of a blank acellular gel. By 24 hours residual matrix tension had increased to a value greater than that for an acellular blank gel indicating permanent matrix remodelling within the collagen gel. There was no significant difference noted between cell types with all retaining between 21 and 35% of the maximum generated force. This is slightly less than that previously noted for rat tendon fibroblasts. The differences observed between our study and that using rat tendon tissue, may simply be a reflection of the tissue of origin. Certainly rat fibroblasts show a higher proliferation in comparison to those from human tissue. Similarly tendon derived fibroblasts are resident within a thick collagenous matrix that is exposed to greater mechanical forces in comparison to the relatively thinner and more pliable palmar fascia.

It is at 48 hours that the changes in residual matrix tension become more marked. For carpal ligament there is very little residual matrix tension to be measured at 22 dynes. It is postulated that this cell type responds little with respect to force generation and matrix remodelling. This may be a result of their location *in vivo*. These cells are

located in a dense collagenous matrix in a region that is not exposed to high mechanical stress on a regular basis, and thus they may be "stress-shielded" (Eastwood *et al* 1998; Tomasek *et al* 2002). By virtue of their relatively protected location these cells may have over time become insensitive to changes in mechanical force, and have less ability to generate a force themselves and hence remodel their surrounding tissue matrix.

It is the Dupuytren's nodule fibroblasts, which have demonstrated the greatest ability to permanently remodel a collagen matrix. Residual matrix tension was 56 dynes, greater than that of the cord (51 dynes), and the dermal fibroblasts (49 dynes), and significantly greater than that for normal palmar fascia from the carpal ligament (22 dynes). However if taken as a percentage of maximum force, matrix remodelling is certainly much less than that of rat tendon fibroblasts at the same time point. It is entirely possible that Dupuytren's fibroblasts do not have any greater ability to remodel a tissue matrix in comparison to their normal counterparts. Matrix remodelling is said to occur as a function of both matrix degradation, plus deposition. It may be in this case, that the laying down of new matrix components does not occur more quickly than normal. Examination of this matrix remodelling process has been looked at over a relatively short period of time of only 48 hours. It has been documented that Dupuytren's disease progresses very slowly over many years duration, in comparison to the relatively short forty eight hour experimental run. Due to this it is possible that changes occurring to the cells and matrix may be occurring later, and thus will have been missed. In order to test this hypothesis, long-running experiments within this system would need to be performed. In the future it would be possible to alter the experimental design in order to do this for example by using a bioreactor system.

What is apparent from this work is not only the difference in remodelling ability between the Dupuytren's and dermal fibroblasts in comparison to carpal ligament fibroblasts, but possibly more importantly is the similarity in behaviour of the Dupuytren's and dermal fibroblasts. Dermal fibroblasts would be expected to display features of matrix remodelling by virtue of their anatomical location and their role in wound healing and scarring. These are cells isolated from clinically normal individuals. In contrast Dupuytren's fibroblasts are isolated from clinically abnormal

tissue, and it is postulated that these cells are behaving in a manner comparable to cells from a healing wound, as described previously histologically (Majno *et al* 1971; Gabbiani *et al* 1971; 1972; Darby *et al* 1990). Carpal ligament cells do not display this permanent pattern of remodelling.

It is postulated that pre-incubation with a pro-fibrotic agent such as TGF-β1 in this model would only serve to increase the residual matrix tension. Work performed by Wilson-Jones (MSc thesis 2002), and Marenzana *et al* (2004) has shown that not only does matrix remodelling increase with time, but in addition it increases when cells are incubated with TGF-β1. Grinnell and Ho (2002) proposed that permanent extracellular matrix remodelling was a result not only of collagen fibril rearrangement, but was also due to deposition of collagen and fibronectin by cells after incubation with TGF-β1. Further work (Marenzana *et al* 2004) has demonstrated that mechanical loading of a cell seeded collagen gel led to a more stable remodelling of the matrix in comparison to TGF-β1 stimulation as judged from the residual matrix tension. From this it was postulated that fibroblasts may be able to produce different matrix material properties in response to different environmental cues, with load promoting collagen synthesis and matrix alignment, while TGF- β1 stimulation increases overall force generation and collagen accumulation.

What is also interesting is the description that RMT is underestimated in all cases due to creep within this experimental model (Marenzana *et al* 2004, in press). As described by Marenzana *et al* (2004) the recoil of the transducer beam applied a force to the collagen gel once the cellular action had been eliminated. This sudden transfer of force was proposed to result in rupture of some new inter-fibril bonds that were contributing to the matrix shortening. The higher the cell generated force, the greater the recoil force applied to the gel, and the rate of fall.

5.7 SUMMARY

- There was no evidence of permanent matrix remodelling after 8 hours in culture for all cell types

- Residual matrix tension was similar in all cell types investigated after 24 hours.

- After 48 hours RMT was significantly greater in Dupuytren's nodules in comparison to carpal ligament fibroblasts, but not significantly different in comparison to cord or dermal fibroblast cell lines.

- In all cell type matrix remodelling increased with time, and this was most significant in the case of Dupuytren's fibroblasts.

- Both Dupuytren's and dermal fibroblasts displayed a similar ability to permanently remodel a collagen matrix in comparison to carpal ligament fibroblasts.

It is postulated that there is a primary abnormality in the process of cellular contraction, leading to the progression of contracture seen in Dupuytren's disease. However the process of remodelling of a collagen matrix is not significantly greater in Dupuytren's cells in comparison to dermal fibroblasts. It is hypothesised that cellular contraction holds the matrix in a newly shortened state, while concurrently the cells act to remodel the surrounding matrix to hold it there permanently. Thus the greater the degree of cellular contraction the greater the permanent shortening of the matrix becomes.

5.8 STAINING OF FIBROBLAST POPULATED COLLAGEN LATTICES FOR COLLAGEN III

5.9 INTRODUCTION

In view of the findings of permanent remodelling of the collagen matrix discussed above, it was proposed to examine collagen in detail on an immunohistochemical level. The enhanced gene expression for collagen III in Dupuytren's fibroblasts, and the permanent matrix remodelling in this system led us to question whether this is translated into collagen deposition over the experimental time period observed.

Other earlier workers have looked at collagen III staining for fixed tissue specimens under light microscopy in renal, dermal and gingival fibroblasts (Hillman *et al* 1999; Kelynack *et al* 1999; Chaussain-Miller *et al* 2002) with the demonstration of positive staining over long time courses. It was hypothesized that Dupuytren's fibroblast seeded collagen gels would stain positively for collagen III in comparison to those from seeded with cells from carpal ligament.

5.10 METHOD OF DEVELOPMENT FOR STAINING COLLAGEN GELS FOR COLLAGEN TYPE III

Only a few studies have documented staining of fibroblast populated collagen gels for collagen III. It was therefore important to establish a procedure that was both sensitive and specific for collagen III staining. An anti-human collagen type III mouse monoclonal antibody (ICN Biomedicals Inc.) was used.

4 six well plates were used to make a total of 24 circular type I rats tail collagen acellular gels. For each gel, 3ml of rats tail type I collagen (First Link, UK) and 0.35 ml of Minimal Essential Media (MEM) were mixed together with a variable dose of collagen type III. The solution was neutralised using drop-wise addition of 1M

sodium hydroxide until a colour change from yellow to orange was noted. Into each well a different dose of collagen III was added (see table 5.1).

6 well plate	**Dose of collagen III**
Well 1	0 mg
Well 2	0.1mg
Well 3	0.05mg
Well 4	0.02mg
Well 5	0.01mg
Well 6	0.001mg

Table 5.1: -dose of collagen type III added to each well of a six well plate

The gels were then placed in an incubator at 37 degrees with 5% CO2 and allowed to set overnight. The next morning each gel was gently freed from the base of the dish using a sterile spatula, and placed into 10% formal saline solution for 24 hours at 4 degrees C.

Following this the protocol outlined in section 2.43 (*Materials and Methods*) was followed for immuno-staining for type III collagen. Various different concentrations of anti-collagen III antibody were tested in order to determine the most appropriate concentration to be used in the experimental gels. A total of 4 plates were used for each of the doses of collagen III seen above, using a different concentration of antibody in each plate (table 5.2). This gave a total of 24 gels.

	Concentration of anti-collagen III antibody
Plate A	5mcg/ml
Plate B	10mcg/ml
Plate C	15mcg/ml
Plate D	20mcg/ml

Table 5.2: Concentration of anti-collagen III antibody used in each plate

5.11 RESULTS

Figure 5.15a: Example of a blank gel with no collagen III added. Similar pictures were seen for each concentration of antibody added. Not the absence of green fluorescence throughout the sample

Figure 5.15b: Example of a gel with 0.1g of added collagen III using an antibody dose of 5μg/ml. Note positive green fluorescent staining of collagen III (arrow)

Figure 5.15c: Example of a gel with 0.05g of added collagen III using an antibody dose of 5μg/ml (arrow)

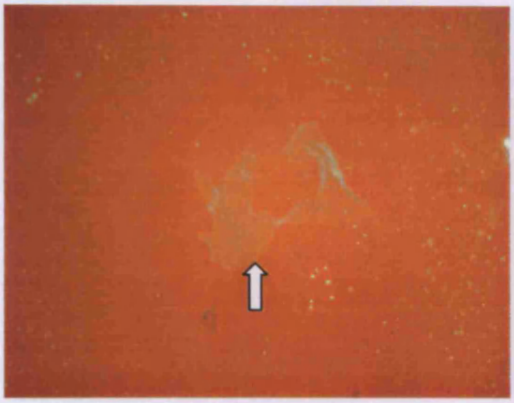


Figure 5.15d: Example of a gel with 0.001g of added collagen III using an antibody dose of 5μg/ml. In figures 4-6 note positive staining for collagen III as shown by green fluorescence (arrow)

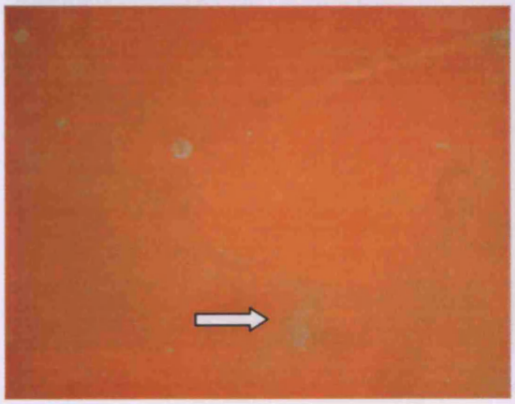


Figure 5.15e: Example of a gel with 0.01g of added collagen III using an antibody dose of 10μg/ml (arrow)

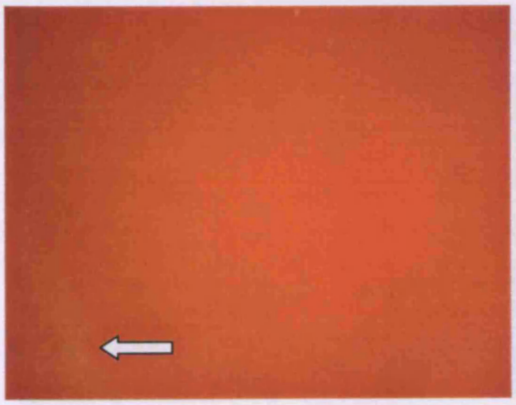


Figure 5.15f: Example of a gel with 0.001g of added collagen III using an antibody dose of 10μg/ml (arrow)

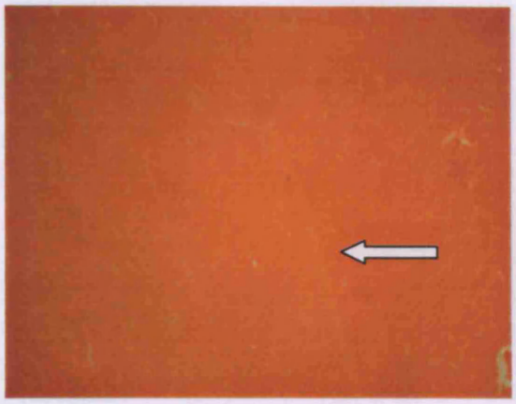


Figure 5.15g: Example of a gel with 0.1g of added collagen III using an antibody dose of 15µg/ml (arrow)

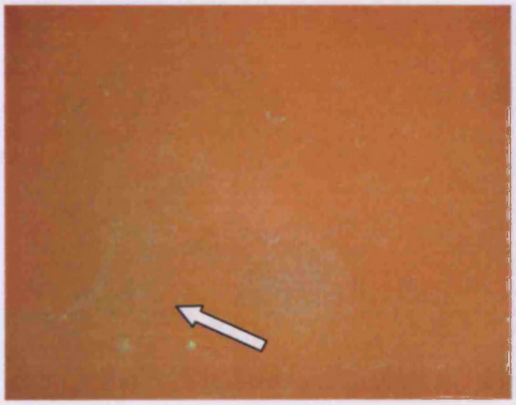


Figure 5.15h: Example of a gel with 0.01g of added collagen III using an antibody dose of 15µg/ml (arrow)

