Collagen Metabolism and Mechanical Loading in Dupuytren's Disease

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January 2005

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ABSTRACT

Dupuytren's Disease results in disabling contracture of the fingers, which often requires corrective surgery, and amputation in the most severe cases. It is a common disease affecting up to 40% of the population over 70 years old. It is caused by a contracture of the palmar fascia, which is an intricate three-dimensional network of connective tissue lying between the tendons and skin in the palm and fingers. The affected tissue is under significant tension, and it is this tension which contracts the fingers into the palm. This tension is thought to originate from fibroblasts within the palmar fascia. The excess fibrotic tissue which accumulates in the disease is composed mainly of collagen. This thesis investigates the relationship between fibroblasts, collagen metabolism and tension in Dupuytren's Disease.

In vitro studies were performed to examine the effect of loading on normal palmar fascia fibroblasts (controls), and fibroblasts affected with Dupuytren's Disease. Fibroblasts were cultured from operative specimens and seeded into collagen gels to mimic the *in vivo* environment. The gels were then either left to float freely in media, or were mechanically loaded in a tensioning culture force monitor. The gels and media were then processed to quantitate collagen, non-collagenous protein, collagenase, and TIMP-1 levels.

Results show that fibroblasts cultured from Dupuytren's Disease have a statistically higher production of both collagen and non-collagenous protein in response to mechanical load. This is an original observation, and helps to explain

various concepts in the understanding of the disease, in particular a historic theory (work hypertrophy) from 1959 suggested by Luck. This understanding opens avenues for further research towards therapeutic targets.

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GLOSSARY OF TERMS

BSA	Bovine serum albumin
^{14}C-proline	Proline incorporating radiolabelled isotope of carbon 14
DMEM	Dulbecco's modified Eagle's medium
FCS	Foetal calf serum
FPCL	Fibroblast populated collagen lattice
MMP	Matrix metalloproteinase
NCP	Non-collagenous protein
PBS	Phosphate buffered saline
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
tCFM	Tensioning culture force monitor
TIMP-1	Tissue inhibitor of metalloproteinase-1

INTRODUCTION

1 Clinical details

1.1 History

Dupuytren's Disease is named after the French surgeon, Baron Guillaume Dupuytren, who first described the condition in a lecture given on 5 December 1831 in Paris (Dupuytren G (1831). Notes of the lecture were recorded by Paillard and Marx, two of his assistants, and the following comments are based on a translation of these notes by Koch (1968). Dupuytren presented a 40-year old coachman, on whom he demonstrated bilateral contracture of the ring and little fingers. There was no history of trauma, or preceding rheumatic condition. Dupuytren then discussed the patients in whom he had observed the condition (usually manual workers), before moving on to the pathological anatomy of the contracture. He made the fundamental observation that the palmar fascia was 'tense, retracted and shortened', and that the tendons and joint capsules were unaffected. Dupuytren then performed palmar fasciotomies through transverse incisions, left the wounds open, and applied a splint. This surgery was conducted without anaesthetics as we know them today.

Elliot has comprehensively reviewed the literature of the period in a series of articles (Elliot 1988, Elliot 1989, Elliot 1990) detailing how debate concerning the aetiology, pathology and treatment of Dupuytren's Disease, also known as Dupuytren's Contracture, quickly developed. Dupuytren himself suggested a

congenital component to the disease, and even a congenital predisposition in patients who acquired the condition after trauma (Elliot 1988).

Surgical treatment of Dupuytren's Contracture spread across Europe over the ten years following Dupuytren's lecture in 1831(Elliot 1989). Elliot (1989) makes particular mention of a surgeon from Aix named Goyrand who, in 1833, suggested that the disease was not limited to the palmar aponeurosis, but may spread to involve the digits. He also performed the first limited fasciectomy by removing loose ends of fascia within the wound after straightening the digits.

Dupuytren described a disease causing contracture of the fingers and attributed the disease process to contracture of the palmar fascia. His ideas were rapidly taken up and modified. He was the first to perform a palmar fasciotomy, whilst others, notably Goyrand, performed fasciectomies. The debate regarding incision or excision of the affected fascia is unresolved to the present day (McGrouther 1988).

1.2 Anatomy of the palmar fascia

Knowledge of the anatomy of the palmar fascia allows the surgeon to understand the distribution of the diseased tissue, as it follows certain anatomical pathways (McFarlane 1974). The involvement of other structures, particularly the digital nerves and blood vessels, within the palm and digits may also be anticipated (McFarlane 1974, Umlas et al 1994).

The palmar fascia consists of a fibrous, collagenous sheet lying between the skin of the palm and the flexor tendons. There were thought to be two main groups of fibres, namely a set of transverse fibres (Skoog 1948, 1967) and a set of longitudinal fibres (Stack 1971, 1973). Microscopic dissection of the fibres within these layers has been performed (McGrouther 1982) and a third group of flimsy, vertical fibres are also present inserting into the skin, particularly at either side of the skin creases, and at the thenar and hypothenar eminences. This third set of fibres shows the palmar fascia structure to be an intricate three dimensional network in which McGrouther observed that the longitudinal fibres have a small degree of mobility in the channels formed from the deep transverse and vertical fibres, allowing some differential movement of the superficial layers of the hand on the deeper structures. Such an arrangement of fibres allows the fascia to resist horizontal shearing forces at any degree of digital flexion and provides firm but mobile skin anchorage. This specialised arrangement of fibres is disrupted in Dupuytren's disease, although the initial cause of the fibrosis is unknown.

The palmar fascia is not a slab of connective tissue laid down in a haphazard manner. Rather it is an intricate three dimensional structure, and as a consequence of its architecture is able to resist shearing forces to which the palm is repeatedly subjected. Dupuytren's Disease follows certain anatomical pathways, and the pattern of disease development may reflect the way in which the specific anatomy of this area responds to the loads applied to the fascial fibres.

1.3 Epidemiology

Dupuytren's Disease was found to be approximately four times more frequent in males than females in a large epidemiological survey undertaken by the International Federation of Societies for Surgery of the Hand on patients who consulted a surgeon specifically about the disease in their hands (McFarlane 1990). McFarlane also noted that the disease appears earlier in males, with an average age of 48 years, and the age at surgery is also earlier in males (57 years).

Most of the surveys this century have been performed on Caucasian populations, particularly those living in or descended from Northern Europeans (hence the term 'Viking Disease'). A recent study in North East Scotland (Lennox 1993) reviewed 200 consecutive geriatric patients over 60 years old and, on the basis of clinical findings, found the prevalence of Dupuytren's Contracture to be 39% in males and 21% in females. A study of male subjects in the Chelsea pensioners home calculated a prevalence of 13.75% (Carson and Clarke 1993).

Recent reports describe cases in non-European populations. Mitra and Goldstein (1994) reported eight cases in the black population in the USA, and Muguti and Appelt (1993) reported four cases in indigenous black Africans. In Asian populations Vathana (1990) reported 19 patients in Thailand and Liu (1991) reported 41 in Taiwan. No general epidemiological studies have been performed in these populations. Egawa (1990) in Japan found a prevalence of 16-25% in males and 3-10% in females over 60 years old. This finding is surprising as it is similar to the European data, although the patients were slightly older, possibly suggesting slower disease progression.

Srivastava et al (1989) reported 10 cases of Dupuytren's Contracture in patients from the Indian sub-continent treated in a hand unit in the Midlands in England. The disease characteristics in this small study (and indeed in the other studies above) are similar to those reported in the Caucasian literature.

Dupuytren's Disease is primarily a disease of the white male of northern European origin who is about 57 years of age and has had Dupuytren's Disease for about 10 years (McFarlane 1990). Case reports are accumulating from the non-Caucasian population, although the disease would seem to be extremely rare in this group.

1.4 Aetiology

Epidemiological evidence is strongly suggestive of a hereditary factor in Dupuytren's Disease, and Ling (1963) suggested that the gene may be inherited in an autosomal dominant fashion. The mechanism of inheritance is presently under investigation in a study in Oxford. However, Lyall (1993) reports on two pairs of identical twins, in each of which only one twin has Dupuytren's Disease and concludes that genetic factors alone are insufficient to develop the disease.

Systemic disorders such as diabetes (Jennings 1989, Chammas 1995) and epilepsy (Arafa 1991) are associated with development of the disease. Alcohol has also been investigated as a causative factor in Dupuytren's Disease. Burge (1997) performed a matched case-control study of 222 patients to investigate the

effects of smoking and alcohol, and showed a strong effect of smoking and a moderate effect of alcohol on the risk of developing Dupuytren's Disease.

Murrell (1989) observed that the microvessels in the palmar fascia of Dupuytren's patients had thickened walls, due to endothelial cell thickening and basal lamina proliferation. This causes narrowing of the microvessel lumen, relative ischaemia in the tissue, and higher levels of free radicals (produced in the conversion of hypoxanthine to xanthine). These free radicals are then thought to stimulate fibroblast proliferation and collagen deposition, which will encourage further microvessel ischaemia. This hypothesis is attractive as it attempts to unify factors which are associated with Dupuytren's Disease, many of which involve microvascular damage, in particular diabetes, alcohol and smoking.

Trauma to the hand may be in the form of an acute, single insult such as a burn, or repeated blows or contusions, such as those experienced by a manual labourer. Fisk (1985) emphasises that the predisposition of the individual must be taken into account when considering if the development of Dupuytren's Disease is a consequence of trauma, because development of the disease is not thought to occur if the patient is not susceptible. McFarlane (1990) suggest several factors (including age of less than 40, absence of epilepsy, diabetes and ectopic deposits) which should apply before an association may be considered between a single injury and disease development.

Hueston (1963, 1966, 1985) suggests that the natural history of the disease is a result of the patient's constitutional tendency (or diathesis). He describes general

and local factors which increase the probability of development of the disease. A patient possessing several of these factors may be described as having a strong diathesis. General factors indicating a strong diathesis include family history (positive), age of onset (young), sex (male), epilepsy and alcoholism. Local factors indicating a strong diathesis include distribution of lesions (ulnar), ectopic deposits (knuckle pads or plantar nodules), rate of progression (rapid) and results of previous surgery (early recurrence).

Dupuytren's Disease is a multifactorial disease. It may be the case that an individual must be genetically susceptible before a trigger causes the fibrosis to occur.

1.5 Pathology

The diseased tissue of Dupuytren's Disease is formed of two distinct macroscopic entities which may be distinguished on examination of the patient, but are more obvious when inspecting the tissue after excision from the palm. The most common structures are cords, which are fibrous bands of contracted palmar fascia, running in line with the digits. Nodules are less frequent than cords, and may be found alone, or in combination with one or more cords.