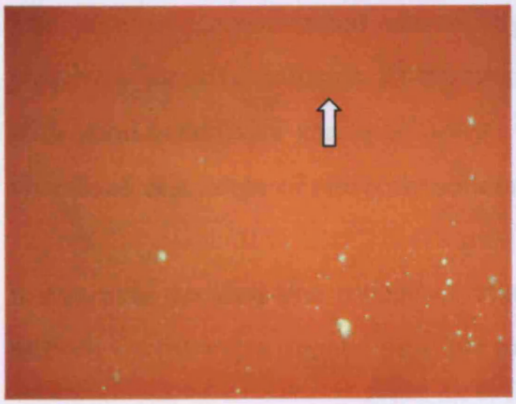


Figure 5.15i: Example of a gel with 0.001g of added collagen III using an antibody dose of 15µg/ml (arrow0

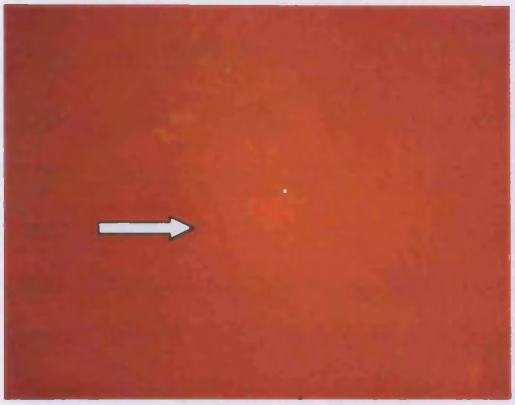


Figure 5.15j: Example of a gel with 0.01g of added collagen III using an antibody dose of 20µg/ml (arrow)

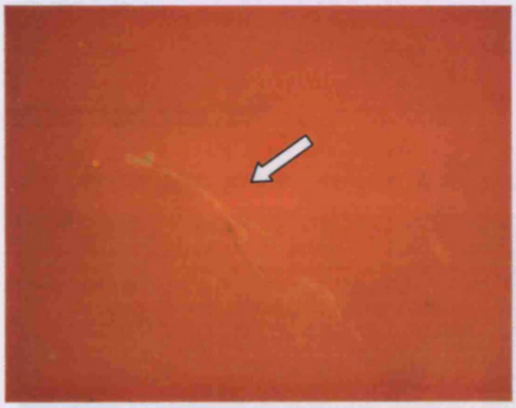


Figure 5.15k: Example of a gel with 0.001g of added collagen III using an antibody dose of 20µg/ml (arrow)

The pictures demonstrated above (figure 5.15a-k) indicate that it is possible to positively identify collagen III deposition using an immuno-histochemical technique with good sensitivity and specificity. Doses as low as 0.001 g of collagen III were visualised at a range of antibody concentrations from 5 to 20 µg /ml.

It was thus decided that a dose of 10µg / ml of anti-collagen III antibody would be utilised for the experimental gels due to the ease of dilution calculation.

5.12 METHODS

Using a dose of 10μg / ml of anti-collagen III antibody as calculated above, a total of 5 gel specimens each from Dupuytren's nodule, Dupuytren's cord and carpal ligament were taken after being allowed to contract for 48 hours on the culture force monitor, and processed as described fully in Section 2.43 (*Materials and Methods).* Specimens were viewed under a fluorescent light microscope at x20 and x40 magnification, in three separate fields for each gel.

5.13 RESULTS

All photomicrographs viewed at x 20 magnification.

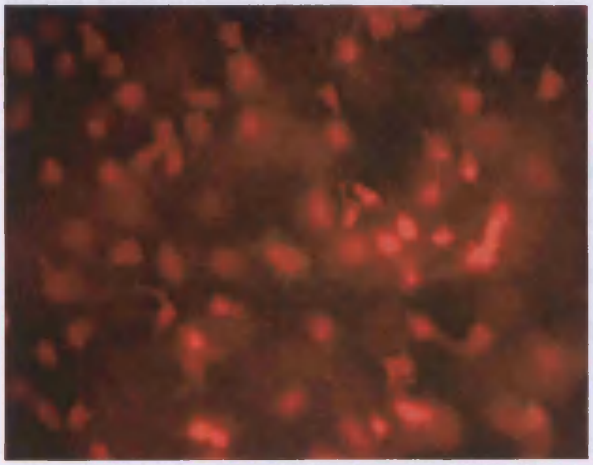


Figure 5.16: a typical Dupuytren's nodule cell line fixed in a 48hr collagen gel and stained for collagen III. There is no evidence of deposition of collagen III within the gel at this time point as shown by the lack of green fluorescence, and positive red nuclear staining

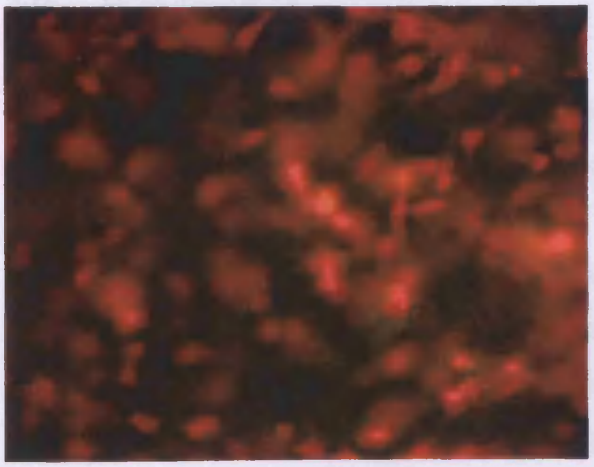


Figure 5.17: a typical Dupuytren's cord cell line fixed in a 48hr collagen gel and stained for collagen III. There is no evidence of deposition of collagen III within the gel at this time point as shown by the lack of green fluorescence, and positive red nuclear staining

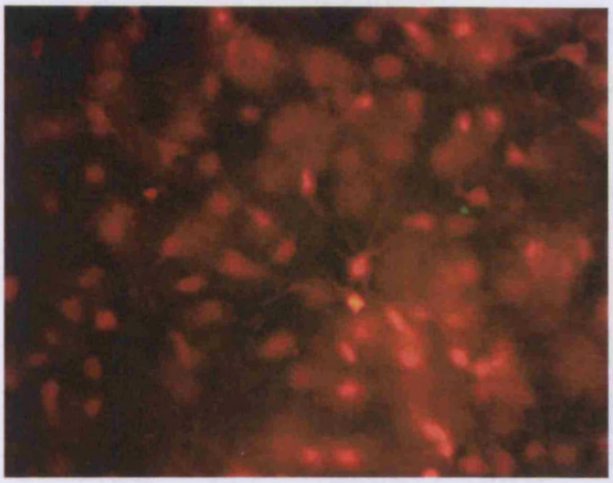


Figure 5.18: a typical carpal ligament cell line fixed in a 48hr collagen gel and stained for collagen III. There is no evidence of deposition of collagen III within the gel at this time point as shown by the lack of green fluorescence, and positive red nuclear staining

As can be seen in the three representative photomicrographs above (figure 5.16-5.18), in each cell type there was no evidence of positive green fluorescent staining that would indicate the presence of collagen III within the collagen I matrix. There is positive red staining for the cell nuclei from the propidium iodide.

These results indicate that there was no collagen III deposition within the extracellular matrix over a 48 hour time period that was detectable by the technique used above. It is certainly possible that smaller amounts than 0.001g may have been produced, but this experimental procedure was not deemed sensitive enough to detect these levels.

5.14 DISCUSSION

This work provides evidence that over the short period of time of these experiments, there was no detectable deposition of collagen III in the surrounding matrix.

It has been shown in previous work that in vivo collagen synthesis is much lower in three dimensional gel systems in comparison to monolayer culture (Nusgens *et al* 1984; Mauch *et al* 1988). A study by Hillman *et al* (1999) on human gingival fibroblasts revealed absent staining for collagen I or III in the first 48 hours of culture with positive staining only apparent after 2 weeks. Chaussain-Miller *et al* (2002) have shown weak positive staining for collagen III in dermal and gingival fibroblasts seeded in type I collagen matrices after a single day in culture, with greater intensity

after 3-7 days. The collagen appeared to be located as thin fibres in a reticular fashion at the basement membrane. Other work has shown that renal myofibroblasts are synthetically active within collagen matrices with the positive detection of intracellular collagen III, however none was detected within the matrix itself (Kelynack *et al* 1999).

There are some limitations to this work, and clearly there are some questions that remain unanswered by these light microscopic studies. Very small quantities of collagen III may have been missed by an immunostaining regime at this magnification. Due to the positive red nuclear staining it was difficult to focus clearly on the cell cytoplasm at magnifications of x40, and thus detection of intracellular collagen III was not possible. It is likely that this would be the first change noticed during this short time period.

5.15 SUMMARY

It is thus likely that the permanent shortening of the collagen matrix (otherwise known as residual matrix tension) seen in experiments run on the culture force monitor are unlikely to be due to the deposition of collagen III, and are more likely to be due to other structural changes in the matrix. Clearly there is some spatial rearrangement of the type I collagen matrix produced by the indwelling fibroblasts, what is as yet unclear is how this occurs. Theoretically it may be that non-covalent fibrillar bonds form first, and that later on, in days rather than hours, collagen is deposited to link these fibrils together to form stronger bonds.

CHAPTER 6

THE RELATIONSHIP BETWEEN FIBROBLAST CONTRACTION AND PATIENT FACTORS

6.1 INTRODUCTION

Recurrence or extension in Dupuytren's disease is a difficult problem both for the patient and for the surgeon. Currently the only therapeutic option is surgical revision with its associated higher morbidity and complication rate in comparison to primary surgery. As documented earlier (*Introduction section 1.7.2*), the recurrence rate increases with time of follow up, but in today's overstretched health care system it is not always possible or cost effective to review all patients that have had Dupuytren's surgery over many years. It would be useful to identify patients who are most at risk of disease recurrence in order that intervention occurs at an appropriate time. For those least at risk, the inconvenience of regular hospital outpatient visits could be delayed.

Some clinical features have provided a means of identifying those patients most at risk, including early age of onset of disease, positive family history, and bilaterality (Hueston 1961; McFarlane 1985). Histological studies have also been used to predict recurrence, with Gelberman (1980) relating recurrence to the findings of myofibroblasts and prominent microtubules in the nodules on electron microscopy. Rombouts *et al* (1989) used light microscopy to subdivide patients into three groups of either proliferative, fibrocellular or fibrotic stages, finding a higher risk of recurrence in the first group.

Given the relative ease of setting up a culture force monitor experiment, it was felt that it may be possible to utilize this experimental model as a possible clinical aid to predict patients most at risk of disease recurrence or extension.

6.1 AIMS

- To establish whether there is any relationship between Dupuytren's fibroblast contractile ability and clinical recurrence in patients with the disease.

6.3 HYPOTHESIS

- Patients that show evidence of the disease by either recurrence or extension will have fibroblasts that generate the greatest forces when allowed to contract a collagen gel on the culture force monitor, in comparison to patients with no evidence of disease re-activation.

6.4 METHODS

All cell lines that had been used to obtain contraction profiles on the culture force monitor were traced back to the original patient after obtaining ethical approval from the local ethics committee. Each patient was asked for permission to review his/her medical notes, and each patient was also invited to attend a review appointment that would be held at their local GP surgery.

Cell lines from the previous researcher in this area (n=11, Bisson MD thesis 2003), and the work conducted in this thesis were reviewed (n=15), giving a total of 26 patients available for follow-up. Of these 2 patients were unobtainable for follow up, and were excluded from the study. For the remainder a full patient questionnaire was filled in based on a review of the medical notes (Appendix XI), and both a telephone interview and patient visit arranged. Further evidence of Dupuytren's disease was documented as either a true recurrence (at the site of original surgery) or as disease extension (new disease elsewhere in the hand) based on the work of previous researchers (Gordon 1957; Millesi 1974; Gelberman *et al* 1980; Tonkin *et al* 1984). Care was taken to distinguish

Dupuytren's disease from scar contracture or joint contracture by careful clinical examination. Maximum force generated from contraction profiles were correlated with patient age, and recurrence. Statistical analysis of results was performed using a Sigma Stat software package.

6.5 RESULTS

The interval between the primary surgery for Dupuytren's disease and research follow up was 19 months on average (range = 13 – 32 months; median = 19 months). Only 2 of the patients were female (8.3%), the remainder being male (91.7%). All of the patients in this group had undergone a standard limited fasciectomy for their Dupuytren's disease. The maximum number of digits involved was two, with the ring and little finger being involved in the majority. 18 patients had undergone surgery on their dominant hand, with 6 on their non-dominant hand. 8 patients showed evidence of an MCPJ contracture, 5 had PIPJ contractures and 8 had both MCPJ and PIPJ contractures, with no documentation of the type of contracture in the notes of 3 patients. All but 3 patients achieved full extension of the affected digit on the operating table (see table 6.1).

6.51 Age Correlations

The maximum generated force for Dupuytren's nodule and for cord cell lines at 24 hours were plotted against patient age in order to determine if there was any association between the two (figure 6.1 and 6.2). Mean age was 64.4years (range 29-77; median 67yrs; Standard Deviation = 10.5).

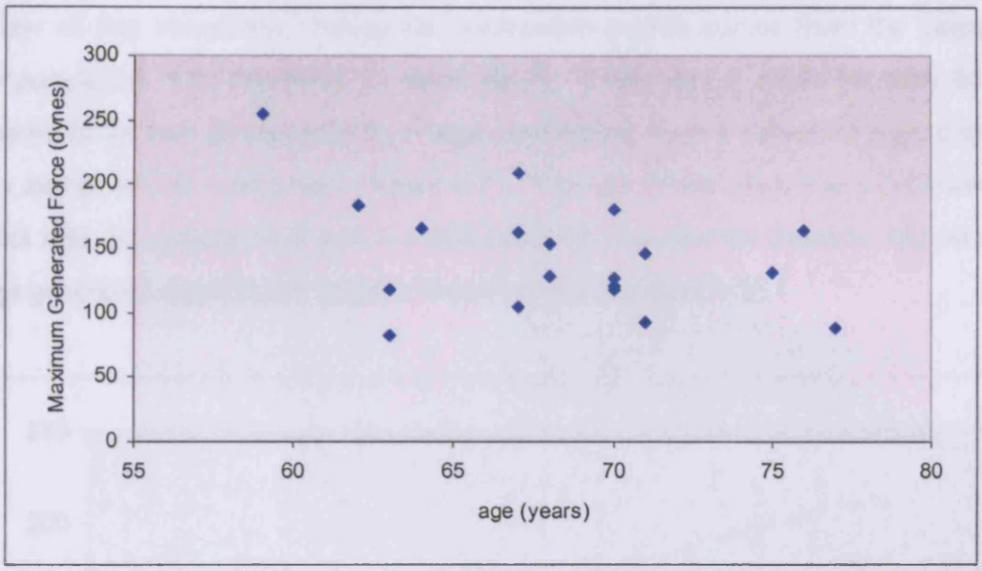


Figure 6.1:- Scatter graph of patient age versus generated force in dynes for Dupuytren's nodule derived fibroblasts. Note that there appears to be no linear correlation between the age of the patient and the generated force.

For Dupuytren's nodule there was no correlation between generated force and patient age (figure 6.1). Measured force was variable when plotted against age. However for cord a correlation was noticed, with increasing force generated as age increased (figure 6.2).

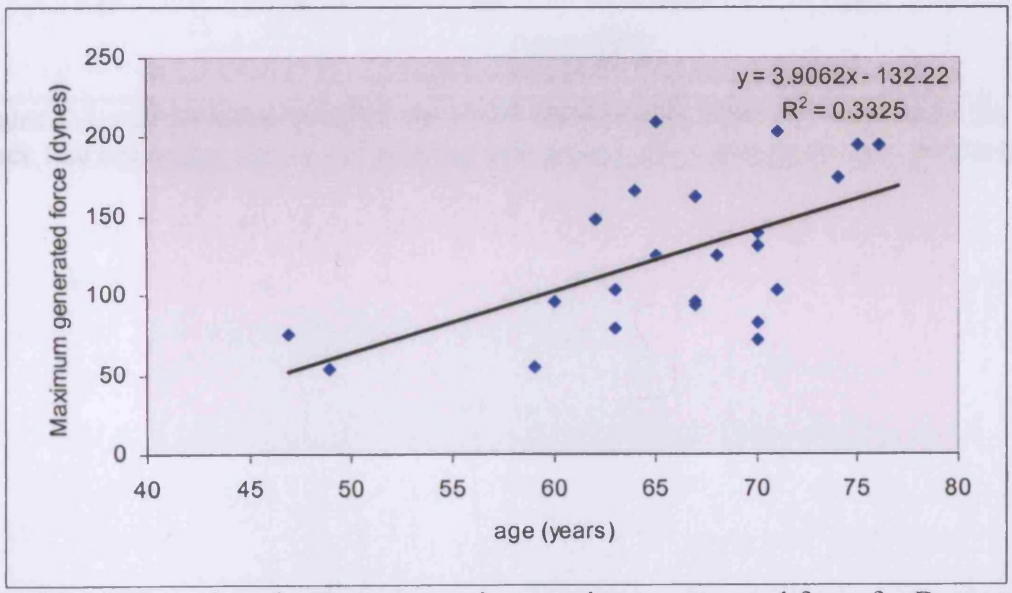


Figure 6.2:- Scatter plot of patient age against maximum generated force for Dupuytren's cord derived fibroblasts. There is a trend for generated force to increase as the age of the patient increases, although this is not highly significant ($R^2 = 0.33$).

In view of this correlative finding the contraction profile curves from the Dupuytren's cord population was examined in more detail. From this it could be seen that there appeared to be two groups within a large continuum, with a subset of high contractors and a subset of low contractors (figure 6.3). The age related data was subdivided based on this into two groups also, and it could again be seen that the patients who were older in age generated significantly greater forces ($p<0.05$) (figure 6.4).

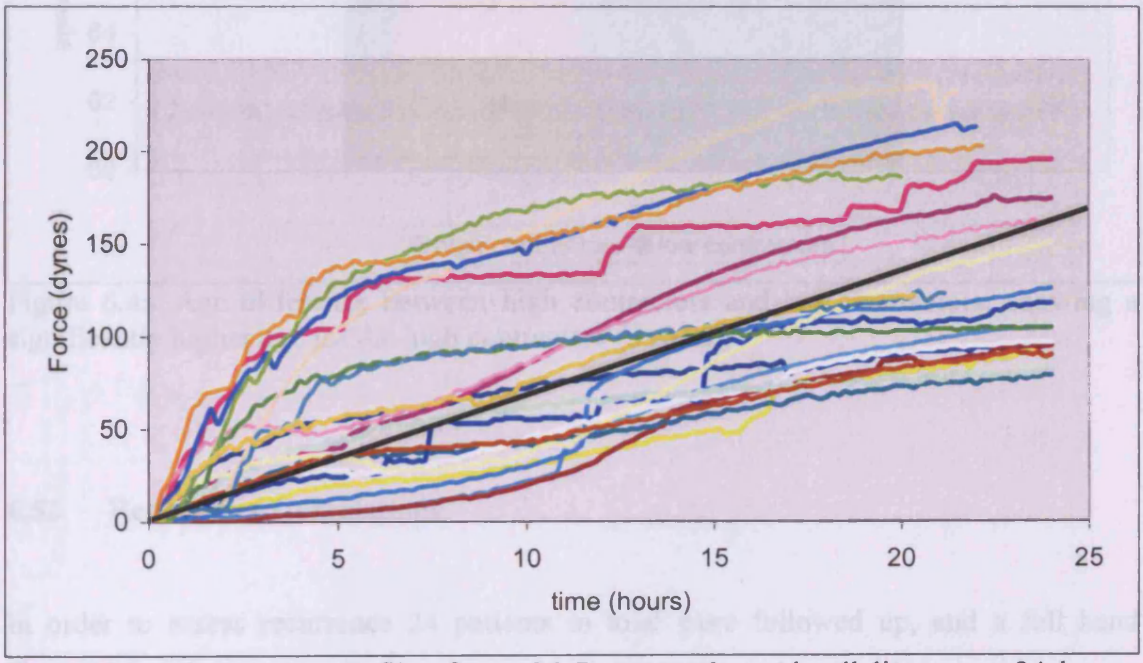


Figure 6.3:- Contraction profiles for n=26 Dupuytren's cord cell lines over 24 hours. Black line represents the cut off between two groups, the high and the low contractors.

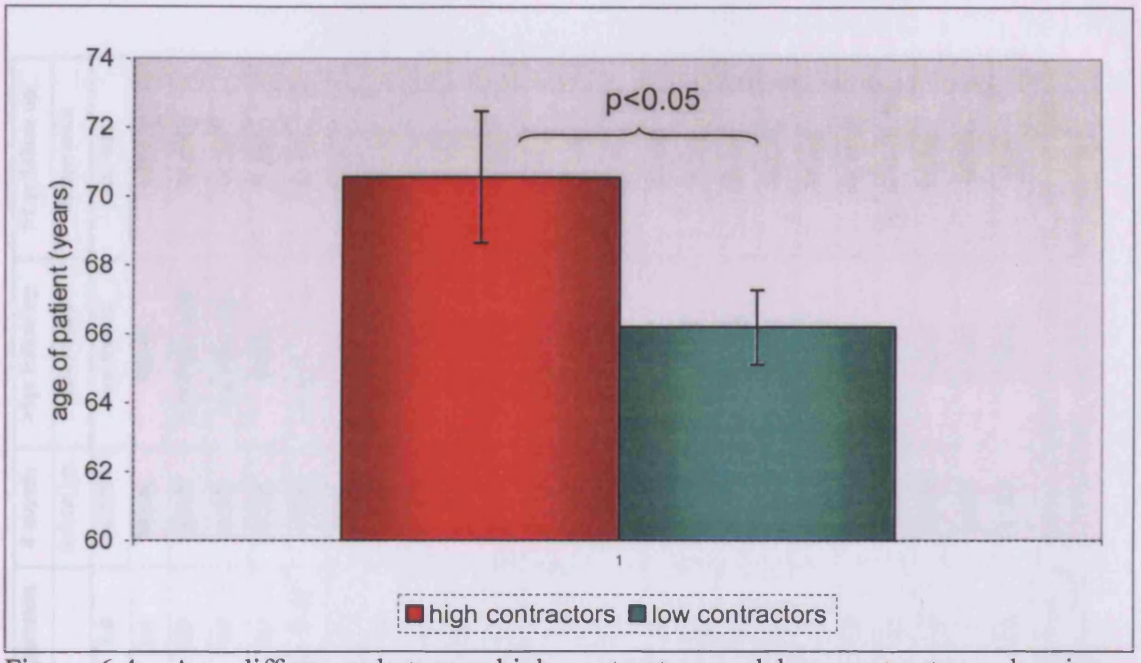


Figure 6.4:- Age difference between high contractors and low contractors, showing a significantly higher age for the high contractors ($p<0.05$).

6.52 Recurrence Correlations

In order to assess recurrence 24 patients in total were followed up, and a full hand examination performed. Each patient was assessed for evidence of a true recurrence, disease extension or both. Patient notes were reviewed to obtain information on 4 month follow up, and a full discussion and patient assessment were performed by the primary researcher on the second follow-up, rather than using patient notes to obtain the information. (Results are summarized in table 6.1).

At 4 months no patients showed evidence of recurrence. At the nineteen month follow-up, there were 7 patients with signs of disease extension, and 5 patients with signs of disease recurrence. Of these there were none with both extension and recurrence. The true recurrence rate was therefore 20.8%, which is slightly higher than that of Hueston (1961) who showed a 12.5% recurrence rate at 2 years. All other studies have looked at longer time periods to assess recurrence rates.

number	age	nodule	cord force	duration	hand	mcp	pip	operation	4 month	>1yr follow up	>1 yr follow up
	(years)	force (dynes)	(dynes)	of disease		(degrees)	(degrees)		follow up	extension	recurrence
DP0502	68	153	124	?	Left	60	30	full	NONE	new thumb	rec little
DP0602	70	120	130	?	left*	0	45	full	NONE	NONE	NONE
DP0702	65	unknown	209	15yrs	right*	0	30	full	NONE	new index slow	NONE
DP0103	64	165	165	4yrs	left*	35	15	full	NONE	new little slow	NONE
DP0203	60	unknown	96	3yrs	right*	0	45	full	NONE	NONE	NONE
DP0303	77	87	unknown	?	right*	45	90	res 10-20	NONE	de novo, no rec	NONE
DP0403	75	130	195	?	right*	15	45	res 5	NONE	NONE	NONE
DP0503	63	81	103	?	right*	45	0	full	NONE	new 1st web	NONE
DP0603	67	104	95	?	right*	45	0	full	NONE	NONE	NONE
DP0703	71	145	unknown	3yrs	left*	20	20	full	NONE	NONE	NONE
DP0803	67	208	93	?	right*	5	45	full	NONE	NONE	NONE
DP0903	74	unknown	174	?	right*	30	0	full	NONE	NONE	NONE
DP1003	63	118	78	12yrs	Left	30	0	full	NONE	NONE	NONE
DP0600	49	unknown	53	sev yrs	left*	? Cont	0	full	NONE	NONE	NONE
DP0700	47	unknown	75	>10 yrs	left	a bit	45	full	NONE	NONE	rec pipj
DP0101	65	unknown	124	?	right*	30	0	full	NONE	NONE	NONE
DP0601	62	183	147	1 yr	right*	a bit		full	NONE	new little	NONE
DP0701	70	126	139	>10yrs	right*	65	55	20 deg	NONE	NONE	NONE
DP0801	68	127	unknown	?	right*	40	0	full	NONE	NONE	NONE
DP0901	70	180	82	>5yrs	left	0	45	full	NONE	NONE	rec slow
DP1001	67	162	162	>5yrs	left	?	?	full	NONE	NONE	rec slow 25 deg
DP1101	29	163	89	2yrs	right*	no	no	full	NONE	NONE	NONE
DP0102	59	254	54	>15yr	left	0	min	?	NONE	ext little	NONE
DP0202	76	164	194	>10yrs	right*	0	45	full	NONE	NONE	slow

Table 6.1: Table documenting patients followed up post surgery for Dupuytren's disease, detailing force generated, patient age, contracture, and evidence of recurrence or extension at 1st and 2nd follow-up. The gaps in the column for force are due to no contraction profiles being available for these patients.

The findings of disease recurrence and extension were correlated with the force generation by the patients' fibroblasts as shown in figure 6.5.