The histological organisation of the nodule and cord is different in terms of fibroblast distribution and collagen subtype (Nezelof 1985). The nodule is a highly cellular structure containing densely packed fibroblasts arranged in an approximately concentric pattern. The nodules contain fibres of newly formed collagen which are thin and tortuous (Nezelof 1985). In contrast, the cord has a

low cellular density, and has a high proportion of thick collagen fibrils which are arranged in a lamellar fashion. Nezelof did not observe any elastic fibres in his study, although Mohr (1994) did note their occasional presence.

Luck (1959) divided the pathogenesis of the disease into three sequential stages, namely the proliferative, involutional and residual. He argues that the fibroblasts form a focus of proliferation in nodules in the proliferative stage, and then contract and produce collagen in the involutional stage, ultimately leaving scar tissue in the final stage. He maintains that nodules are the origin of contracture, and that cords have resulted from nodules which have been subjected to tension stresses, and are therefore results of 'work hypertrophy'.

Histological studies performed since 1959 have made use of Luck's histological descriptions of the stages of the disease. Pasquali-Ronchetti (1993) found that the stages of the disease were not always as clear cut as Luck suggested when comparing clinical stage and structural alteration. Pasquali-Ronchetti (1993) also noted that apparently normal branches of palmar fascia in patients with Dupuytren's Disease were in fact affected by the pathological process. These findings, Pasquali-Ronchetti noted, were consistent with biochemical data (Bailey 1977) that showed collagen changes in both the affected and apparently normal areas of fascia in a patient with Dupuytren's Disease, and suggesting that Dupuytren's Disease may be a diffuse disorder of the palmar fascia.

Fibroblasts are the only cell seen in the tissue of Dupuytren's Disease, and investigation has focused on their microscopic, metabolic, and contractile

characteristics in vitro. Contractile studies of these fibroblasts have lead to the description of a subtype of fibroblast known as a myofibroblast (Gabbiani 1972).

Type I and Type III collagen are the predominant collagens laid down in Dupuytren's Disease, whilst their fibrillar structure resembles that of a hypertrophic scar (Bailey 1994).

The physical characteristics of Dupuytren's tissue are not only due to their cellular and collagenous constituents, but also to the glycosaminoglycan content (Flint 1982).

The mechanism of contraction in Dupuytren's Disease is still unsolved, although it is generally agreed to be cell mediated by the fibroblasts, as collagen itself does not contract until it has denatured, and such conditions do not exist in vivo (Bailey 1994). Luck (1959) believed the fibroblasts in the nodule played a critical role in contraction, particularly if the nodule was situated at a position enjoying mechanical advantage eg. directly over the metacarpophalangeal or proximal interphalangeal joint. Hueston (1965) believed that the disease originated as a nodule in the subdermal fibrofatty layer and became attached to the fascia, which it contracted. Chiu and McFarlane (1978) dispute the model of a nodule acting as a motor which winds in the tissue. Rather, they suggest, the contraction occurs throughout the diseased tissue as the myofibroblasts align the collagen fibres as they are formed.

There are three curious pathologies associated with Dupuytren's Disease which are examples of ectopic fibromatosis. Knuckle pads over the dorsum of the proximal interphalangeal joints are the most common site, followed by plantar nodules on the feet, and finally penile lesions in Pyronie's disease (McFarlane 1985). The histology of these areas is identical to that of Dupuytren's tissue (McFarlane 1985). The presence of these deposits increases the likelihood of developing Dupuytren's Disease (Hueston 1963), and they are therefore included in the diathesis criteria.

Dupuytren's Disease is a fibrotic disorder, occasionally associated with other sites of fibrosis. Macroscopic examination of the fascia appears to show two discrete entities, the nodule and the cord. Microscopic findings, however, demonstrate a tissue which may be universally affected, despite being clinically apparent only at certain sites.

1.6 Treatment

The treatment of Dupuytren's Contracture is surgical, the aim being to restore function and prevent recurrence (Tubiana 1985). Some non-surgical techniques such as enzyme injection (McCarthy 1992), topical steroid preparations (Shelley and Shelley 1993), and interferon injection (Pittet et al 1994) have been attempted, although none yet have shown superior results to those treated surgically. There are a wide variety of surgical procedures, and this section aims simply to outline the principles.

The surgeon may use one or a combination of four techniques. The palmar fascia may be divided (fasciotomy), removed (fasciectomy), or removed and covered with a full thickness skin graft (dermofasciectomy). When the disease is very advanced, amputation of the affected digit may be necessary. All of these techniques may be performed through a wide variety of skin incisions, and on completion of the operation, the wound may be left open or closed.

The fasciotomy, first performed by Dupuytren (1831), is the most straightforward procedure. Incision of the affected fascia allows the tension to be released, and may produce dramatic correction of metacarpophalangeal joint deformity (Hueston 1963). In 14 patients with an average age of 71, Hueston (1963) noted that recurrence required repeated surgical correction in only two cases. He also noted that, in eight cases, the bands of tissue totally disappeared after the fasciotomy. He therefore suggests that the operation is particularly suitable in elderly patients with severe metacarpophalangeal deformity. Burge (1994), in his review of the fasciotomy, suggests that, in the present day, cases treated by fasciotomy are just as suitable for treatment by limited fasciectomy due to the safety of axillary block anaesthesia. The patient also benefits from the decreased recurrence rate of the limited fasciectomy. The fasciotomy operation, nevertheless, gives an interesting insight into the role of tension in Dupuytren's Disease.

Removal of the fascia may be partial as in a limited fasciectomy where the affected fascia is removed with a small margin of normal aponeurosis (Hueston

1961), or complete as in a radical fasciectomy, an operation rarely performed now (Clarkson 1963)

Dermofasciectomy is performed in patients who have recurrent, aggressive disease, and also those in whom aggressive disease may be expected eg. younger patients with a strong diathesis (Varian 1994). Hueston (1985) states that skin replacement prevents recurrence, although subsequent studies have proved this incorrect (Kelly 1992, Searle 1992).

Surgical treatment is routinely performed for Dupuytren's Disease, and the trend is towards conservative, rather than radical, procedures. Skin grafting is thought to be particularly appropriate for cases of aggressive disease.

1.7 Recurrence

Recurrence of Dupuytren's Contracture after surgery is a major problem. Recurrence is defined as appearance of diseased tissue in an area already cleared by surgery, whereas extension is defined as appearance of the disease in an unoperated area of the hand (Leclercq 1994).

Table 1 Recurrence rates after surgery.

YEAR	FIRST AUTHOR	PATIENT NUMBER	TECHNIQUE	AVERAGE FOLLOW UP PERIOD	RECURRENCE RATE
1994	Brotherston	34	Dermofasciectomy	100 months	0%
1992	Kelly	24	Dermofasciectomy	13 years	47%(? definition)
1992	Searle	32	Dermofasciectomy	38 months	4%
1984	Tonkin	100	Fasciectomy/Dermofasciectomy	38 months	46.5%
1992	Adam	85	Partial fasciectomy	?	34%
1992	Vigroux	56	Fasciectomy	12 years	45%
1991	Moermans	175	Segmental aponeurectomy	2.6 years	35.7%
1991	Andrew	46	Segmental aponeurectomy	1 year	34%
1994	Cools	28	Open palm	2.5 years	33.5%
1992	Foucher	107	Open palm	5.6 years	41%
1997	Duthie	82	Fasciotomy	10 years	66%
1976	Rodrigo	47	Fasciotomy	2 years	100%

Table 1 shows that recurrence rates are high in many of the longer term follow up reviews, despite different surgical procedures being performed by dedicated hand surgeons.

The natural history of Dupuytren's Contracture varies from patient to patient. Hueston (1963, 1966, 1985) suggests that the natural history of the disease is a result of the patient's constitutional tendency (or diathesis), as described in section 1.4. Hueston concludes that patients with a stronger diathesis should, in general, have more radical surgery. McFarlane (1985) presents a world-wide study on 736 patients and shows that those patients who have a strong diathesis

have a 78% recurrence rate, compared with those who have no factors contributing to a diathesis having a 17% recurrence rate.

Adam and Loynes (1992) analysed 85 patients in a retrospective study to describe prognostic factors. They found that family history, age and alcoholism had no prognostic significance (as well as occupation, gender, and metacarpophalangeal joint involvement). Bad prognostic factors included proximal interphalangeal joint involvement, little finger involvement, a second operation, more than one ray affected, and long periods between operations.

Why does recurrence occur? The most likely reason is that not every affected cell can be surgically removed from the palm, even after fasciectomy and skin grafting. If this is the case, then the question may be asked, why is the recurrence rate not higher? Hueston (1963) would argue that it is the diathesis of the patient which predicts the chance of recurrence, rather than the type of operation selected. This argument, however, does not give any insight into the cellular mechanisms of the disease progression.

Recurrence is the main problem post operatively in Dupuytren's Disease. It is thought that the patient, rather than the surgeon, is the cause of this recurrence, although the mechanisms are not clear.

1.8 Discussion: The role of tension

Several observations make it likely that tension plays an important role in the development of Dupuytren's Disease. The important fact that the fascia is subjected to repeated compressive and shearing forces means that it must be able to absorb the energy without damaging itself or underlying structures. The specialised anatomical design of fascial fibres running perpendicular to each other allows secure but flexible skin anchorage. These fibres transmit the load to which they are exposed from the skin to their deep attachment sites. It has been noted that the disease process of Dupuytren's Disease follows certain anatomical pathways, which pick out favoured channels of tension transmission in the multitude of routes which may be taken through the palmar fascial fibres. The disease, once initiated by a trigger, may be following preferred lines of loading within the fascia.

When examining a lesion of advanced Dupuytren's Disease, it is clear that the mass of diseased tissue is under significant tension. Cords of Dupuytren's tissue contract the digit and may bowstring, lifting the overlying skin. It is not possible to straighten the finger by applying external force, although a gentle attempt at extension of the diseased fascia makes the contracted tissue more prominent whilst allowing the examiner to appreciate the tension within. The origin of the tension is debated, although it is generally accepted that it is cell driven.

The palmar fasciotomy is one of the simplest surgical interventions in Dupuytren's Disease as it involves incision of the affected fascia and a consequent release of tension within the tissue. Results of the fasciotomy are

comparable to other standard procedures such as the fasciectomy, despite the fact that very little if any tissue is removed. It has been observed that the diseased fascia may disappear after the tension has been released after a fasciotomy (Hueston 1963).

Rather than releasing tension on the diseased fascia, a technique has been developed which involves exerting additional, supraphysiological tension on the tissue (Messina and Messina 1991). This technique was developed to be used preoperatively on severely contracted digits to avoid amputation. The principle of the technique is the application of an external fixator device across the contracted joints, and continuous passive traction may then be applied over two to three weeks to correct the flexion deformity. Surgery is then much easier, as the finger is extended. In this case an overload of tension, rather than a release, causes the fascia to partially resolve. If the fixator is removed and no surgery is performed, the contracture reappears in a few days.