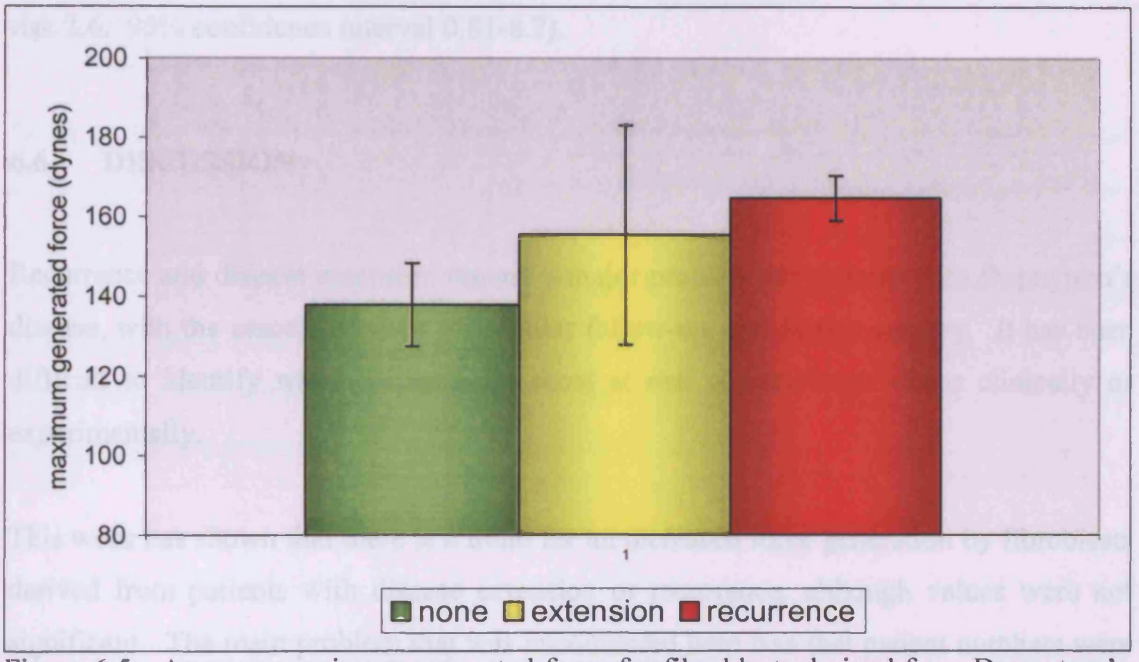


Figure 6.5:- Average maximum generated force for fibroblasts derived from Dupuytren's nodule with no recurrence (n=12),nodule with extension (n=7) and nodule with recurrence (n=5). There is no significant difference in force generation between sub-groups.

Of note is that there was no significant difference in force generation between each group, with the average force generated from fibroblasts in patients with no disease recurrence being 138 dynes (SEM ± 10 dynes), 155dynes (SEM ± 28 dynes) for extension and 165 dynes (SEM ± 6 dynes) for recurrence. Although these differences were not significant, what is of note is the trend for an increase in generated force for those fibroblasts isolated from patients with a recurrence. The numbers utilized in this work are too small to make any firmer conclusions.

Similarly for Dupuytren's cord derived fibroblasts, a trend for increasing force generation in relation to recurrence was noted, but once again these values were not significant. Force generated for fibroblasts with no recurrence was 115 dynes (SEM ± 13 dynes),

force for extension was 134 dynes (SEM ± 22 dynes), and force for recurrence was 127 dynes (SEM ± 23 dynes). A Fisher's exact test was performed to establish that there was no significant relationship between force generation and recurrence ($p>0.156$) (relative risk 2.6. 95% confidence interval 0.81-8.7).

6.6 DISCUSSION

Recurrence and disease extension remain a major problem for patients with Dupuytren's disease, with the associated need for regular follow-up, and further surgery. It has been difficult to identify which patients are most at risk of recurrence either clinically or experimentally.

This work has shown that there is a trend for an increased force generation by fibroblasts derived from patients with disease extension or recurrence, although values were not significant. The main problem that was encountered here was that patient numbers were very low in order to come to any definite conclusions. The only prognostic test available clinically at the current time is good clinical history and examination in order to identify the Dupuytren's diathesis. Recent work has identified bilateral hand involvement, ectopic lesions, and early age of onset as the best predictors of recurrence and extension (Abe *et al* 2004), and these authors have composed a scoring system in order to evaluate risk. However results of a prospective cohort study are awaited to verify the system.

Rombouts *et al* (1989) utilized a histological classification as a predictor for recurrence finding greater recurrence in the proliferative group. They defined the proliferative group as areas on histology with mitotic figures visible within areas of high cellularity. The main limitation with their study was that examination of specimens individually usually revealed more than one histologic subtype making it difficult to grade the specimen. Certainly in our work where primary cell cultures were set up, it was apparent that the majority of specimens obtained from the operating theatre had two main components that we have grossly defined as nodule and cord, and which in Rombout's study would be

defined as proliferative type and fibrotic type. Thus it would be difficult to utilize their grading system clinically due to the problems with grading. Similarly electron microscopic examination as used by Gelberman *et al* (1980) is time consuming, and expensive.

None of these methods developed have been ideal to ascertain those patients most at risk of recurrence. This work is currently only preliminary with a small patient number and short follow-up period available from which to draw any conclusions. At least 40 more specimens would need to be processed in order to obtain results from which powerful statistical analysis may be performed. The overall feeling is that it has promise as a diagnostic aid to identify patients most at risk, although it is doubtful that it will ever replace regular patient review in the out-patient clinic.

CHAPTER 7

GENERAL DISCUSSION

7.1 BACKGROUND

The main clinical problem in Dupuytren's contracture is not only the formation of disabling flexion contractures of the digits, but also the high recurrence rates seen post-operatively. At each operation for recurrent disease the operative risks increase, and with them the chances of a fully functional hand are much reduced. Since the advent of surgery for Dupuytren's disease in 1831, few technological advances have been made which improve prognosis for patients.

Many aetiologic factors have been associated with Dupuytren's disease, but the trigger that induces a fibroproliferative reaction and progressive matrix shortening remains unknown. This thesis has concentrated mainly on the effect of mechanical stimulation on Dupuytren's fibroblasts. This is an area that is of great importance in an organ that is constantly moving in flexion and extension and which within a normal day is exposed to many externally applied forces. It is also an area of interest when considering post-operative rehabilitation regimes in the hand where both passive and active flexion and extension exercises are used to restore function.

Work has looked at the contractile properties of the diseased fibroblasts, their responses to mechanical stimulation, both in terms of contractile behaviour and in terms of molecular behaviour, and at the ability of these cells to remodel a collagen matrix. Finally the relationship between these findings and clinical recurrence has been investigated. It is hoped that this will lead on to allow a better understanding of the natural history of the disease itself, and of the normal behaviour of the palmar fascia.

7.2 THE USE OF A CONTROL TISSUE IN THE INVESTIGATION OF DUPUYTREN'S DISEASE

There has been much debate on the ideal control tissue for use in the investigation of Dupuytren's disease. Previous workers have used tendon fibroblasts (Bulstrode MD thesis 2001), clinically normal adjacent fascia from hand affected by the disease (Alioto *et al* 1994), and the carpal ligament (Badalamente *et al* 1996; Tomasek *et al* 1995; Bisson *et al* 2004). There are pros and cons to the use of each. It was elected in this case to continue using the distal free edge of the carpal ligament as a control, firstly in order to provide some continuity to the work commenced by the previous research fellow in this area, and secondly as it is, of all the controls identified, likely to be closest in biochemical structure and function to the palmar fascia of a normal hand. The use of adjacent clinically normal fascia to the disease was thought to be unsuitable based on the abnormal biochemical findings seen from work by Brickley-Parsons *et al* (1981), and the fact that cells from this area may already be acting in an abnormal manner. An alternative would have been the use of normal fascia taken from patients undergoing trauma surgery to the hand, although this idea was rejected due to the reduced likelihood of obtaining tissue from patients of the same age range as in the Dupuytren's group.

7.3 THE INVESTIGATION OF SEPARATE NODULE AND CORD DERIVED CELL LINES

The separation of Dupuytren's tissue into nodule and cord derived fibroblasts has been performed very rarely in the past in tissue culture based experiments. Some have used undefined tissue (Murrell *et al* 1991), and the majority have focused on the nodule alone (Hurst *et al* 1986; Rayan *et al* 1996; Tarpila *et al* 1996). Bisson *et al* (2003; 2004) demonstrated that there is a difference in the phenotype of fibroblast cultures from nodule and cord, and hypothesized that due to the higher myofibroblast content in nodules that

this was likely to be the most active contractile element of the lesion. Similarly, Vande Berg *et al* (1984), and Moyer *et al* (2002) demonstrated differences in phenotype and activity between nodule and cord. Due to the above, it was deemed important to continue to investigate these two structurally different components of the Dupuytren's lesion separately.

7.4 EXPERIMENTAL EVIDENCE

In section 3.1, Dupuytren's fibroblasts were shown to display no evidence of tensional homeostasis over a 48 hour time period in the culture force monitor model. This is a significant finding, especially when comparing the contraction profiles of the Dupuytren's cells to two separate controls, the carpal ligament and dermal fibroblasts in which an obvious plateau period was observed after 15 hours. The ability of Dupuytren's cells to generate higher levels of tension within a collagen matrix than normal fibroblasts, and the progression of this contraction with time provides a possible explanation for the slow continued shortening of the matrix seen clinically. Morphologically it appears that fibroblast alignment within a collagen gel correlates well with overall force generation (section 3.3), with the nodules displaying greatest alignment along the long axis of the gel.

Within the same experimental model subsequent loading regimes were applied in order to provide a clearer picture of the response of Dupuytren's fibroblasts to changes in their mechanical environment (section 3.2). Given earlier work showing a significant abnormal contractile response to a mechanical overloading regime (Bisson *et al* 2004), suggestive of a positive feedback response with exacerbation of cell mediated contraction, it was important to determine these cells response to a loss of mechanical tension. Interestingly underloading resulted in cellular contraction for all cell types with no difference in response between Dupuytren's and control cells. From this it appears that Dupuytren's fibroblasts will tend to contract irrespective of the mechanical stimulus

applied to them. This finding may go some way to explaining the recurrence of a contracture after fasciotomy. A reduction in perceived tension after contracture release is likely to evoke increasing cellular contraction to a higher level of homeostatic equilibrium than normal, thus tending to return the hand to its pre-operative flexed state.

Morphological changes in response to mechanical stimulation were found to be minimal for both overloading and underloading when compared to static load for both control and Dupuytren's fibroblasts, with little change in overall cell alignment. However force changes introduced were relatively small, and thus may reflect the absence of a morphological change. In view of this, it was interesting to observe and discuss the genetic findings regarding matrix degrading enzymes released in response to mechanical stimulation, as it appears that changes in cell orientation are not responsible for the alterations in MMP gene expression or alterations to the shortened fabric of the matrix.

Firstly expression of MMPs and TIMPs was obtained from fibroblasts exposed to static loads only, in order to provide a baseline with which to compare loading regimes to. The striking finding was that there was no difference in gene expression between control and Dupuytren's cells, with a higher TIMP expression for both in comparison to the MMPs, which was extrapolated to indicate little matrix turnover in a non stressed environment. When a mechanical overload was applied there was a markedly different response by Dupuytren's nodules in comparison to cord and carpal ligament. For nodule a distinct up-regulation of all investigated MMPs occurred, with no up-regulation of TIMP-1 or 2. For carpal ligament and cord derived cells there was a converse effect, with up-regulation of TIMPs and no up-regulation of MMPs. It can be hypothesized that there is an increase in matrix turnover by the nodule, with a reciprocal "mopping-up" of turnover by the TIMP up-regulation by cord and carpal ligament in response to strain. Thus it can be theorized that there is a complex interplay of events when mechanical strain changes in the Dupuytren's nodule– this begins with localised matrix breakdown, which can then allow structural alterations in the ECM, further tissue contraction, and eventually long term net matrix deposition leading to contracture. The other important finding here is that there is a distinct difference in behaviour between the nodule and the cord

fibroblasts, reflecting the likelihood that they come from distinct separate cell populations, each with a different role within the disease. On a molecular level the nodule appears most active in relation to matrix turnover, with the cord acting in a quiescent manner very similar to that of carpal ligament, with no major role in remodelling activity.

Finally, having already established the abnormal contraction profiles of Dupuytren's fibroblasts with their altered MMP expression, and contraction in response to load, the next logical step was to measure the amount of permanent shortening of a collagen matrix that diseased cells were capable of producing (section 5). Over a short time period a complete loss of tension followed disruption of the actin cytoskeleton by cytochalasin-D in all fibroblasts under investigation, indicating that no spatial remodeling of the collagen had occurred. However by 48 hours a permanent shortening of the collagen network was seen which was most marked for Dupuytren's nodule fibroblasts, but not significantly greater than dermal fibroblasts. It may be hypothesized that although matrix remodeling is important for the permanence of a contracture, it is the abnormal cellular contraction that lends to the progression of the disease, while incremental remodeling occurs at each step.

Collagen was investigated as a possible structural element responsible for remodeling of a collagen matrix during the experimental period, both on a molecular level (section 4) and on a histological level (section 5). Certainly overall collagen III expression was greater in Dupuytren's fibroblasts than in controls in statically loaded specimens. Mechanical loading or TGF-β1 stimulation resulted in little change in collagen expression, although as summarized earlier the time course of 24-48 hours may be sufficiently small to note changes in collagen expression here. Similarly, no evidence of collagen III deposition was demonstrated by immunohistochemical staining in either Dupuytren's or control cells. Thus it is felt that the permanent shortening of the collagen matrix as discussed above is likely to be due to other factors, and not the deposition of collagen III. Theoretically it may be that non-covalent fibrillar bonds form first, and that

later on, in days rather than hours, collagen is deposited to link these fibrils together to form stronger bonds.