Attempts to pinpoint the origins of Dupuytren's Disease in the palm have proved impossible as the disease is already reasonably advanced by the time the patient presents with digital deformity. Skoog (1948) suggested that rupture of the fascial fibres due to trauma may cause a focus of proliferating cells which initiate the disease. Luck (1959) postulated that reactive, functional hypertrophy may play an important role in the disease development as the palm is subjected to repeated mechanical stresses.

Tension within normal palmar fascia is transmitted through a specialised network of fibres. In Dupuytren's Disease, this arrangement is lost along certain anatomical pathways on a macroscopic level, and throughout the palmar fascia on a microscopic level. Tension plays a role in the development and treatment of the disease, and possibly in its initiation.

2 The Fibroblast

2.1 The myofibroblast

The myofibroblast was first described by Gabbiani et al (1971) when his group examined the ultrastructure and contractile characteristics of granulation tissue. They observed that fibroblasts in granulation tissue could be responsible for contraction because cytoplasmic fibrils similar to those in smooth muscle cells were noted, along with nuclear changes which had been noted in other contractile cells (myocardium, vein endothelium, smooth muscle). They also demonstrated that strips of granulation tissue contracted like smooth muscle when stimulated with pharmacological agents.

Subsequent studies have suggested that myofibroblasts are derived from fibroblasts and express actin filaments within the cytoplasm (Grinnell 1995). They are thought to be central to contraction of the wound in normal wound healing.

2.2 Fibroblasts and contraction of collagen lattices

An important study by Stopak and Harris (1982), who were particularly interested in embryonic connective tissue morphogenesis, showed that fibroblasts cultured from chick embryo explants were capable of aligning collagen fibres in a collagen mesh of random fibre arrangement. The explants were circular in shape and therefore the collagen fibres aligned in a radial pattern around the explant.

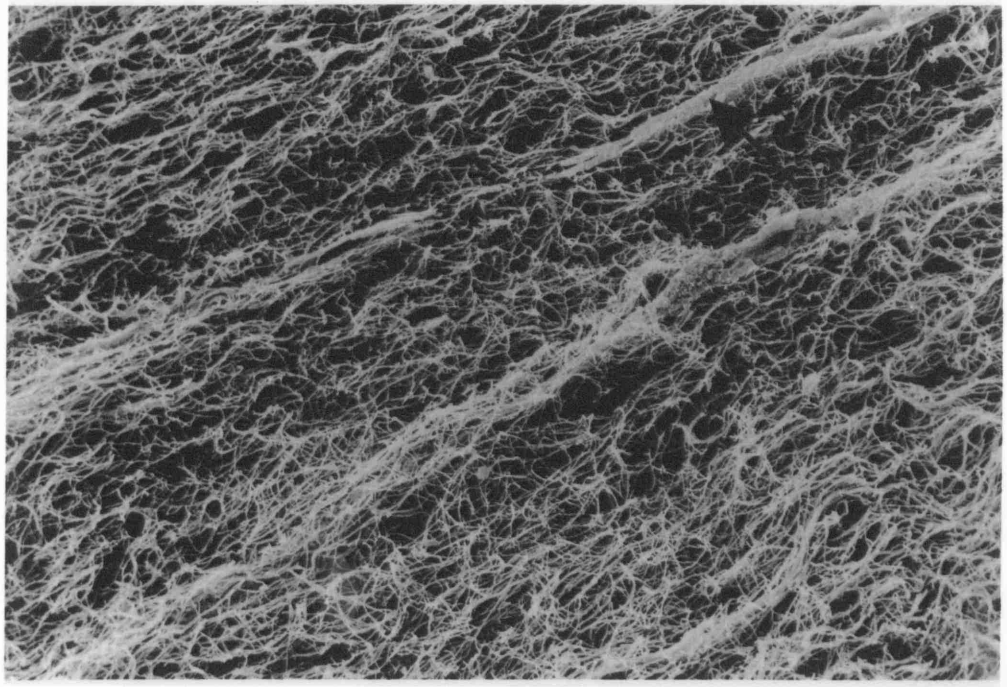


Figure 1.1 Scanning electron microscope image of dermal fibroblasts on a collagen lattice. An elongated fibroblast is indicated by the arrow. (Courtesy of Miss Kirsty Smith)

Various arrangements of explants and tethering devices within the collagen gel preventing alignment and contraction led to different, but predictable, patterns of gel deformation due to collagen fibre rearrangement. They proposed that collagen gel contraction was due to a mechanical effect of cell contractility, and that the cells do not simply shorten and contract the underlying collagen fibre, but

exert a traction force by elongating (rather than contracting like a muscle cell) and rearranging the underlying collagen. They proposed the term 'tractional structuring' to describe this mechanism.

Grinnell and Lamke (1984) examined collagen lattice contraction by human dermal foreskin fibroblasts because of their interest in wound contraction. They also observed that collagen fibres were reorganised by fibroblasts and suggested a balance is ultimately reached between the contractile force exerted by the cell on the collagen fibre and the tensile strength of the collagen fibre itself. They also suggested that the fibroblasts, in contracting the collagen lattice (collagen concentration 0.2%), may be attempting to obtain an *in vivo* environment of collagen concentration - 7% by contraction and compaction.

Tomasek et al (1992) studied human palmar fibroblasts in collagen gels, specifically the effect of releasing tension from the gels after five days, which resulted in rapid contraction of the collagen lattice. They found that the contraction of the gel after release was dependent on an organised actin cytoskeleton, by treating the gels with cytochalasin D. It was also shown that contraction was dependent on the presence of serum, and it was suggested that growth factors within the serum were the vital constituents which enabled contraction. Ehrlich et al (2000) investigated contraction of collagen gels by human fibroblasts and investigated the role of gap junction channels, which are composed of an array of six transmembrane proteins (connexins). They found that gel contraction was dependent on intact gap junctions. Ehrlich and Rittenberg (2000) have also shown that the contraction of collagen gels by human

foreskin fibroblasts is affected by cell density and is reduced by disrupting microtubules, inhibiting tyrosine kinase, and blocking $\alpha 2\beta 1$ collagen integrin.

Fibroblasts cultured from palmar fascia affected with Dupuytren's Disease have not been studied in terms of their synthetic or contractile properties. It is not the aim of this study to examine mechanisms of contraction of collagen gels by fibroblasts, but it is necessary to know the background of the proposed mechanisms of contraction of collagen fibres by fibroblasts and, based on the clinical observation that Dupuytren's Disease causes an extremely strong contraction of the affected digits, it may be reasonable to propose that the contractile abilities of the fibroblast in Dupuytren's Disease are not normal, and are upregulated.

The most recent work on contraction of FPCL has been performed by Bisson et al (2004). An *in vitro* study examined the force produced by fibroblasts cultured from control (carpal ligament) tissue, and nodule and cord cell lines from individual patients affected with Dupuytren's Disease. The fibroblasts were seeded in FPCL, and the contractile forces generated were quantitated using a culture force monitor (the same device used in this thesis). The results showed that Dupuytren's fibroblasts (particularly those derived from the nodule) exhibited increased force generation when compared to the control samples. Data showed that the Dupuytren fibroblasts had an inherently higher basal tension and contractile ability than controls, although no mechanism for this has been suggested.

The cellular pathway of fibroblast contraction of a collagen gel from nucleus, through cytoplasmic structures such as microtubules, cell membrane receptors, and their interaction with the collagen substratum is not fully understood. It is reasonable to assume that at one or more points in this pathway, fibroblasts affected with Dupuytren's Disease are upregulated in terms of their ability to contract, and there is likely to be a genetic component in coding for the proteins involved in the pathway.

3 Collagen

The collagens are a group of fibrous proteins constituting about 25% of the total protein mass in mammals (Alberts et al 1994). All members of the collagen family possess a triple helical structure, made from three polypeptide chains known as α-chains (Alberts et al 1994). The α-chains of the collagen molecule have a particular amino acid sequence, which may be described as a triplet Gly-X-Y, although this sequence is not unique to collagen (Chambers and Laurent 1997). Glycine occurs every third amino acid because it is the smallest amino acid (its side chain is one hydrogen atom) and so it allows the three α-chains to wind tightly around each other to form the triple helix. Every third X in the Gly-X-Y structure is proline, and every third Y is hydroxyproline.

Each α-chain forms a left handed helix, and three α-chains form a right handed helix, stabilized by inter α-chain hydrogen bonding. In the triple helix, glycines are positioned in the centre of the helix, and peptide bonds are also deep in the interior (Chambers and Laurent 1997). Proline and hydroxyproline confer rigidity to the helix because of their restricted rotation and bulk. Hydrogen bonds stabilize intra- and inter-chain helices, and the X and Y amino acid side chains point outwards from the helix and form lateral interactions with other collagen helices or proteins.

At present, 33 distinct α-chains have been described, and these may be arranged into 19 collagen types (Chambers and Laurent 1997).

Fibril forming collagens include Type I, III and XI. The fibrils are formed from different fibrillar collagens. These collagens are abundant in skin and tendon and their main function is thought to be mechanical support. Type III collagen contains three identical α-chains (α1), but Type I collagen (the most abundant) is made of two types of α-chain (two α1 chains, one α2). The fibrils are formed from triple helices in a 'quarter staggered' alignment which is stabilized by noncovalent forces and crosslinks between chains derived from lysine groups. The quarter-staggered arrangement is so called because each collagen chain is displaced from its neighbour by a quarter of its length, and gives the banded appearance of collagen under the electron microscope (Baynes and Dominiczak 1999).

Non-fibrillar collagens are a heterogeneous group including basement membrane collagens (Type IV) and fibril associated collagens with interrupted triple helices (FACIT, Type IX in cartilage and XII in embryonic tendon and skin).

3.1 Collagen synthesis

Collagen genes contain coding sequences of exons interrupted by intervening sequences of introns, which are excised. The resulting strands of exons are ligated in the nucleus to form mRNA which undergoes transcription in the rough endoplasmic reticulum. Preprocollagen possesses a hydrophobic signal sequence which helps the molecule bind to the endoplasmic reticulum and directs the growing peptide chain into the endoplasmic reticulum (Baynes and Dominiczak 1999). Post translational modification involves removal of the signal peptide, leaving procollagen. Hydroxylases then add hydroxyl groups to proline and

lysine residues yielding 3- and 4-hydroxylysine and hydroxylysine. O-linked glycosylation occurs by addition of galactosyl residues to hydroxylysine. Disulphide bonds (intra and inter chain) are formed by a disulphide isomerase and the formation of a triple helix is achieved. The procollagen is then transported to the Golgi apparatus where it is packed into secretory vesicles and exported to the extracellular space by exocytosis, where non-helical extensions are removed by proteinases yielding a tropocollagen molecule. These molecules then form insoluble collagen fibrils by the action of lysyl oxidase which creates aldehyde crosslinks within and between triple helices.

It is important to understand that collagen is a dynamic protein in a state of flux (Laurent 1987). Degradation may occur intracellularly or extracellularly. Intracellular degradation may occur within the lysosome/cisternae of the endoplasmic reticulum, and the Golgi apparatus. It has been suggested that the Golgi pathway may fulfil a basal turnover role, whereas the lysosome pathway is increased if defective collagen production increases. Extracellular degradation may occur by the action of various proteases such as collagenases produced by fibroblasts, and also by macrophages and neutrophils which are capable of phagocytosis and protease synthesis. Extracellular collagen may also be internalised by fibroblasts before breakdown.

3.2 Collagen in Dupuytren's Disease

Bailey (1990, 1994) has reviewed the changes in type, proportions and nature of collagen in Dupuytren's Disease. The major differences from the normal palmar fascia will be outlined.

Hunter et al (1975) performed one of the first studies on collagen in palmar fascia affected with Dupuytren's Disease. They showed that the Dupuytren's tissue was very similar, in terms of amino acid composition, to tendon collagen. They also analysed the extent, but not the nature, of the collagen cross links within the Dupuytren's tissue, and found that the collagen in areas of active disease was much more susceptible to depolymerisation, and was therefore thought to be less highly crosslinked, than collagen from mature areas of disease.

Bailey et al (1977) made further fundamental observations on the basis of the chemical composition of the diseased tissue, noting that normal aponeurosis consists almost entirely of Type I collagen, but Type III collagen was noted in Dupuytren's tissue in much higher levels than in normal aponeurosis. The increased levels of Type III collagen were also found in apparently unaffected palmar fascia of patients affected with Dupuytren's Disease. This suggested that the disease process was present throughout the palmar fascia. This observation was supported by evidence of reducible cross links (an indication of new collagen synthesis) in both the affected tissue and apparently unaffected tissue, in contrast to evidence of mature collagen in the normal aponeurosis (hexosyl-lysine). The findings of Bailey et al (1977) were confirmed by Gelberman et al (1980) and Bazin et al (1980). Bazin also noted the location of Type III collagen fibres (by

immunostaining) in both the affected and apparently unaffected fascia of Dupuytren's tissues.

Brickley-Parsons et al (1981) conducted a large study on the diseased fascia of 400 patients with Dupuytren's Disease, and 100 controls. Results of biochemical analysis of these samples were in keeping with the observations of Bailey (1977). Brickley-Parsons suggested that the collagen present in Dupuytren's tissue was typical of new, rapidly synthesized collagen produced in wound healing, and therefore the disease was behaving like a 'local, active repair of the palmar fascia'. They also suggested that the contracture of the tissue may be due to the same mechanism as the contraction that normally occurs in a dermal wound healing response as the edges of the wound are drawn together. The power of contraction, Brickley-Parsons suggests, is of fibroblast origin.

There is only one paper at present (Murrell et al 1991) which attempts to describe collagen synthesis in vitro by fibroblasts derived from Dupuytren's tissue. Fibroblasts from explant culture were incubated in multiwell plates and the collagen synthesis was quantitated over a 20 hour period using a ^{3}H proline incorporation method. No difference was found in the collagen production in terms of amount and proportion of collagen type (I and III) between control and Dupuytren's fibroblasts. A fall in total collagen was noted as the seeding densities of the fibroblasts increased, the fall being due to a decrease in Type 1 collagen. Type III collagen synthesis is unaffected by cell density in this study. Murrell concludes from this in vitro work 'These and recent morphological studies by Gabbiani and Montandon (1985) indicate that the fibroblasts in

Dupuytren's contracture are not abnormal: they merely produce less Type I collagen at high cell density'. Murrell suggests that the reason for the fibrosis in Dupuytren's Disease is simply that the fibroblasts are in greater numbers than in normal palmar fascia, and the resultant excess collagen is a reflection of the greater proliferative rate of the fibroblasts.

Bailey (1994) comprehensively reviewed the literature of collagen in Dupuytren's Disease. He makes several important points. Tissues which are developing fast and may be said to possess a high degree of plasticity typically have excessive hydroxylation and the presence of Type III collagen. Dupuytren's tissue has these properties. Biochemically, the tissue is much more akin to hypertrophic scar which fails to mature.

Tissue affected with Dupuytren's Disease is different to normal tissue in terms of composition (more collagen, higher levels of hydroxylation), collagen type (more type III), and cross links (more immature cross links).

3.3 Collagen synthesis in FPCL

Collagen synthesis within FPCL has not been extensively studied. Collagen synthesis by human dermal fibroblasts, over a short period of four hours using a radiolabelled incorporation method, shows a decrease in collagen synthesis over this period when compared with monolayer culture (Mauch 1988). The decrease was not related to enhanced collagen degradation. The decreased synthesis was consistent at both mRNA and protein levels, and was seen in both collagen I and

III. 90%of newly synthesized collagen was found in the contracted gel, compared with 75% in monolayer culture.

A report by Lambert et al (1992) attempted to quantitate collagen and collagenase from human dermal fibroblasts within FPCL. The lattices were cast in bacteriological dishes either within a wire ring, or simply on the base of the dish. Lattices cast alone in the dish floated freely and were contracted by the fibroblasts. Lattices cast within the metal ring were also allowed to float freely, but were subjected to tension as the wire ring restrained retraction of the collagen lattice by the fibroblasts. The tension set up in the lattice was therefore endogenously developed. Under these conditions, collagen synthesis (as measured by radiolabelled proline incorporation) was higher in the bound lattices than in the free floating lattices on each day of the five day experiment. Collagenase activity was very low and could not be accurately quantitated in the first 24 hours, although it greatly increased by day 3 in both free floating and bound lattices. By day 5 the collagenase level was twice as high in the free floating lattices as in the bound lattices.

4 Aims of thesis

Dupuytren's Disease often results in severe deformity. The diseased tissue is composed of an excess of extracellular matrix components, particularly collagen. The main collagens identified are Type I and Type III. The diseased tissue is under tension, which causes the digits to be drawn into the palm, and loss of function slowly develops.

The reason for excess collagen deposition is uncertain. There are several possibilities.

Firstly, the fibroblast may be genetically different in individuals affected with Dupuytren's Disease. From birth, the fibroblast may be destined to synthesise excess collagen, although, for an unknown reason, this synthesis does not occur in most affected individuals until later in life in the fifth or sixth decade. The mechanism for the increased synthesis is not known. The observation that, in affected individuals, apparently clinically unaffected areas of palmar fascia are affected with microscopic disease, supports the concept of a 'field change' throughout the palmar fascia, which lends weight to a fundamental genetic difference in the fascia.

Secondly, the excess collagen synthesis may result from a particular insult, which changes the metabolic synthetic properties of the fibroblast. Trauma has been suggested as a trigger, either in the form of a single impact, or repeated smaller events. The fibroblast may be attempting to respond to ruptures of the intricate

three-dimensional structure of the palmar fascia by initiating a wound healing response. It may be that this repair mechanism restores the normal structure in unaffected individuals, but results in an excessive, hypertrophic, response in those with Dupuytren's Disease.

The origin of the tension in the affected tissue is uncertain. The main contenders are the fibroblast cell itself which contracts the collagen fibres, or, possibly, the collagen fibres themselves which contract of their own accord. Contraction due to collagen shrinkage alone can probably be discounted because it only occurs at 65 ^{0}C (Bailey 1994).

The discussion in section 2 on the myofibroblast following the work of Gabbiani and Majno (1972) leads to the conclusion that the fibroblasts are capable of contraction of tissues, and are the most likely origin of the tension within the collagen fibrils because of their structural similarities to muscle cells. However, this raises the question of whether the increased collagen synthesis by the fibroblast is autonomous, whatever the tensional environment, or whether it is a *response* to the tension generated by the Dupuytren's fibroblasts.

There has been no work undertaken on the response of fibroblasts from Dupuytren's Disease to mechanical loading. There is a clear gap in our knowledge of their behaviour under such conditions, and this seems to be a fundamental step in understanding the disease.

This thesis was undertaken to examine the relationship between fibroblasts, tension and collagen metabolism. *In vitro* experiments were performed to investigate the collagen metabolism of normal palmar fascia fibroblasts cultured from patients undergoing carpal tunnel release (control fibroblasts), and compare the results to fibroblasts cultured from tissue excised at operation from individuals with Dupuytren's Disease.

The fibroblasts were seeded into a collagen gel to mimic, as closely as possible, the physical environment of the extracellular matrix and the three dimensional attachments formed by fibroblasts *in vivo.* In order to apply mechanical loads to the collagen matrix, a tensioning culture force monitor (tCFM) developed in the laboratory at University College London, and validated by Dr Mark Eastwood, was employed to apply loads to the collagen gels in a standardised, reproducible manner. The gels which were loaded were compared with gels which were left to float freely on medium, and their response in terms of protein synthesis was examined. The collagen synthesized during the experiments was identified using a standard radiolabelling technique with proline incorporating ^{14}C.

The analytical work was performed at Addenbrooke's Hospital, Cambridge under the supervision of Dr Graham Riley in the Rheumatology Research Unit. Standard biochemical techniques were used to quantitate proteins incorporating ^{14}C labelled proline. Electrophoresis gels were run to demonstrate products of collagen metabolism during the experiment. ELISA techniques were employed to quantitate activity of collagenase and TIMP-1.

MATERIALS AND METHODS

1 Introduction

Methods are divided into four sections. The first details general methods, the second details culture of fibroblasts from operative specimens. The third section describes seeding of fibroblasts into collagen gels (or fibroblast populated collagen lattices-FPCL), and application of mechanical loads to the gels. The final section describes analysis of gels and media.

2 General Methods

Medium was made up using the same supplements in all experiments. Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Paisley, Scotland) was supplemented with 10% foetal calf serum (First Link FCS 1044 APP Batch AF1611, Sutton Coldfield, UK), 1% L-glutamine (Life Technologies, Paisley, Scotland), and 1% penicillin/streptomycin (Life Technologies, Paisley, Scotland).

Collagen gels were prepared from chloroform sterilised rat tail Type I collagen in acetic acid (First Link), and 10x DMEM. TGFβ-1 (human recombinant) was obtained from Sigma (Poole, Dorset UK).

3 Cell Culture

3.1.1 Explant culture

Explant cultures were established from tissue obtained from the operating theatre. Normal, proximal palmar fascia (control tissue) was obtained from patients undergoing surgery for carpal tunnel syndrome. Dupuytren's tissue was obtained from fasciectomy procedures. Consent was obtained from all patients.