On a more clinical level, patients were followed up at around nineteen months after their primary surgery in order to assess whether the measurement of contractile force by fibroblasts could act as a useful clinical tool to assess those most at risk of recurrence. The main limitation here was the small number of patients within the study. Although a trend for an increased force with recurrence was noted this was not significant, and thus it was concluded that this test would be unreliable at this stage to aid clinicians when conducting patient reviews.

7.5 RELATION TO THE RECENT LITERATURE

The majority of connective tissues are characterized by a small number of quiescent fibroblasts embedded within large amounts of extracellular matrix. These cells act to establish a resting tension within the ECM by the cross-linking of proteins, and by the synthesis of new protein (Hinz and Gabbiani 2003). Earlier work has found that fibroblasts resident within the ECM are usually stress-shielded, and do not develop stress fibres (Tomasek *et al* 2002). Myofibroblasts are thought to form when the ECM alters such as in the case of a wound in the dermis. The loss of mechanical stress seems to be an important stimulus for differentiated myofibroblasts to dedifferentiate or disappear (Hinz and Gabbiani 2003). This has been seen in wound healing models where at the time that the ECM is reconstituted and takes over mechanical load, the myofibroblasts are released from stress and become apoptotic (Desmouliere *et al* 1995; Grinnell *et al* 1999; Fluck *et al* 1998).

In Dupuytren's disease a shift occurs from the behaviour of the normal palmar fascia where cells are acting as described above, and are stress shielded, to the marked change seen in Dupuytren's fibroblasts which have the ability to generate significantly greater

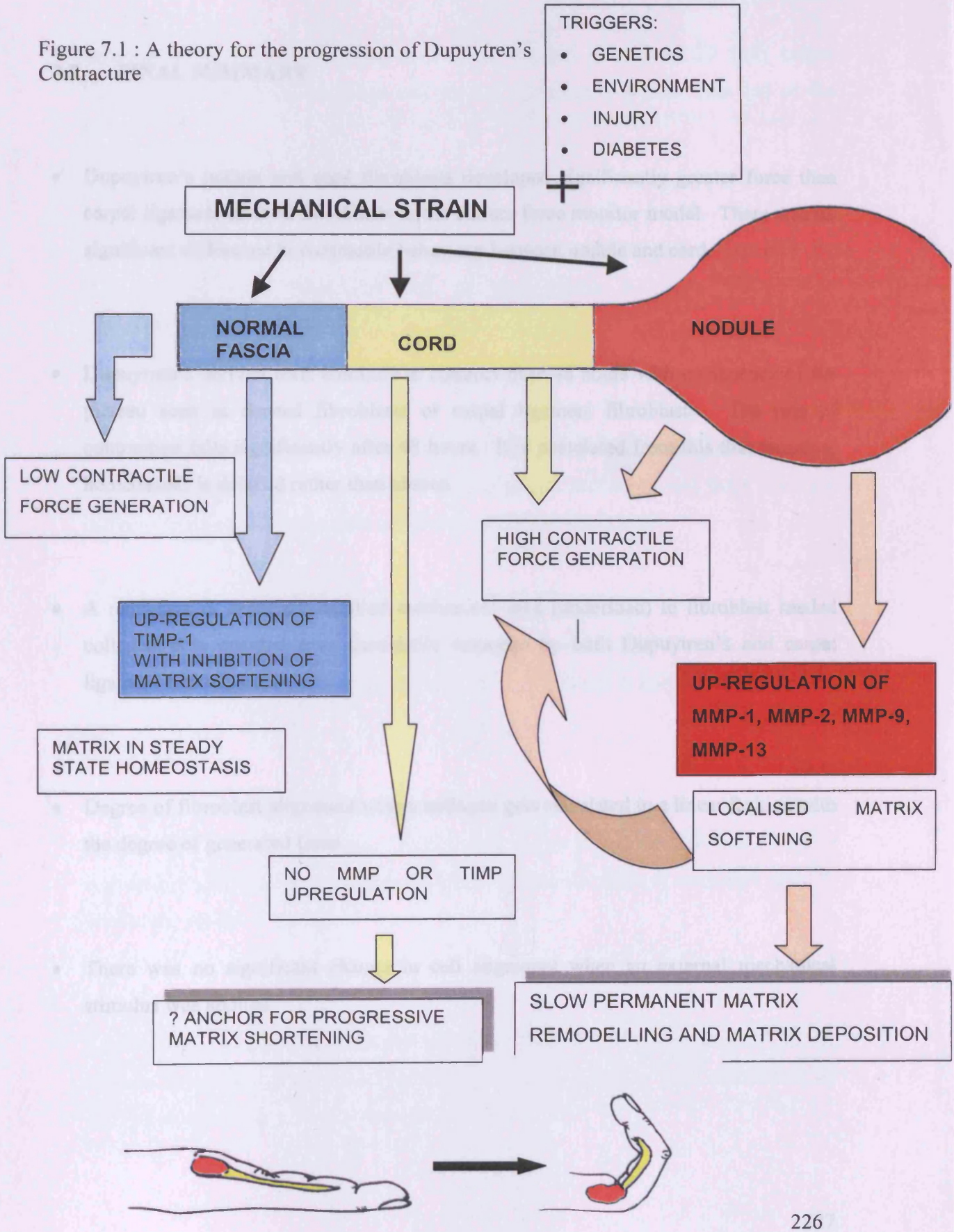
force and form many stress fibres. What is as yet unknown is the trigger by which this occurs. The generation of contractile force results in mechanical loading, which is thought to lead to a synthetic cell phenotype characterized by increased matrix synthesis and decreased degradation (Kessler *et al* 2001), plus cellular proliferation (Rosenfeldt and Grinnell 2000). The up-regulation of MMPs seen here in the Dupuytren's nodule would reflect matrix remodeling activity taking place. The difference between wound healing and Dupuytren's contracture is the lack of an end point in this remodeling activity, with matrix changes continuing over many years.

7.6 THEORY OF THE PROGRESSION OF DUPUYTREN'S CONTRACTURE

From the results of the work presented here in combination with current knowledge of the fibrocontractive disorders in general, a hypothetical theory for the progression of a clinical contracture has been formulated (figure 7.1). It is hypothesized that a number of triggers act together to allow the change from normal palmar fascia to Dupuytren's disease. Having switched from normal to "pathological", a number of changes occur that are related to mechanical tensional homeostasis of the Dupuytren's nodule fibroblast in particular. These cells lose the ability to maintain a tensional equilibrium within the surrounding extracellular matrix, which results in an abnormal excessive cellular contraction. Any external mechanical force applied across the matrix results in a cascade of matrix metalloproteinase up-regulation. One can theorise that this may lead to a localized pericellular matrix softening, which will then allow further cellular contraction, which may then hold the matrix in a shortened state. This tissue contraction produced by the cells is then translated into permanent remodeling, initially by changes in the overall spatial arrangement of the matrix, and later on by the deposition of collagen and other structural elements of the matrix. This is similar to the slip and ratchet theory discussed in previous work (Tomasek *et al* 2002). For the Dupuytren's cord, despite the generation of a high contractile force with the absence of tensional homeostasis, there is no major

change in the expression of the matrix metalloproteinases in response to changes in matrix tension. It is proposed (like previous workers (Vande Berg *et al* 1984; Hueston 1985; Moyer *et al* 2002; Bisson *et al* 2004)), that the cord is more quiescent than the nodule, and is composed of a different cell population. These cells, located in their dense collagenous matrix may, in fact act as an anchor against which the nodular tissue can "pull" as a contracture progresses.

Figure 7.1 : A theory for the progression of Dupuytren's Contracture



7.7 FINAL SUMMARY

- Dupuytren's nodule and cord fibroblasts developed significantly greater force than carpal ligament derived fibroblasts in the culture force monitor model. There was no significant difference in contractile behaviour between nodule and cord.

- Dupuytren's derived cells continue to contract over 48 hours with an absence of the plateau seen in dermal fibroblasts or carpal ligament fibroblasts. The rate of contraction falls significantly after 48 hours. It is postulated from this that tensional homeostasis is delayed rather than absent.

- A reduction in externally applied mechanical load (underload) to fibroblast seeded collagen gels resulted in a contractile response by both Dupuytren's and carpal ligament fibroblasts

- Degree of fibroblast alignment within collagen gels correlated in a linear fashion with the degree of generated force.

- There was no significant change in cell alignment when an external mechanical stimulus was applied

- Baseline TIMP gene expression in statically loaded fibroblasts for both carpal ligament and Dupuytren's nodule and cord is significantly greater than that of the MMPs

- Mechanical stimulation results in up-regulation of MMP gene expression by Dupuytren's nodule fibroblasts, with no up-regulation by carpal ligament or Dupuytren's cord fibroblasts

- Dupuytren's cord fibroblast MMP gene expression responses to mechanical stimulation broadly mimic those by carpal ligament, indicating that these cells are likely to represent a separate cell population to those from nodule.

- Carpal ligament fibroblasts display an up-regulation of TIMP-1 expression when exposed to mechanical stimulation, a response that is absent in nodule derived cells.

- The action of TGF-β1 appears similar regarding MMP expression between carpal ligament and Dupuytren's tissue. TGF-β1 acts to downregulate MMP-1 and MMP-2 expression, and acts to up-regulate TIMP-2 expression which is consistent with its profibrotic action.

- The finding of a reduction in TIMP-1 expression in all fibroblasts exposed to TGF-β1 may be explained by a feedback inhibition response resulting from stimulation by both mechanical and growth factor derived cues.

- Dupuytren's fibroblasts demonstrate a greater baseline gene expression of collagen III in comparison to normal palmar fascia.

- Neither TGF-β1 nor mechanical stimulation appeared to affect collagen gene expression within this experimental set up.

- There was no evidence of permanent matrix remodelling apparent after 8 hours in culture for all cell types investigated on the culture force monitor model

- After 48 hours residual matrix tension was significantly greater in Dupuytren's nodules in comparison to carpal ligament fibroblasts, but not significantly different in comparison to cord or dermal fibroblast cell lines. In all cell type matrix remodelling increased with time.

- As described in previous work (Marenzna et al 2004), it is likely that RMT and thus matrix remodelling is underestimated due to the influence of "creep" on the collagen gel

- It is postulated that the permanent shortening of the collagen matrix (otherwise known as residual matrix tension) seen in experiments run on the culture force monitor are unlikely to be due to the deposition of collagen III, and are more likely to be due to other structural changes in the matrix.

7.8 FUTURE WORK

There are a number of areas that would merit further research after reflecting on the findings discussed here. Two ideas work on expanding the investigation of the cells' contractile and molecular behaviour that was focused on previously.

The first is the investigation of fibroblast contractile behaviour and of matrix remodeling over prolonged time courses. Due to the associated problems of infection, and nutrition when allowing gel's to contract within an open culture system it was difficult to obtain accurate data beyond 48-56 hour time periods. It would be of interest to study the time taken for Dupuytren's fibroblasts to reach tensional homeostasis, if indeed this happens at all. Similarly, it would be exciting to analyse the fixed changes in the matrix by remodeling over several days rather than hours.

Secondly analysis of matrix metalloproteinases only occurred at a gene expression level, due to the time constraint of the thesis. It would have been of interest to directly measure protein levels within the media by a combination of Western blotting and Northern blot techniques. In addition it would also have been useful to look at gene expression over several different time points rather than after a static load or a single mechanical stimulus.

When taking all of the results obtained in this thesis together it seems that there is one avenue in particular which would be of relevance in developing a future therapeutic strategy for Dupuytren's disease – by further investigation of the abnormal MMP and TIMP expression. Future work on the Dupuytren's project would seek to study ways in which the aggressive fibroblast contraction and proliferation can be arrested in Dupuytren's disease using chemotherapeutic agents, and secondly: to extend this knowledge into the setting of clinical medicine by devising ways to administer the agent safely and effectively to patients in order to halt disease recurrence.

Production of natural inhibitors of MMPs (TIMPs) is one of the usual mechanisms by which MMP expression is controlled (Visse and Nagase 2003). Due to the fact that MMPs are overexpressed in some cancers and in wounds, there has been much interest in using matrix metalloproteinase inhibitors as therapeutic agents, in order to limit tumour invasion, and to facilitate normal wound healing. Both natural TIMPs and the synthetic TIMPs or MMPIs have been investigated.

Scott *et al* (1998) investigated the role of marimastat, a synthetic MMP inhibitor, in the contraction of dermal fibroblast seeded collagen gels. It was seen that Marimastat inhibited MMP-I activity, and fibroblast collagen lattice contraction, and this effect was reversible upon removal of the inhibitor. The viability and ability of cells to migrate and spread was not affected by the drug. A study on human retinal pigment epithelial cells demonstrated similar findings using the broad spectrum MMP inhibitor Galardin (Sheridan *et al* 2001). Galardin inhibition was dose-dependent, reversible, and dependent on cell number. Ilomastat has been shown to inhibit inflammatory protease activity in human middle ear effusions (Antonelli *et al* 2003), and has been very successful in the treatment of bacterial keratitis in human eyes within the remits of a clinical trial (Galardy *et al* 1994). Recent work in the field of glaucoma surgery has found the MMPIs to be effective in the inhibition of scar tissue formation in the eye (Wong *et al* 2003). By targeting the actions of these proteolytic enzymes, it is felt that a more physiologic and controlled method of modulating fibrosis may be achieved.