After excision, the tissue was wrapped in a swab soaked in 0.9% saline, and immediately stored at 4^0C. Tissues prepared under such conditions were transported to the laboratory and processed for explant culture within 48 hours. Explant cultures were performed in sterile conditions in a laminar flow hood.

The gross pathological specimen was subjected to one wash in 70% alcohol, and a further two washes in phosphate buffered saline (PBS). Small cubes of tissue approximately 2mm x 2mm x 2mm were cut from the operative specimen using a forceps and scalpel. The samples were gently pressed onto the base of a 75 cm^3 flask (Corning) with a glass pipette. Approximately 10 pieces of tissue from the same patient were placed in each flask. The flask was incubated at 37^0C for one hour.

After incubation, the flask was gently flooded with 6 ml medium, and gassed with 5% CO_2 for one minute and sealed. Flasks were incubated at 37^0C and checked daily. Medium was changed twice each week.

Explants of normal palmar fascia were set up using the method described. Dupuytren's tissue was prepared in the same way, but in addition an attempt was made to separate nodular tissue from cord tissue, depending on the macroscopic appearance of the sample. Only tissue which was clearly appeared to be entirely nodule or entirely cord was used for explant culture.

3.1.2 Cell passage and feeding

When explant flasks had reached confluence, cells were passaged into further flasks. Cells were passaged by pipetting off the medium, and washing the flask once with 10ml PBS. The PBS was discarded and a 10% solution of trypsin-EDTA (Life Technologies) was added to the flask in sufficient quantity to cover the floor of the flask (eg. 5ml trypsin solution in a 75 cm^3 flask). The flask was placed on a rocking plate for 10 minutes in an incubator at 37^0C and gently knocked by hand to dislodge any remaining attached cells. The trypsin suspension containing the cells was pipetted into a Universal container and an equal volume of complete medium was added. The suspension was centrifuged at 1500 rpm for three minutes, and the supernatant discarded. The precipitated cells were resuspended in a known volume of media and distributed into new flasks. Flasks were gassed with 5% CO_2 for one minute following each passage.

Cells were fed with new medium twice each week, each feed was preceded by a single rinse with 10 ml PBS. Flasks were gassed with CO_2 after each feed.

The passage number of cells was kept as low as possible throughout the experiments (Moyer et al 2002). Experiments were performed on cells between passage three and six.

4 Preparation of FPCL and application of mechanical load

4.1 Introduction

The tensioning culture force monitor (tCFM) was used to apply a specific, quantifiable loading pattern to the fibroblast populated collagen lattice (FPCL). Two types of FPCL were compared in these experiments. The first was attached to the tCFM by attachment bars which allowed mechanical loads to be applied to the FPCL (*Figure 2.1*). The second was a free floating gel which floated unattached to the tCFM.

There were five steps in the set up of the experiment:-

- cell preparation and counting
- preparation of cell chamber
- bar and A-frame preparation
- gel preparation and setting
- addition of the radioactive proline and gel floatation

All steps were carried out under sterile conditions in a laminar flow hood.

4.2 Cell preparation

Flasks of cells to be tested were washed with PBS and removed from the flask using 10% trypsin solution (as described in **3.1.2**). Cells were counted using a

haemocytometer slide (Improved Neuberge). Four million fibroblasts were used to seed each FPCL. After counting, four million fibroblasts were resuspended in 0.5ml of medium.

4.3 Preparation of cell chamber

A cell chamber was made by casting silicone elastomer (Dow Corning, Milton Keynes, UK) into a polymethylpentene petri dish around a rectangular stainless steel block mould of dimensions 75 x 25 x 10mm. The silicone elastomer was degassed before and after casting for one hour. The petri dish and silicone elastomer were then incubated at 37^0C for 24 hours to harden. After 24 hours the block was removed to leave a rectangular culture well. The well was hydrophobic in nature to inhibit collagen attachment.

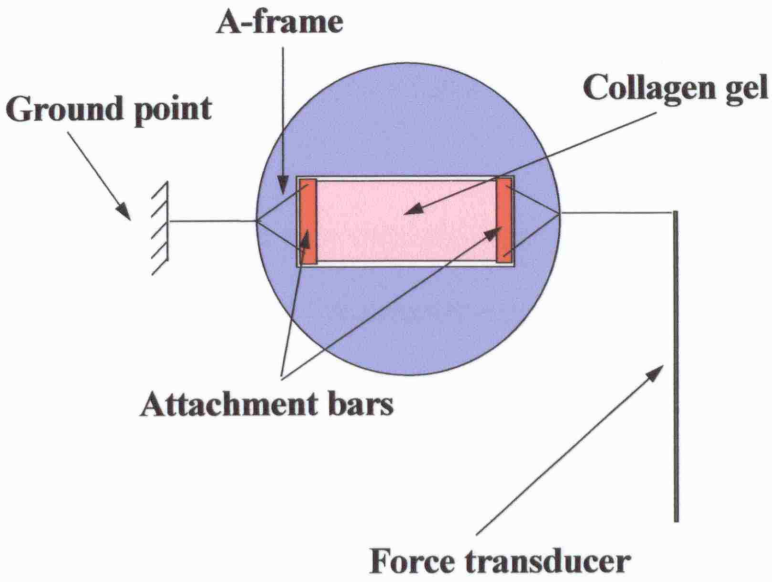
4.4 Bar and A-frame set up

Each end of the gel was to be attached to a bar (see *Figure 2.1*). In each bar an A-frame was inserted. The bars were made of four 25mm lengths of nylon mesh stitched together with clear nylon thread. A-frames were made from 0.375mm stainless steel suture wire (28SWG) (Downs Surgical Supplies, London UK). The A-frames were pushed into the bars, and then placed in the channel in the silica mould awaiting addition of the collagen gel solution.

Immediately prior to addition of the collagen-cell suspension, the bars were coated with a cell free solution of collagen. The bar coating solution comprised 2ml Type I rat tail collagen in acetic acid and 0.25ml 10x DMEM. The resulting solution was neutralised in dropwise fashion with 5M and then1M NaOH (as described in **4.5** below). The bar coating solution filled the spaces within the bars with cell free collagen solution. Therefore when collagen suspension (containing

cells) was added, a minimal number of cells would enter into the bars, maximising and attempting to keep constant the number present in the collagen gel. The final dimensions of the collagen gel were 65 x 25 x5mm.

Figure 2.1. *High aspect ratio culture well. In this format the aspect ratio is 3:1.*



4.5 Gel preparation and setting

The gel was prepared by mixing 4ml of Type I rat tail collagen in acetic acid with 0.5ml 10x DMEM in a Universal container. This solution had an orange colour due to indicator in the 10x DMEM. It was necessary to neutralise the solution in order for the gel to set (pH dependent polymerisation of collagen). Neutralisation was achieved by dropwise addition of 5M NaOH until tinges of pink were seen in the solution. At that point, 1M NaOH was added in dropwise fashion until the solution became completely pink.

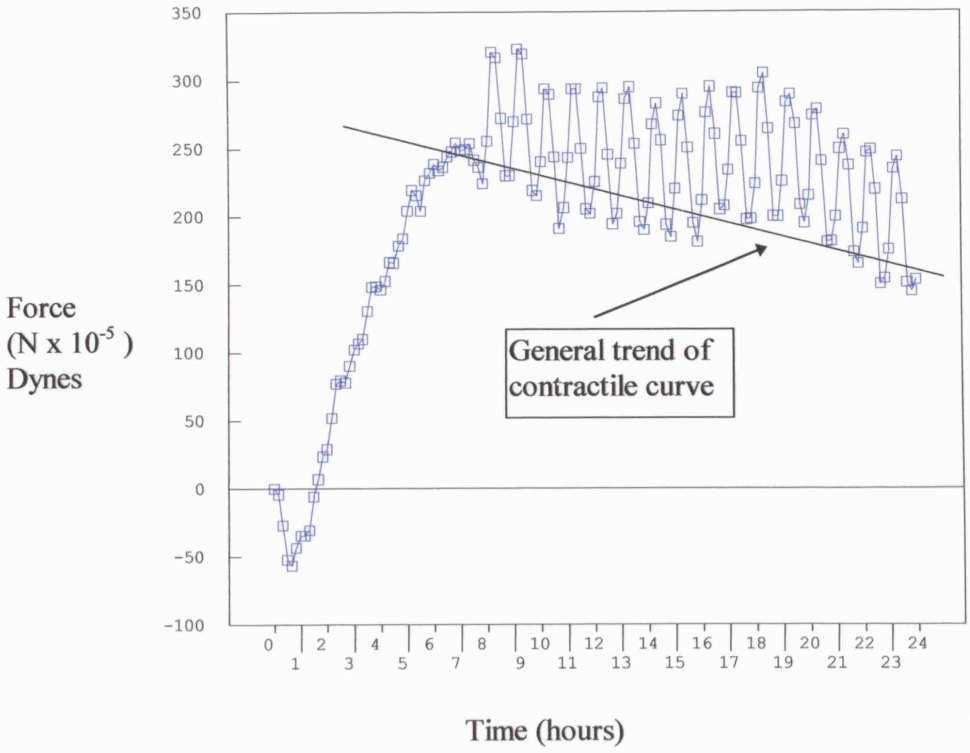
The neutralised collagen solution was added to the Universal containing four million fibroblasts in 0.5ml of medium (section **4.2**) to give a solution with a final collagen concentration of 1 mg/ml. This cell suspension was gently taken up and down once in a 10ml pipette to mix the cells, and was gently poured into the silicone mould and a lid was added. The petri dish was placed in a humidified 5% CO_2 incubator at 37^0C for 15 minutes to allow the gel to set.

After 15 minutes incubation, the petri dish was removed from the incubator and returned to the laminar flow hood. A solution containing 25ml DMEM, 25μCi ^{14}C proline (Amersham, UK), 1.25mg β aminopropronitrile (Sigma) (to give a final concentration of 50 μg/ml), and 1.25mg L-ascorbic acid (Sigma) (to give a final concentration of 50 μg/ml), was gently flooded over the gel surface. The gel was gently teased from the floor of the petri dish with a pin so that it floated to the surface attached to the bars and A-frames.

4.6 tCFM set up

The petri dish was transferred to a 37^0C incubator gassed with 5% CO_2 housing the tCFM. The A-frames were attached to the fixed point and force transducer. The loading pattern was programmed and the experiment was run for 24 hours. The loading pattern was the same in all experiments. No load was applied for the first eight hours, to allow cell attachment and cell process elongation (Eastwood et al 1996). Thereafter an hourly cyclical overload at 120 dynes above the endogenously generated force at eight hours was applied for the remaining 16 hours (*Figure 2.2*).