Many of these MMP inhibitors have been safely and effectively utilized in the treatment of neoplasia in order to limit metastatic spread all within closely monitored clinical trials. Marimastat has been most closely investigated (Praga-Wojtowicz *et al* 1998; Belotti *et al* 1999). It has been seen in human patients to be well tolerated, with reported side effects of musculoskeletal myalgia and arthralgia, which in all patients settled after a 2-week drug holiday. Phase III trials are ongoing in patients with glioblastoma and gastric carcinoma. It would be very interesting to investigate the ability of a broad spectrum matrix metalloproteinase inhibitor to arrest Dupuytren's fibroblast contractility and remodeling ability. If successful this may be put to some use in the future clinically.

APPENDIX

APPENDIX 1

Cell Lines Established from Patients undergoing Surgery for Dupuytren's Disease and Carpal Tunnel Syndrome

Dupuytren's patients

Patient	Cell line	Sex	Age	Digit affected
1	DP0302	M	71	R little
2	DP0402	M	71	L ring and little
3	DP0502	M	74	L little
4	DP0602	M	72	L little
5	DP0702	F	65	R little
6	DP0103	M	64	L ring
7	DP0203	M	60	R little
8	DP0303	M	77	R ring and little
9	DP0403	M	75	R ring and little
10	DP0503	F	63	R ring
11	DP0603	M	60	R little
12	DP0703	F	71	L ring
13	DP0803	M	67	R middle and ring
14	DP0903	M	74	R ring
15	DP1003	M	63	L ring
16	DP1103	M	67	L ring
17	DP1203	F	81	L little and middle
18	DP1303	M	78	R ring and little
19	DP1403	M	77	R little
20	DP0104	M	63	R little
21	DP0204	F	67	L ring and middle
22	DP0304	M	56	R little

Carpal ligament patients

Patient	CELL LINE	Sex	Age	Hand affected
1	NC0302	M	64	Right
2	NC0103		65	Left
3	NC0203		77	Left
4	NC0303		71	Right
5	NC0403		84	Right
6	NC0503		53	Right
7	NC0603		40	Right
8	NC0703		82	Right
6	NC0104	F	76	Left

APPENDIX II

FORMULATIONS OF CELL CULTURE SOLUTIONS USED

Normal fibroblast Growth Media (NGM 10%FCS)

Reagent	Final Concentration	Volume
Fetal calf serum (Gibco)	10%	50ml
Penicillin / Streptomycin (Gibco)	1U / ml	5ml
L-glutamine 200MM (Gibco)	1mM	5ml
HEPES Buffer (1M pH8)	7mM	3.5ml
Dulbecco's Modified Eagle's Media (Gibco)		500ml

Trypsin : Versene Solution (1:10)

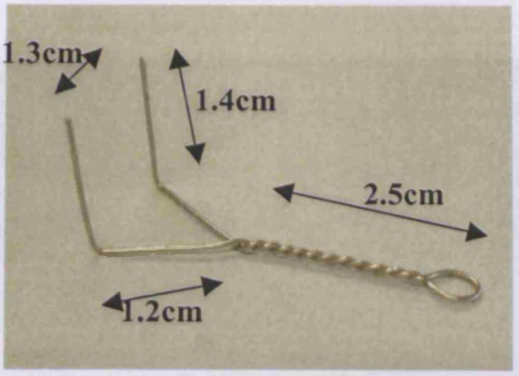
Reagent	Volume
Trypsin (2.5%) (Gibco)	2ml
Versene (Gibco)	18ml

Buffered Formal Saline

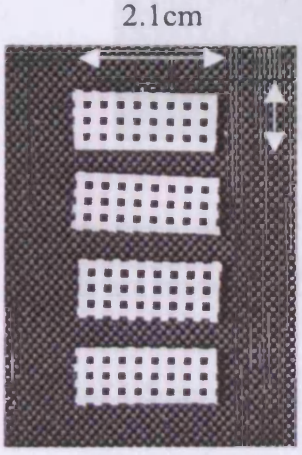
Reagent	Amount
Formaldehyde (BDH)	1 litre
Sodium Dihydrogen Phosphate (dehydrate)	45g
Di-sodium hydrogen phosphate (anhydrous)	65g
Distilled water	Made up to 10 litres

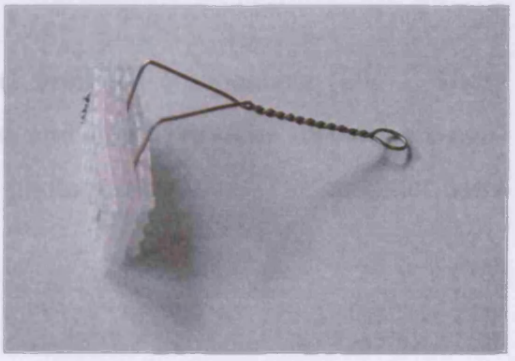
APPENDIX III

Composition of floatation bars and "A"frames for the Culture Force Monitor



"A" frames were constructed from lengths of surgical wire, bent and twisted to the dimensions shown above.



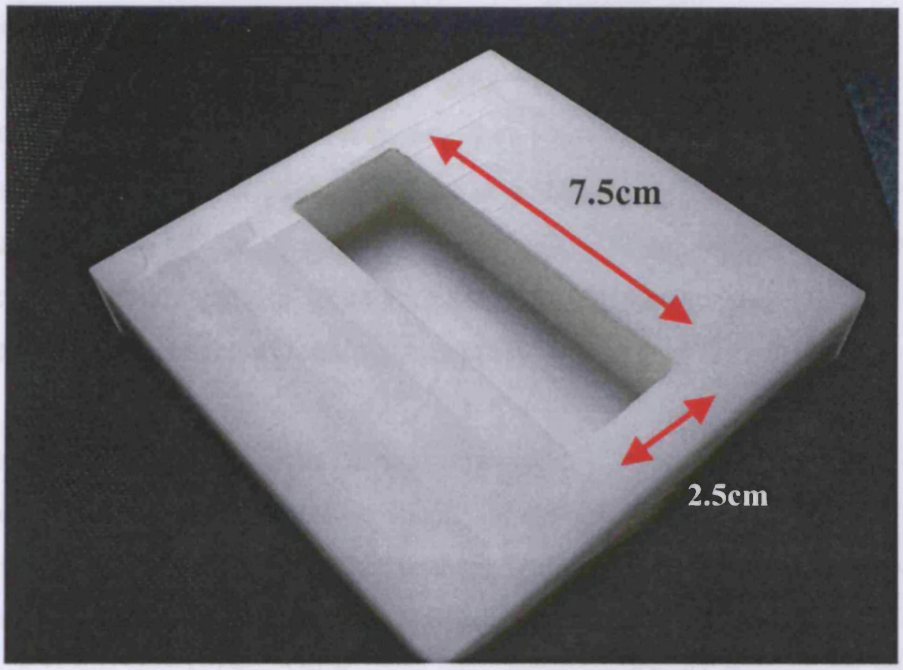


Completed floatation bar shown above

Floatation bars (A and B)were constructed from No.10 Clear Mesh (Cat 33030-1, Haberdashery department, John Lewis Department Store). Four 8 by 3 square rectangles were joined together to form each bar using stainless steel sutures.

APPENDIX III(b)

Composition and Dimensions of Mould for the Culture Force Monitor



Culture force monitor moulds were purchased from RS components (UK). These moulds are made of PTFE, and are easy to clean and autoclave. The surfaces are non-stick. Dimensions are shown on the annotated photograph above. The chamber has a depth of 1.5cm.

APPENDIX IV

Calibration of the Culture Force Monitor

Displacement of the force transducer in a horizontal direction occurs as a result of contraction of cells within a collagen gel. The cell filled gel acts to pull the transducer lever inwards and this is recorded as a positive force reading. Any manual forces on the transducer or prolonged time at a temperature different from 37 C may cause inaccuracies of the transducer's readings. Thus calibration of the force transducer within the culture force monitor was performed on a monthly basis to ensure continued accuracy of readings obtained.

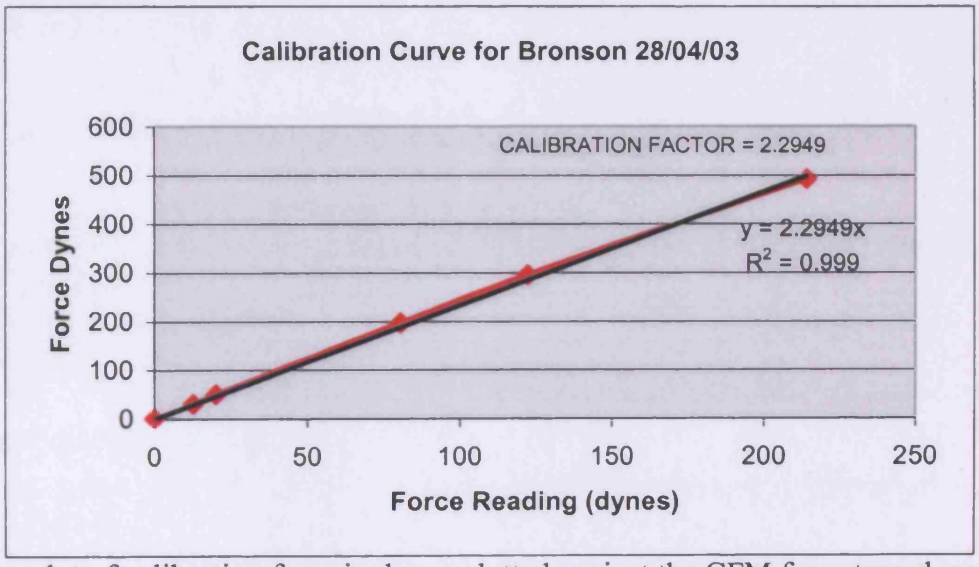
The transducer is removed from its retaining clamp and placed in a vertical direction on top of the micrometer mounting stage. The transducer sits in a neutral position when no weight is applied to it. The incubator door is shut, and the system allowed to equilibrate for temperature and CO_2 levels. The calibration factor on the labview program is set to 1. A one minute recording is then made of the measured force by the transducer in the neutral position. At this stage a small known weight is applied to the transducer, which causes a deflection of the arm, and an increase in the transmitted force reading. Once again the door is closed and the incubator allowed to equilibrate before recording the force reading for a one-minute period. The process is repeated for a series of 5 known weights in total. Each one-minute period allows the software to record 60 readings of force, and the mean of these readings is calculated to provide a relative mean force transducer reading for each weight.

The data is tabulated against each weight used, and the force in dynes that this weight corresponds to (1 dyne = 10^{-5} Newtons). A sample table is shown overleaf. From this the corresponding scatter plot of each computer reading, and the equivalent standard force applied can be generated. A line of best-fit is drawn, and providing the correlation coefficient (R^2) is good, the slope of the graph will correspond to the calibration factor. This value is then entered into the computer software. (i.e. the value of the actual reading of the force transducer needs to be multiplied in order to

convert the figure to dynes). A sample graph and the relevant calculations are shown below.

Weight number	Actual weight (g)	Corresponding force in Dynes	Mean force transducer reading
0	0	0	0
1	0.03	29.4	12.48
2	0.05	49	19.84
3	0.2	196	80.82
4	0.3	294	122.24
5	0.5	490	214.17

Table above demonstrates each weight used to calibrate the CFM, with its corresponding force in dynes that it related to, plus sample readings from a calibration run.



Scatter plot of calibrating force in dynes plotted against the CFM force transducer reading. The slope of the curve gives the calibration factor to be used.

Force = mass times acceleration
Force (Newtons) = Weight (kg) times Gravity (9.8)

APPENDIX V

Composition of DABCO

DABCO Mixture – Anti fade agent for immunofluorescence

Stored at 4°C wrapped in silver foil.

REAGENT	Amount
14-Diazodicyclo 2,2,2 Octane (Sigma)	1g
Phophate Buffered Saline (PBS) (Gibco)	4ml
Glycerol	36ml

APPENDIX VI

T.E.M fixatives and buffers

2.0% GLUTARALDEHYDE

- A stock solution of 25% glutaraldehyde is taken (EM Sciences Ltd. Cat no:16210)
- 8ml of the stock solution is mixed with 42 ml of distilled water
- To this is added 50ml of 0.2M Sodium cacodylate buffer pH 7.2

PARAFORMALDEHYDE (PA) – GLUTARALDEHYDE (GA) FIXATIVE

To make a 0.2%GA in 4% PA in a 0.2 M sodium cacodylate buffer

- 2 g of PA is dissolved in 25 ml of distilled water (heated to 60-70°C and stirring)
- Solution is cleared with 1M sodium hydroxide (slight milkiness may persist)
- Solution is cooled
- 0.4ml of 25% GA is added to 25ml of PA
- Solution is made up to 50ml with the addition of 0.2M sodium cacodylate buffer
- All fixation is carried out at 4°C

0.2M SODIUM CACODYLATE BUFFER

- Sodium cacodylate ($Na(CH_3)_2As\ O_2.3H_2O$ – M.W. 214.03)
- Take 4.280g of sodium cacodylate and dissolve in 66.6ml of distilled water in a volumetric flask
- Adjust to pH 7.4 using an appropriate volume of 0.1M hydrochloric acid
- Make up to the final volume of 100ml using distilled water

OSMIUM TETROXIDE

- 2ml ampoules of 2% osmium tetroxide were used individually (SPI chem. – 02595-BA)

RESINS

To make a hard Spurr's resin, the following were mixed together.

- ERL 4206 – 20g
- DER 736 – 8g
- NSA – 52g
- S-1 - 0.8g

APPENDIX VII

GENERAL PRECAUTIONS USED FOR RNA EXTRACTION

A separate clean "RNA area" lab bench was designated for RNA extraction. This was cleaned with IMS, and then cleaned further with DEPC water (see below). A clean lab coat and fresh gloves were utilised on a regular basis. All equipment and apparatus was designated for RNA use only and was pre-incubated with DEPC-water before autoclaving. (DEPC water is ultrapure water i.e. DNAase and RNAase free).

REAGENTS FOR RNA EXTRACTION

DEPC WATER

- Add 2ml diethylprocarbonate (0.2%) (Sigma P-1037) to 1 litre ultrapure (18MΩ) water shaking vigourously
- Incubate overnight at 37°C
- Autoclave
- Dry bottle

GT EXTRACTION BUFFER
(Store at 4°C for 2 months)

FORMULATION	SOURCE	For 500ml
4M guanidium thiocyanate (GT)	Sigma	236.4g
0.5% sarkosyl (free acid)	Sigma	2.5g
0.1% antifoam A	Sigma	0.5ml
25mM sodium citrate pH 7.0	BDH	50ml of 250mM stock
0.1M β-mercaptoethanol	BDH	3.49ml
0.2% DEPC	Sigma D5758	1ml

- Dissolve GT in 200ml of DEPC-water at 60°C, stirring thoroughly for 30 minutes
- Separately dissolve Sarkosy in the sodium citrate stirring thoroughly at room temperature, for 30 minutes
- Mix the citrate-sarkosyl plus the GT in the fume cupboard
- Add antifoam A
- Make solution up to 500ml using a measuring cylinder
- Adjust pH to 7.0
- Add 0.2% DEPC, shaking vigourously to dissolve.
- Incubate at 37°C overnight
- Autoclave
- Add β-mercaptoethanol

WATER SATURATED PHENOL

- Melt 100g of molecular grade phenol (Sigma (D5758)) at 60°C, then allow to cool
- Fill bottle to the top with an equal volume of DEPC water and shake vigourously
- Leave overnight to allow phase separation
- Remove the top layer, the aqueous phase
- Transfer or convert to a foil wrapped bottle
- Store at 4°C

ISOAMYLALCOHOL

- Isosamylalcohol (BDH 100383L)
- Chloroform (BDH 100774W)
- Mix chloroform with isoamylalcohol at a ratio of 24:1 in a clean autoclaved container

SODIUM ACETATE

- 2M sodium acetate adjusted to pH 4 with acetic acid.
- 12.31g sodium acetate
- 48.75ml of acetic acid, made up to 500ml with DEPC water.

SODIUM CITRATE

- 36.76g of tri-sodium citrate made up to 500ml in DEPC water, and adjusted to pH 7.0 with acetic acid.

70% ETHANOL

- 70ml of absolute alcohol was taken, and to this was added 30ml of DEPC water.

TAE

To make 1 litre stock solution of 50x TAE

- 242g Tris base (Sigma Cat T1378-500G)
- 57.1ml glacial acetic acid
- 18.5g EDTA (ethylenediaminetetacetic acid) (Sigma E-5134)
- 20g Sodium Acetate
- Add 700ml of distilled water to the above in a conical flask swirling gently
- Make up to pH 8

To make a stock solution of 1x TAE, take 20ml of 50xTAE in a one litre beaker and add 980ml of distilled water.

APPENDIX VIII

Reagents for Reverse Transcriptase Reaction

REAGENTS	QUANTITY	VOLUME	SOURCE
Extracted RNA	5mcg	8µl	-
Oligo-DT primers	200µg/ml	1µl	Gibco
0.1M DTT		2µl	Gibco
5 x RT buffer		4µl	Gibco
DEPC water		1µl	-
10mM dNTPs		2µl	Pharmacia (100mM kit)
RNA guard	30U/µl	1µl	Pharmacia (porcine)
MMLV Reverse transcriptase (RT)	200U/ml	1µl	Gibco
TOTAL VOLUME		20 µl	-

Table showing reaction substrates for the RT reaction for cDNA synthesis

CYCLE NUMBER	TEMPERATURE (°C)	DURATION (mins)
1	65	10
1	0	5
1	37	60
1	75	10

Table demonstrating reverse transcriptase reaction conditions

APPENDIX IXa

Reagents for the Polymerase Chain Reaction

REAGENT	Quantity	Origin
CDNA (from RT reaction)	2µl	-
10 x PCR buffer	2µl	Finnzymes
2mM dNTP	2µl	Invitrogen
DEPC water	8.75µl	-
DMSO	1µl	Sigma
Primer1 (F)	2µl (10pmol)	Applied Biosystems
Primer 2 (Rev)	2µl (10pmol)	Applied Biosystems
GAPDH primer (F)	2µl (10pmol)	Applied Biosystems
GAPDH primer (Rev)	2µl (10pmol)	Applied Biosystems
DNAzyme	0.25µl	Finnzymes
TOTAL VOLUME	**20µl**	

Table showing reaction substrates for the polymerase chain reaction

PCR CONDITIONS

Number of cycles	Temperature °C	Duration / minutes
30	95	1
	Annealing temp minus 2°	2
	72	3
1	72	7

APPENDIX IXB

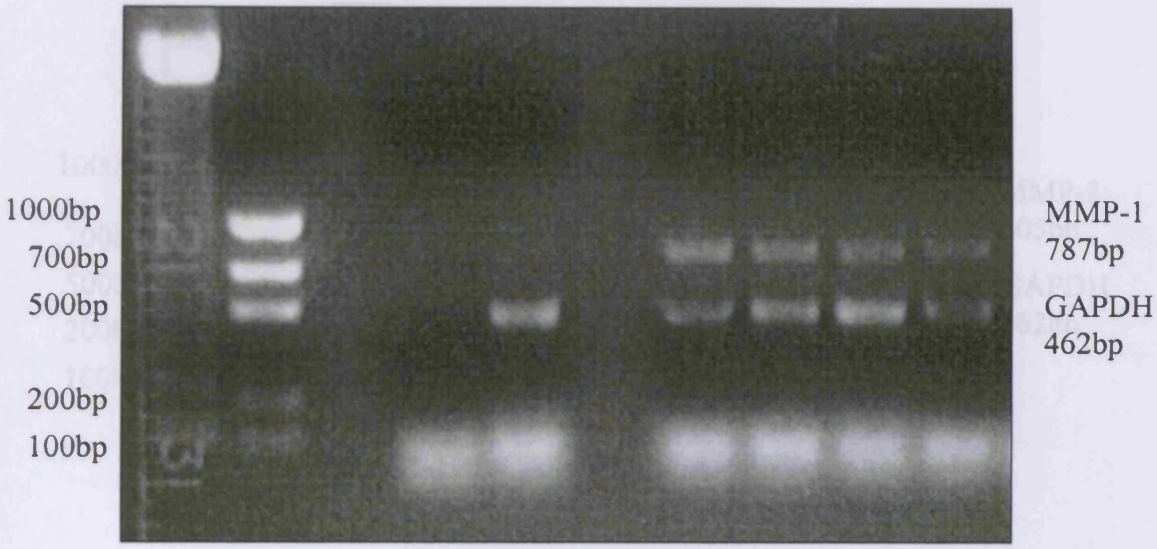
Table showing primer sequences for target genes for PCR, their expected PCR product size, and annealing temperatures for each gene investigated.

TARGET PROTEIN PRIMER	SEQUENCE	PCR product size (base pairs (bp))	Tm °C	Annealing temp °C
MMP-1	F - 5' CGA CTC TAG AAA CAC AAG AGC AAG A 3'	787	72	70
	R – 5'AAG GTT AGC TTA CTG TCA CAC GCT T 3'		72	
MMP-2	F – 5' GTG CTG AAG GAC ACA CTA AAG AAG A 3'	605	70	68
	R – 5' TTG CCA TCC TTC TCA AAG TTG TAG G 3'		72	
MMP-9	F – 5' CAC TGT CCA CCC CTC AGA GC 3'	263	66	62
	R – 5' GCC ACT TGT CGG CGA TAA GG 3'		64	
MMP-13	F – 5' TGC TGG CTC ATG CTT TTC CTC 3'	273	64	62
	R – 5' GGT TGG GGT CTT CAT CTC CTG 3'		66	
TIMP-1	F – 5' ACC ACC TTA TAC CAG CGT TAT GAG 3'	363	70	68
	R – 5' GAG GAG CTG GTC CGT CCA CAA GCA 3'		78	
TIMP-2	F – 5' CGC TGG ACG TTG GAG GAA AGA AGG 3'	358	76	74
	R – 5' GGG TCC TCG ATG TCG AGA AAC TCC 3'		76	
COLLAGEN-I	F – 5' CCC CCT CCC CAG CCA CAA AG 3'	361	68	64
	R – 5' TCT TGG TCG GTG GGT GAC TCT 3'		66	
COLLAGEN-III	F – 5' GGC TCC TGG TGA GCG AGG AC 3'	530	68	63
	R – 5' CCC ATT TGC ACC AGG TTC TCC 3'		66	
GAPDH	F – 5' AAG AAG ATG CGG CTG ACT GTC GAG CCA CAT 3'	462	92	80
	R – 5' TCT CAT GGT TCA CAC CCA TGA CGA ACA TG 3'		82	

APPENDIX X (a)

Densitometric analysis of PCR products from RT-PCR

MMP-1

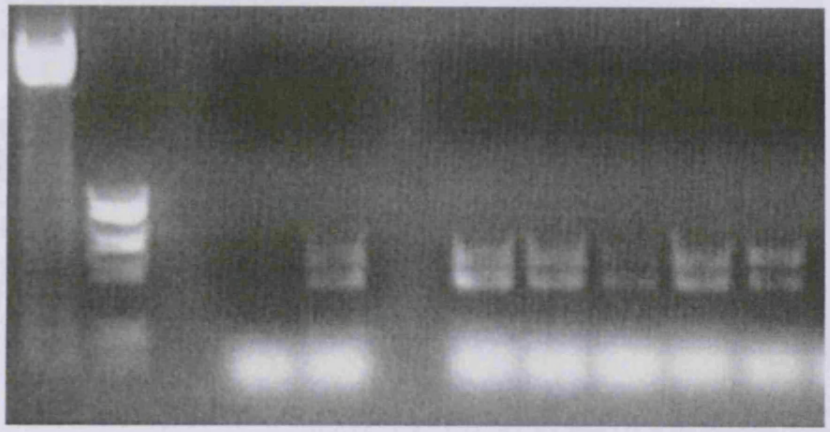


Photograph depicting MMP-1 expression by a representative selection of 4 different cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of MMP-1 (787bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and in addition a precision mass ruler to its immediate right. Note the absence of bands for the negative control, and the presence of GAPDH for the positive control. Note also the constant expression of the control gene GAPDH in all cases.

APPENDIX X (b)

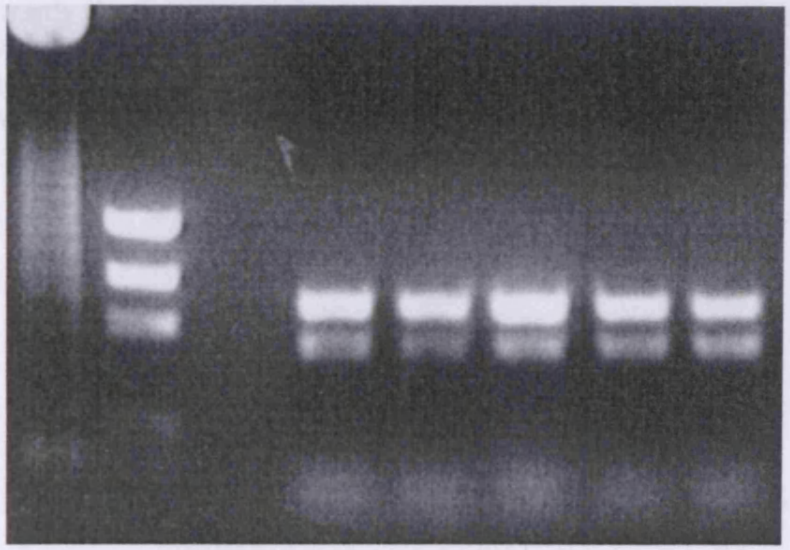
Densitometric analysis of PCR products from RT-PCR

MMP-2



CONTROL CARPAL LIGAMENT

(i)



DUPUYTREN'S NODULE

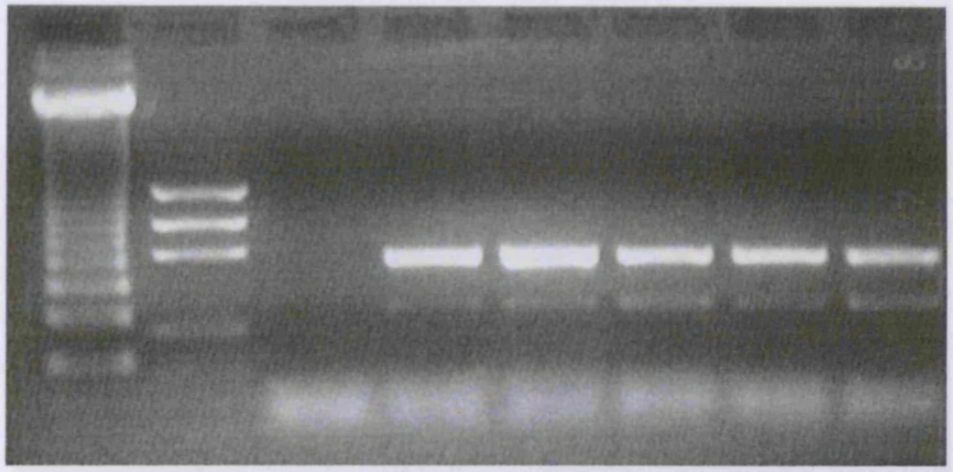
(ii)

Photograph depicting MMP-2 expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of MMP-2 (605bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and in addition a precision mass ruler to its immediate right. Note the constant expression of the control gene GAPDH in all cases. Note the enhanced band intensity for MMP-2 for Dupuytren's nodule (ii) in comparison to controls (i).