Figure 2.2 *Cyclical overloading regime used in each loading experiment*



4.7 Free floating FPCL

The free floating gel was made following exactly the same methodology as the tCFM gel. After the medium, radiolabelled proline, β aminoproprionitrile and ascorbate had been flooded over the set gel, the attachment bars were cut off the gel. The gel therefore simply floated freely on the medium. The petri dish was placed in the same incubator as the tCFM so that both the free floating and mechanically loaded FPCLs were exposed to the same conditions simultaneously.

4.8 End of experiment

All experiments lasted for 24 hours. After this time, the gel was lifted into a Universal container. The media was removed with a pipette and placed into a separate Universal. Both Universals were immediately frozen at -20 ^{O}C.

5 Analysis of gels and media

5.1 Introduction

Gels and media were subjected to analysis to quantitate synthesis of collagen and non-collagenous protein. The media alone were also subjected to ELISA assays for collagenase and tissue inhibitor of metalloproteinase (TIMP-1). All analysis was performed at 4^{0}C unless stated.

5.2 Initial treatment of media and gels

Samples of both media and gels from the experiments were defrosted and kept at 4^{0}C.

Gel samples were immediately subjected to an overnight pepsin digestion by adding an equal volume of 2 mg/ml pepsin (Sigma) in 0.5M acetic acid to the sample. These samples were left overnight at 4^{0}C. Media samples were not subjected to pepsin digestion.

The purpose of digestion with pepsin was to degrade all non collagenous protein. Pepsin does not degrade the triple helical structure of collagen.

5.3 Addition of protease inhibitor solution

A solution of protease inhibitors was made up to inhibit enzymes which would break down molecules under investigation. The solution contained 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc SC, Boehringer Mannheim), 2 mM benzamidine (Sigma), 2.5 mM N-ethyl maleimide (Sigma), 2.5 mM EDTA (Sigma) in water to give a resulting solution of 20 times the required concentration. These inhibitors block the action of serine proteases (4-(2-aminoethyl)-benzenesulfonyl fluoride and benzamidine), cysteine proteases (N-ethyl maleimide), and matrix metalloproteinases (EDTA). 0.2 ml protease inhibitor solution was added to the gel samples (sample volume 4ml) which had been digested by pepsin solution the previous night. 1.2 ml protease inhibitor solution was added to the media samples (sample volume 24ml).

5.3 Dialysis

Dialysis of the resulting solutions was necessary to remove free, unincorporated ^{14}C-proline, which, if not removed from the samples, would lead to falsely high results. Each sample was tied into 24/32 dialysis tubing with a bijou float at one end. The samples were placed in a 5l flask of distilled water (not exceeding 10 samples per 5l flask) at 4^0C for 48 hours to remove free label and salts. The water was changed six times over 48 hours. The samples were dialysed until no free label remained in the sample, and this was confirmed by checking radioactivity levels of cell free samples containing only free ^{14}C-proline at regular intervals.

5.4 Freeze drying

Freeze drying was used as a technique to concentrate the samples so that they would be easier to manipulate by taking up the resulting solid in small volumes of solvent. Samples were freeze dried in 100ml round bottomed flasks overnight. 2ml 50mM TRIS 1M NaCl was added to the dried residue of the media samples. 2 ml 0.5M acetic acid was added to the dried residue of the gel samples.

Aliquots were taken from the media samples at this stage for collagenase and TIMP-1 ELISA assays.

5.6 Collagen measurement

5.6.1 Bacterial Collagenase assay

200µl of ice cold 10% TCA/0.1% tannic acid was added to 100 µl of sample and left overnight at 4^0C. The next day the sample was centrifuged at 10000rpm for 15 minutes. The supernatant was carefully aspirated from the pellet and duplicate aliquots taken for counting (50µl) to check that no free ^{14}C-proline was present. The pellet was subjected to a bacterial collagenase digestion for 90 minutes on a shaker at 37^0C in 0.2 ml of a solution containing 40 µg/ml bacterial collagenase 1A (Sigma), 0.1M HEPES pH 7.4, 1.25µM N-ethyl maleimide (a cysteine protease inhibitor), and 0.25µM calcium chloride (an essential cofactor for collagenase).

20µl of 2.0mg/ml bovine serum albumin was added to each digest to act as a carrier protein to aid ^{14}C protein recovery during precipitation. The proteins were then precipitated with 400µl ice cold 10% TCA, 0.1% tannic acid and left overnight at 4^0C.

Samples were centrifuged the next day at 10000rpm for 15 minutes. The supernatant was carefully aspirated and duplicate aliquots (100µl) were counted. The supernatant count was taken to represent collagenase soluble counts, and therefore mainly represented newly synthesized collagen. The collagenase insoluble pellet was solubilized in 250µl 0.8M NaOH and duplicate aliquots (100µl) were removed for counting. These counts were taken to represent non collagenous protein.

5.6.2 SDS PAGE and autoradiography

The aim of electrophoresis and autoradiographic analysis of the media samples was to demonstrate that the counts quantitated on the β scintillation counter represented collagen. By running pepsin digested samples and collagen standards on a gel and then performing autoradiography, it was possible to check that radiolabelled proline had being incorporated into collagen α-chains. The method followed was that of Sykes et al (1976).

5.6.2.1 Sample preparation

Duplicate 200µl aliquots of the freeze dried media samples in 50mM TRIS 1MNaCl were taken. Duplicate 100 µl aliquots were also taken for counting. 200µl 0.1mg/ml pepsin in 1M acetic acid was added to half the samples. 200µl 1M acetic acid was added to the other half of the samples. The reason for addition of acetic acid was twofold. Pepsin requires an acidic environment to function, and this is provided by the presence of acetic acid. Secondly, collagen is not normally very soluble in standard buffers, but has greater solubility in acetic acid. The samples were then incubated for six hours at 4^0C and agitated occasionally. After six hours 40µl 1.1mg/ml pepstatin was added to all samples, followed by an a double volume of 0.5M acetic acid 4M NaCl (4.7g NaCl in 20ml 0.5M acetic acid). The samples were left overnight at 4^0C.

The following day samples were centrifuged at 13000 rpm for 15 minutes at 4^0C. Duplicate 100µl aliquots of the supernatant were taken for counting, and the remainder of the supernatant was discarded. 250µl of final sample buffer (see

section **5.6.2.2** for composition) was added to the pellet and agitated. The resulting solution was heated to 100^{O}C for 3 minutes on a dry heating block.

5.6.2.2 Preparation of SDS gel and running buffers

Duplicate gels were prepared for samples, one for staining with Coomassie Brilliant Blue (0.25%) and the other for autoradiography. Gels were prepared using the following:-

Resolving gel (6%) 3.75 ml acrylamide, 6.25 ml 4x lower gel buffer (LGB)(1.5M TRIS and 0.4% sodium dodecyl sulphate-SDS), 15 ml H_2O, 25 µl tetramethylethylenediamine (TEMED), 250 µl ammonium persulphate (APS).

Stacking gel (4.5%) 12.5 ml stacking buffer, 25 µl TEMED, 250 µl ammonium persulphate (APS)

Running buffer (5x)0.125M TRIS-HCl, 0.1M glycine, 0.5% SDS

Stacking buffer 4.5% upper gel buffer (UGB-0.5M TRIS, 0.4% SDS), 4.5% acrylamides

Final sample buffer(5x) 0.625M TRIS-HCl, 5% SDS, 40% w/v glycerol, 0.01% bromophenol blue

The resolving gel solution was poured into a gel cassette of a Bio-Rad Mini-Protean II apparatus, and a thin layer of propan-1-ol was poured over the top of the gel. The gel was left to polymerise (about 10 minutes), and the propan-1-ol was poured off. The stacking gel solution was then poured on top of the polymerised resolving gel and left to polymerise. A Teflon comb was used to define the wells.

5.6.2.3 Loading of sample onto SDS gel

100 µl of sample was loaded in duplicate using a Hamilton syringe. 100µl type I and type III collagen standards were also run on each gel.

5.6.2.4 Gel running and delayed reduction

Gels were run at 200V. As the bromophenol blue tracking dye reached the stacking gel-resolving gel interface, the current was switched off and 10 µl β mercaptoethanol (100 µl β mercaptoethanol, 300 µl glycerol, 600 µl distilled H_2O) was added to each well. The current was restarted after 10 minutes and continued until the tracking dye reached the bottom of the resolving gel. The addition of β mercaptoethanol causes reduction of inter-chain disulphide bonds in type III collagen, and therefore enables it to enter the resolving gel. Inter-chain disulphide bonds are not present in type I collagen.

5.6.2.5 Autoradiography

Gels for autoradiography were fixed overnight in 10% methanol/10% acetic acid, and rinsed in distilled water. After 30 minutes of soaking in Amplify (Amersham), gels were dried on filter paper for 70 minutes at 70^0C in a gel vacuum dryer.

Films (Amersham MP) were preflashed prior to use. Preflashing of the films increased their sensitivity, and also improved the linearity of response, particularly important at extremes of light and dark. Gels were placed on top of the film and securely sealed in a film cassette. The cassettes were stored in a freezer at -70^0C and developed at 120 days.

5.7 ELISA assays for collagenase and TIMP-1

5.7.1 General methods

Both ELISA assays used the underlying principle of a sandwich technique. Nunc immunosorp 96-well plates were used in both ELISA assays. Solutions common to both assays included:-

Phosphate buffered saline	8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g NaN_3
Blocking buffer	10 mg/ml BSA (Sigma) in PBS
Wash buffer	0.1% Tween 20 (a detergent solution of polyoxicethylene (20-sorbitan monolaurate) (BDH) in PBS/Thimerosal
Protein diluent	0.5 mg/ml BSA (Sigma) in wash buffer
Phosphate-citrate buffer	1 capsule (Sigma)/100 ml distilled water

5.7.2 Collagenase ELISA

96 well plates were coated overnight at 4^0C with 100µl/well of 3µg/ml RRU-CL1 in PBS. This was a mouse monoclonal antibody to collagenase. The following day, antibody solution was washed off and plates were blocked for one hour at room temperature with 150µl/well blocking buffer. After blocking, the plates were washed three times with wash buffer.

Samples were vortexed and diluted accordingly in protein diluent and vortexed again. Standard dilutions (0,2,5,10,20,30,40,50 ng/ml collagenase standard in protein diluent-100µl/well) and samples (100µl/well) were applied to the plate which was then covered with film and left overnight at 4^0C.

Samples were removed from the plate and the plate washed three times with wash buffer. 100µl/well of 3µg/ml biotinylated anti-collagenase (a polyclonal rabbit antibody) was applied to each well and incubated at room temperature for two hours.

The biotinylated anti-collagenase was discarded and the plate washed three times with wash buffer. 100µl/well of streptavidin-HRP (horse radish peroxidase) (3µl streptavidin-HRP in 15ml protein diluent) was applied to the plate and incubated for 30 minutes at room temperature.