APPENDIX X (c)

Densitometric analysis of PCR products from RT-PCR

MMP-9

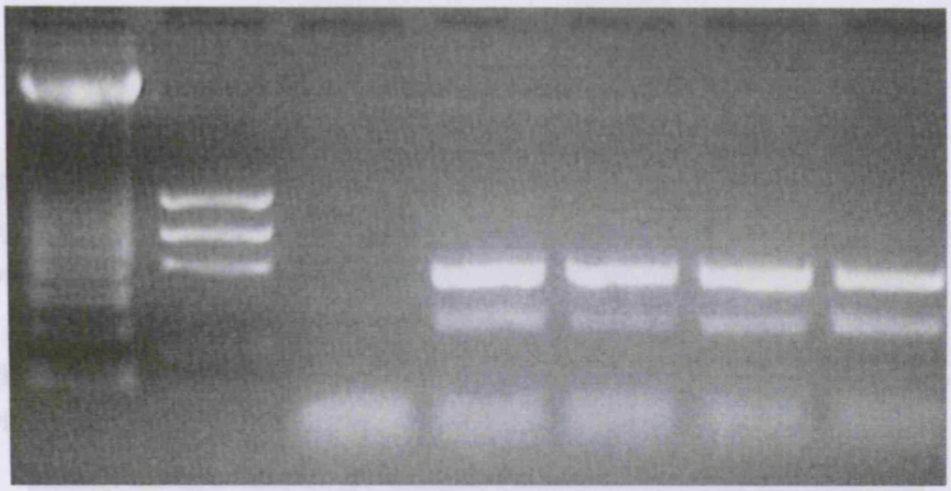


Photograph depicting MMP-9 expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of MMP-9 (263bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and in addition a precision mass ruler to its immediate right. Note the constant expression of the control gene GAPDH in all cases. Note the low band intensity for MMP-9 in all cases.

APPENDIX X (d)

Densitometric analysis of PCR products from RT-PCR

MMP-13

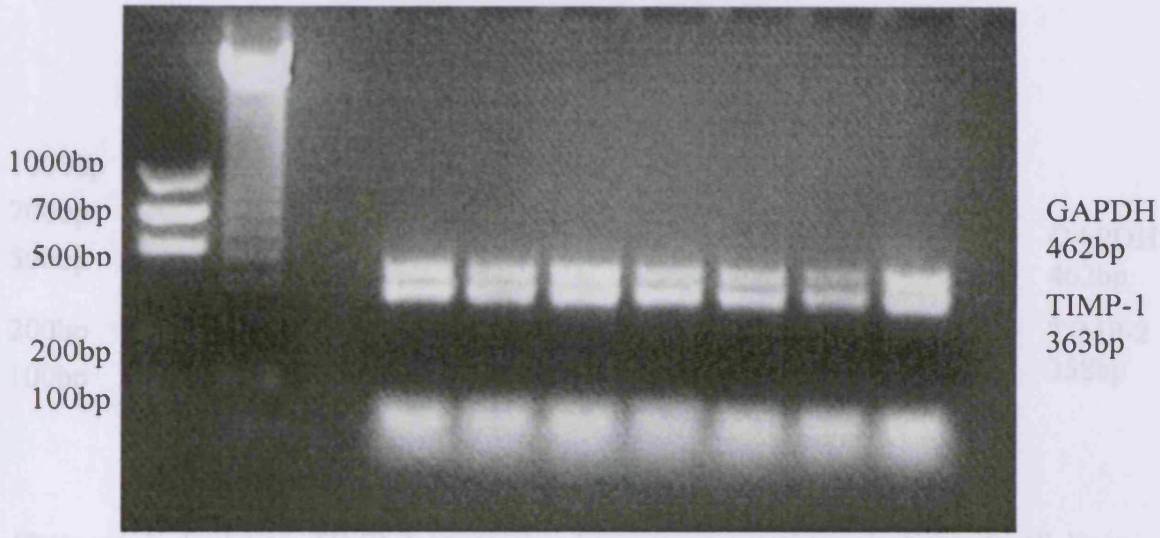


Photograph depicting MMP-13 expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of MMP-13 (273bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and in addition a precision mass ruler to its immediate right. Note the constant expression of the control gene GAPDH in all cases. Note the low band intensity for MMP-13 in all cases.

APPENDIX X (e)

Densitometric analysis of PCR products from RT-PCR

TIMP-1

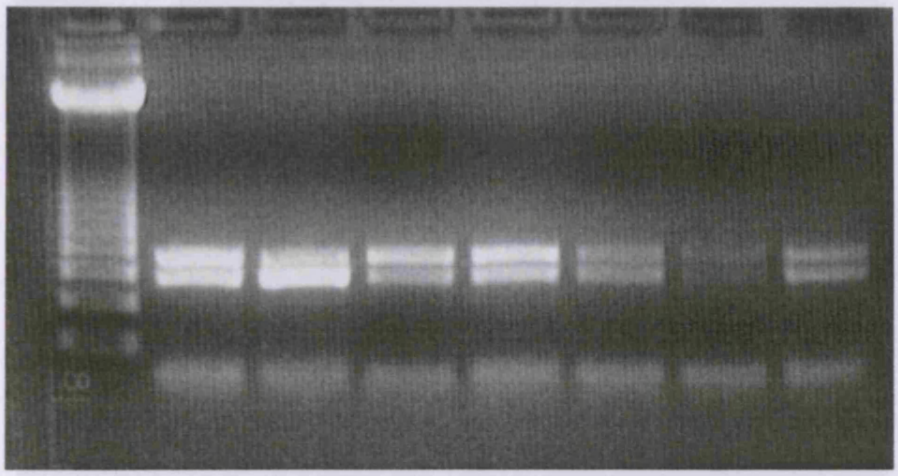


Photograph depicting TIMP-1 expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of TIMP-1 (363bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and in addition a precision mass ruler to its immediate left. Note the constant expression of the control gene GAPDH in all cases.

APPENDIX X (f)

Densitometric analysis of PCR products from RT-PCR

TIMP-2

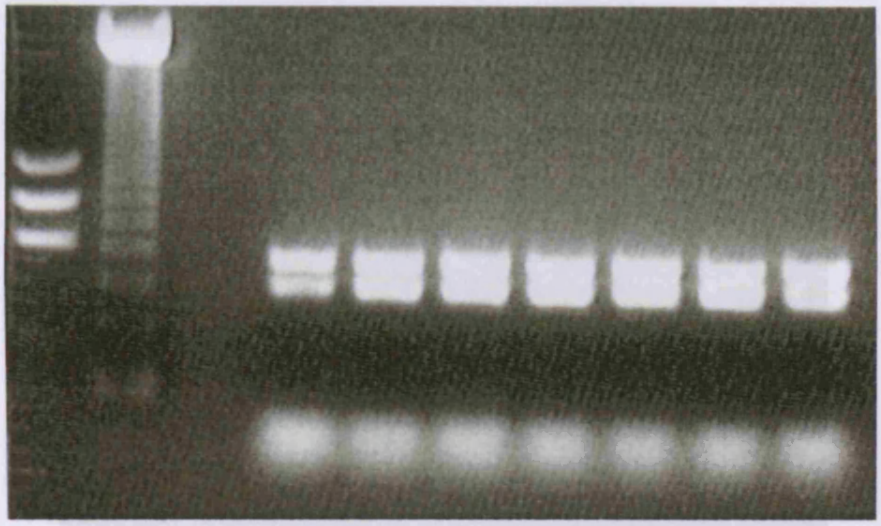


Photograph depicting TIMP-2 expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of TIMP-2 (358bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel.

APPENDIX X (g)

Densitometric analysis of PCR products from RT-PCR

COLLAGEN I

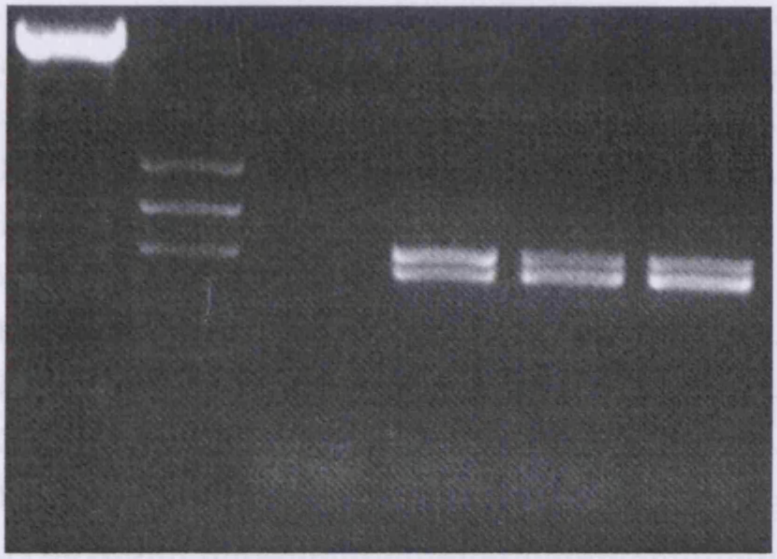


Photograph depicting collagen I gene expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of collagen I (361bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and to the left of that a precision mass ruler.

APPENDIX X (h)

Densitometric analysis of PCR products from RT-PCR

COLLAGEN III



Photograph depicting collagen III gene expression by a representative selection of cell lines from Dupuytren's tissue or carpal ligament. A 2% agarose gel stained with ethidium bromide shows the transcription of collagen III (530bp) and GAPDH (462bp). A 100bp marker is placed to the left of the gel, and to the right of that a precision mass ruler.

APPENDIX XI

PATIENT QUESTIONNAIRE FOR DUPUYTREN'S DISEASE

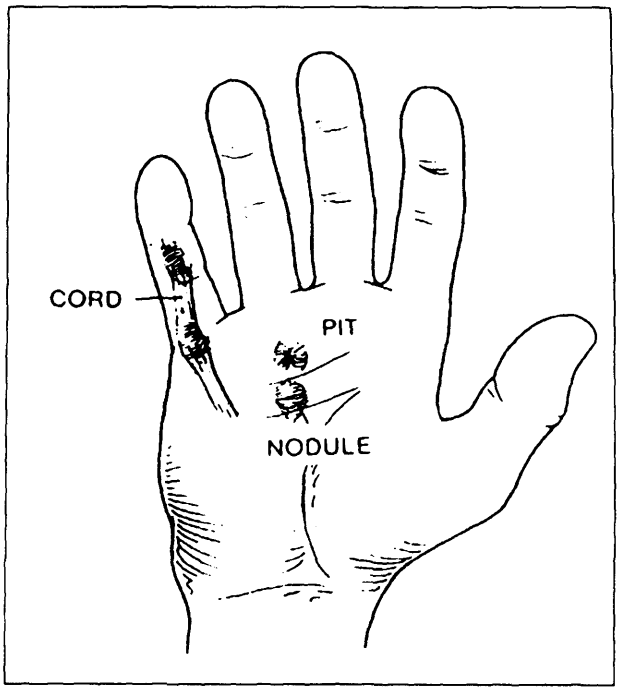
1. FEMALE / MALE
2. AGE.....
3. D.O.B.....
4. UNIT NUMBER.....
5. CELL LINE NUMBER.....
6. RIGHT HANDED / LEFT HANDED
7. OCCUPATION.....
8. PREVIOUS INJURY.....
9. SMOKING.....
10. ALCOHOL.....
11. DIABETES.....
12. HAND AFFECTED.....
13. TIME BEGAN.....
14. TIME CONTRACTURE 1ST NOTICED.....
15. TIME CONTRACTURE AFFECTED DAILY LIFE.....

CLINICAL EXAMINATION

DIGITS - THUMB INDEX MIDDLE RING LITTLE

SKIN CHANGES PITS THICKENING

CONTRACTURE – DEGREES FOR MCPJ AND PIPJ



SURGERY

DATE

TYPE OF OP

GRAFT?

CORRECTION?

RECURRENCE

TIME SINCE SURGERY

DEGREE OF RECURRRENCE

NEW DISEASE

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