Plates were washed three times in wash buffer and 100µl/well of OPD solution (one OPD tablet (Sigma) in 12 ml phosphate-citrate buffer) was added. Plates were incubated at room temperature on the benchtop and regularly observed to check the colour change.

When the standards and samples were estimated to have sufficient colour, the reaction was stopped by adding 50 µl/well 2M H_2SO_4. Plates were read on a Multiscan at 492 nm, using appropriate calibration formats.

5.7.3 TIMP-1 ELISA

96 well plates were coated overnight at 4^0C with 100µl/well of 4µg/ml RRU T5 in PBS. This antibody was a monoclonal anti-TIMP antibody. The following day, antibody solution was washed off and the plates were blocked for one hour

at room temperature with 150µl/well blocking buffer. After blocking, the plates were washed three times with wash buffer.

Samples were vortexed and diluted accordingly in protein diluent and vortexed again. Standard dilutions (0,2,5,10,20,30,40,50 ng/ml TIMP standard in protein diluent-100µl/well) and samples (100µl/well) were applied to the plate which was then covered with film and left overnight at 4^0C.

Samples were removed from the plate and the plate washed three times with wash buffer. 100µl/well of 2µg/ml biotinylated G10C6 (a monoclonal antibody to TIMP) was applied to each well and incubated at room temperature for two hours.

The biotinylated G10C6 was discarded and the plate washed three times with wash buffer. 100µl/well of streptavidin-HRP (15µl streptavidin-HRP in 15ml protein diluent) was applied to the plate and incubated for 30 minutes at room temperature

Plates were washed three times in wash buffer and 100µl/well of OPD solution (one OPD tablet (Sigma) in 12 ml phosphate-citrate buffer) was added. Plates were incubated at room temperature on the benchtop and regularly observed to check the colour change.

When the standards and samples were estimated to have sufficient colour, the reaction was stopped by adding 50 µl/well 2M H_2SO_4. Plates were read on a Multiscan at 492 nm, using appropriate calibration formats.

RESULTS

4.1 Introduction

Dupuytren's Disease causes the palmar fascia to thicken, a finding which is clinically consistent when examining a patient with Dupuytren's Disease. The thickened fascia is also under tension, and gradually contracts the fingers into the palm as the disease progresses. The diseased tissue has been extensively studied in terms of extracellular matrix components, and contains an excess of collagen. The main collagens identified are Type I and Type III.

The reason for excess collagen deposition is unknown. The fibroblast may be genetically different, and produce excess collagen autonomously, but the disease occurs late in life, not affecting the palm and digits until the fifth or sixth decade. The trigger for this change is unknown. Alternatively, the excess collagen deposition may result from a disordered wound healing response following a fracture of one or more microfibrils of palmar fascia, which is arranged in an intricate three dimensional structure. The development of the thickened palmar fascia may therefore be analogous to that of a hypertrophic scar (although the fascia is situated beneath the skin, rather than within it).

The tension in the diseased tissue may originate from the fibroblast, or the excess collagen which has been deposited. The diseased fascia then acts like a scar contracture, gradually drawing the digit into the palm over time. Therefore, the question arises whether excess collagen synthesis is an inherent property of the Dupuytren fibroblast whatever the tensional environment, or if excess collagen

synthesis is a pathological fibroblast response to tension generated in the Dupuytren's tissue. The latter theory may result in a positive feedback loop, where increased tension causes further collagen synthesis, which leads the fibroblast to contract the extracellular matrix further.

The aim of this thesis was to examine the relationship between tension, by application of mechanical loads, and protein synthesis, particularly collagen, in fibroblasts derived from normal palmar fascia and fascia affected with Dupuytren's Disease. The reason for quantitating the protein, that is collagen, rather than genes coding for collagen, is that collagen undergoes extensive post translational modification, and an increase in mRNA coding for Type I collagen may not actually result in an increase in Type I collagen itself.

Although this is an *in vitro* study, the conditions were designed to mimic the *in vivo* environment as closely as possible. The responses of fibroblasts to the surrounding matrix on which they were attached could, therefore, be assumed to be similar to their responses *in vivo*.

Conditions of fibroblast culture for both control and Dupuytren fibroblasts were identical. The passage number at which the fibroblasts were harvested was kept as low as possible in an attempt to maintain their cellular phenotype as close as possible to that in the surgically resected tissue.

In a further attempt to mimic *in vivo* conditions, fibroblasts were seeded in a collagen gel (or fibroblast populated collagen lattice-FPCL), rather than

monolayer culture. The FPCL closely resembles the extracellular matrix, permitting fibroblasts to form attachments on fibres of collagen in a three dimensional manner. Mechanical loads applied to the FPCL were therefore transmitted to fibroblasts through the collagen fibres, and the response to these mechanical cues could be quantitated. The experimental conditions attempt to mimic the environment of the palmar fascia as closely as possible, so that the fibroblasts experience mechanical loads *transmitted through* the collagen fibres as in the extracellular matrix. This is in contrast to applying loads directly onto the fibroblasts, for example, in a monolayer culture.

The application of mechanical loads was administered using the tensioning Culture Force Monitor, which applies loads to the FPCL through a computer controlled micromotor in a reproducible, cyclical manner. Analysis of the resulting FPCL, and the media in which the FPCL floated, was performed at Addenbrooke's Hospital in the Rheumatology Research Unit using techniques based on a bacterial collagenase assay to quantitate ^{14}C-proline incorporation into collagen and non-collagenous protein. ELISA was used to quantitate collagenase and TIMP-1 in the media samples.

4.2.1 Method development

A significant problem in the development of the collagen assay was separation of a relatively very small amount of newly synthesised collagen which was labelled with ^{14}C, from a vast excess of unincorporated free ^{14}C. Some ^{14}C would also have been incorporated into proteins other than collagen over the course of the experiment, but these were not specifically studied in this thesis. Previous studies on collagen metabolism in the literature (Murrell et al 1989, Melling et al 2000) have been based on the assumption that incorporation of radiolabelled proline (using either ^{14}C-proline or ^{3}H-proline as the label) provides a valid measure of collagen synthesized.

4.2.2 Dialysis

It was decided to remove the free, unincorporated ^{14}C-proline by employing a dialysis technique. Each sample was therefore tied into 24/32 dialysis tubing with a bijou float at one end. The samples were placed in a 5l flask of distilled water (not exceeding 10 samples per 5l flask) at $4^{0}C$ for 48 hours to remove free label and salts. The water was changed six times over 48 hours. This final protocol was arrived at by running several trial samples, and measuring the amount of label remaining at intervals over a 48 hour period.

4.2.3 Confirmation of collagen synthesis

It was essential to confirm that detectable levels of collagen were being synthesized within the 24 hours of the experiment. It was also necessary to verify that free ^{14}C-proline had been incorporated into collagen. Electrophoresis gels were therefore prepared and run, as described in the materials and methods, to

demonstrate that free ^{14}C-proline added to the medium at the beginning of the experiment had been incorporated into collagen synthesised by both control and Dupuytren fibroblasts. Type I and Type III collagen standards were run alongside the samples on each electrophoresis gel so that it was possible to make some qualitative assessment of the collagen type synthesized by performing delayed reduction of the gel samples.

4.2.4 Background radioactivity levels

Cell free samples were run to assess background radioactivity. If background levels of radioactivity were significant, then falsely high results would be obtained. If the background levels were very low or zero, then no signal would be obtained on the gel and it would not show any black line. Gel 6 shows no such signal. It also confirmed that the dialysis had been effective in removing free unincorporated ^{14}C-proline.

4.3 Sample nomenclature

The first three letters are either FAS, indicating fibroblasts derived from normal palmar fascia (controls), or DUP, indicating fibroblasts derived from Dupuytren's tissue. The first three letters are then followed by two further letters specific to the specimen eg. FASVG or DUPIC. Some samples derived from Dupuytren's tissue may also have a suffix C or N (eg. DUPICC) indicating that the tissue was derived from cord or nodule tissue from within the Dupuytren's tissue sample.

The following page shows an example of an autoradiograph run to confirm collagen synthesis.

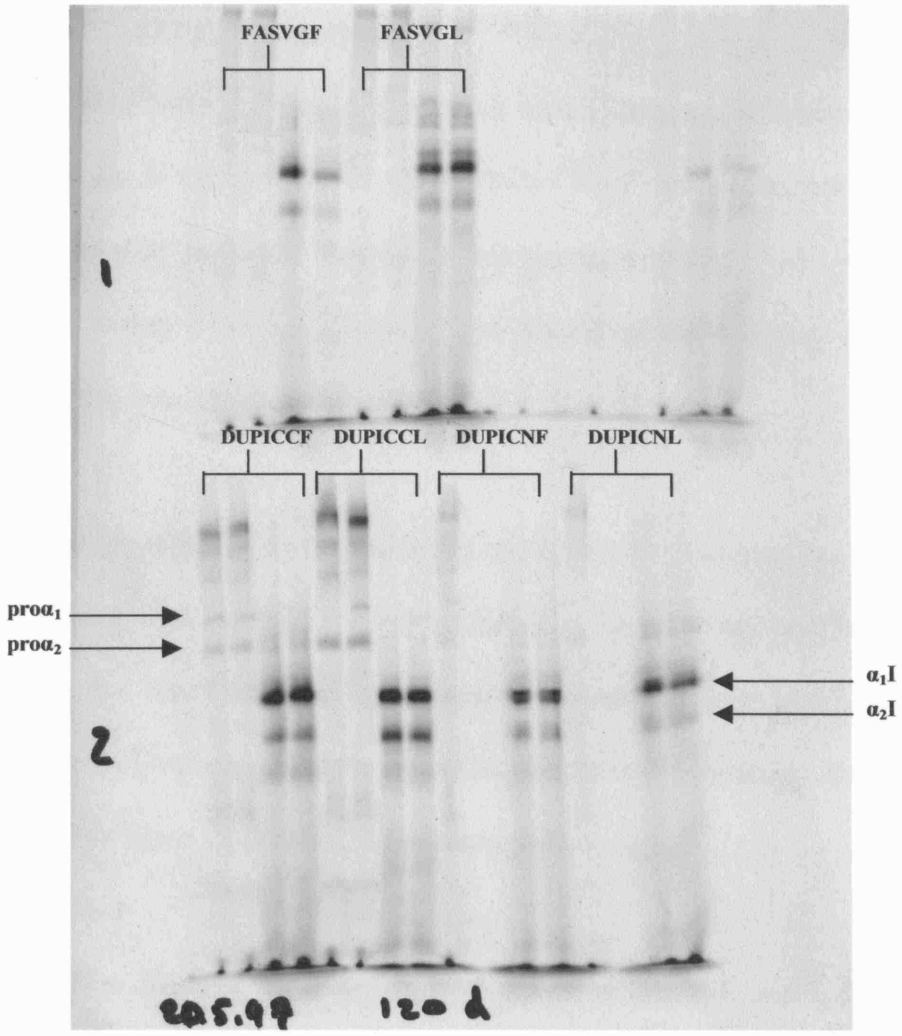


Figure 4.1 Autoradiographs of control and Dupuytren samples. The samples are run in duplicate. Samples are labelled with sample name, and last letter denotes free floating gel (F) or loaded gel (L). Lanes 1,2,5,6,9,10,13,14-no pepsin. Lanes 3,4,7,8,11,12,15,16-pepsin digest.

Figure 4.1 shows two autoradiographs which demonstrate important findings. The second, lower gel has developed better than the first, upper gel. Samples were loaded in duplicate, lanes1 and 2 in acetic acid only, 3 and 4 in acetic acid and 0.1 mg/ml pepsin solution.(section 5.6.2.1 in the materials and methods). The pattern was repeated along the gel.

It is clear, particularly in the lower gel in ***Figure 4.1***, that samples which had not been treated with pepsin did not show bands at the α_1I or α_2I positions. It may be the case that there are large aggregates of insoluble protein which are unable to enter the gel, or the collagen is in the pro form (seen above the α_1I and α_2I positions in lanes 1,2,5 and 6 in the lower gel).

The pepsin digested samples showed bands at the α_1I and α_2I positions as expected from the standards. The α_2 band is, as expected, approximately half as dark as the α_1 band, clearly seen in lanes 7 and 8 in gel 1 (FASVGL) and in gel 2 in lanes 3 and 4, 7 and 8, 11 and 12, 13 and 14. (In gel 1, half the volume of sample was loaded into lane 3 compared to lane 4 due to sample shortage). Type 1 collagen was therefore present in all samples on gels 1 and 2.

The importance of these results is that it can be demonstrated that fibroblasts are synthesising collagen within the relatively short timeframe of the experiment (24 hours). This is extremely important because the assumption of the method is that ^{14}C-proline is incorporated into newly synthesised collagen. These autoradiographs demonstrate that collagen is being synthesised. The

autoradiographs were run to confirm this finding, and were not intended to quantify collagen synthesis in any way.

FASVG when loaded shows extra bands in the region where Type III collagen may be expected. These bands are not present in other samples in gel 1 or 2, and only present in two other samples (DUPJCN and DUPPWN). These bands may be fragments of Type III collagen, or may be peptide fragments of larger proteins such as fibronectin. Type V collagen is known to appear just above the α_1I position, but no Type V standards were run in these experiments.

Cell free control samples were run on a separate gel, both pepsin and non-pepsin digests, and showed no signal of any kind. This demonstrates that background levels of radioactivity are not recordable, and therefore not contributing to sample counts. It may be assumed, therefore, that the counts recorded in the results have all been incorporated into either collagenous or non-collagenous proteins.

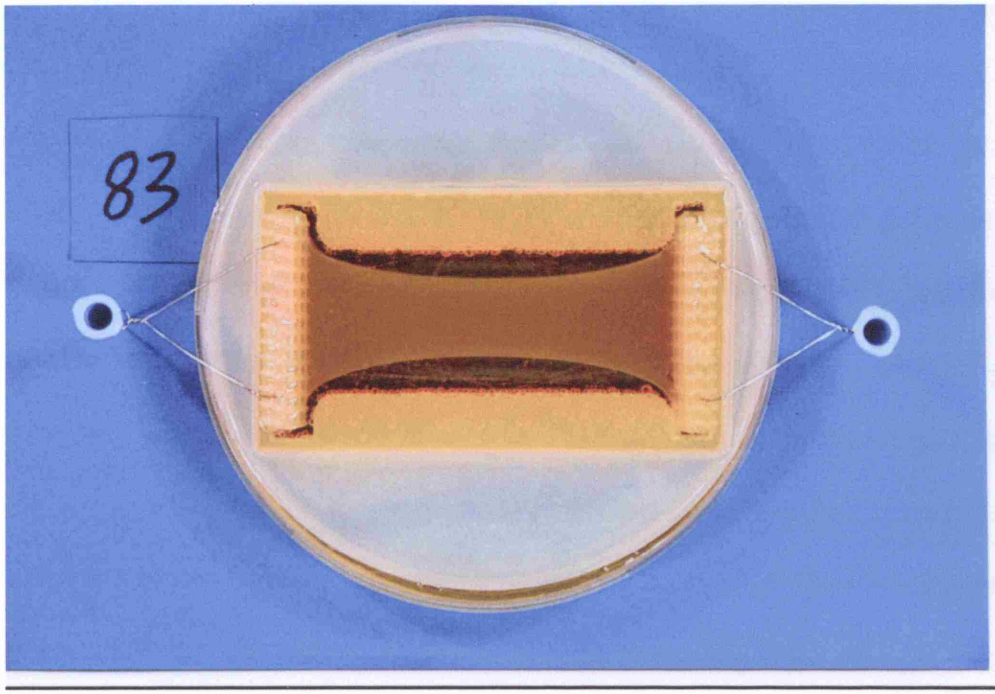
4.4 Loaded and Free Floating Gels at the end of 24 hour experiment

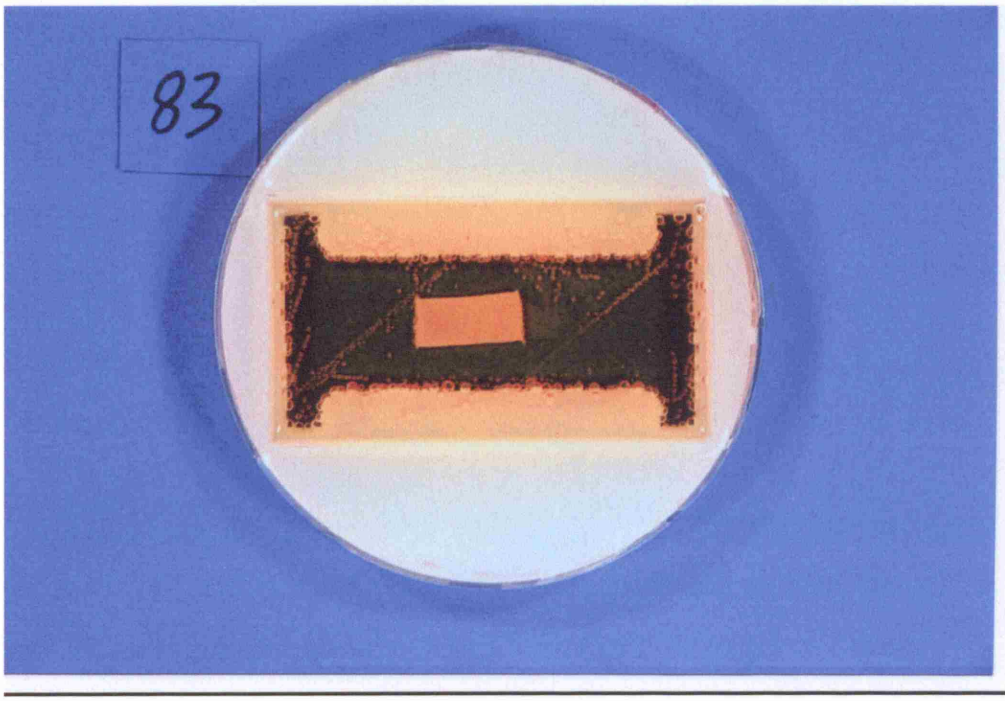
Figures 4.4a and 4.4b show the appearances of a mechanically loaded gel and a free floating gel at the end of a 24 hour experiment.
The mechanically loaded gel has retained its length due to firm attachment to the bars floating in medium at either end of the petri dish. The lateral edges of the gel which were straight and parallel at the beginning of the experiment have become concave over the 24 hour period.

The free floating gel has undergone a striking reduction in length and breadth during the course of the 24 hour experiment.

The reason for the change in shape of the collagen gel over the course of the experiment is the result of the fibroblast contraction on the collagen fibrils. The fibroblasts attach themselves to the collagen fibrils which comprise the gel, and then apply contractile forces. From an initially completely rectangular shape, the loaded gel undergoes a change, causing the longer sides of the rectangle to become concave. The unloaded gel retains the same rectangular shape after 24 hours, but has dramatically decreased in size.

Figure 4.4 The top figure shows a mechanically loaded gel at the end of a 24 hour experiment. The gel is connected to bars at either end, and A-frames can be seen inserted into the bars. The gel, bars and A-frames are all floating in media. The lower figure shows a free floating gel at the end of the experiment.





The mechanism for contraction of the collagen gel by fibroblasts has been extensively studied. It is thought that the fibroblasts exert tension on the collagen fibrils, until they reach a steady state at which point the actin cytoskeleton of the fibroblast, and the forces transmitted through the collagen fibril itself, are at equilibrium. This may be the reason that fibroblasts in an unloaded gel contract the gel to a small size. The concept of 'tensegrity' (Ingber 1994) has been developed by Brown et al (1998) to one of 'tensional homeostasis'. This theory suggests that fibroblasts actively change their surrounding environment until they achieve a steady state in terms of tension perceived by the cell through the extracellular matrix, and that exerted on the matrix by the cell. This is a dynamic situation, and the cell rapidly responds to significant tensional changes in the extracellular matrix.

4.5.1 Effect of loading on total collagen synthesis

The major aim of this thesis was to examine the relationship between mechanical loading and collagen synthesis in fibroblasts derived from Dupuytren's Disease, and compare them to normal palmar fascia fibroblasts. Significant measures have been taken to mimic the *in vivo* cellular environment. The results already presented confirm that collagen is being synthesised by fibroblasts of both control and Dupuytren cell lines within the 24 hour duration of the experiment, and that excess radiolabel is being successfully removed prior to the quantitation of the collagen. The results have also shown that radiolabel has been incorporated into proteins other than collagen, but it is not the aim of this work to identify these individual proteins.

These results are original because collagen production by fibroblasts from Dupuytren's Disease has only been studied in monolayer culture (Murrell 1991), without application of mechanical load. Murrell's results show Dupuytren's fibroblasts produce less Type I collagen than control tissue.

Total collagen synthesis by control and Dupuytren fibroblasts in free floating and mechanically loaded gel experiments has been calculated by summing results in counts per minute (cpm) of 100 µl aliquots of supernatant following bacterial collagenase digestion of gel and media samples. Results for gel samples alone and media samples alone are discussed in sections 4.5.2 and 4.5.3 respectively. *Figure 4.5* shows the effect of loading on total collagen synthesis in control and Dupuytren samples. Individual changes for each sample are shown in *Figure 4.5a*, and results of a Mann-Whitney U test in *Figure 4.5b*.

Two control samples show small changes on loading. FASCW shows a small increase in total collagen synthesis and FASRM shows a slight decrease. Technical problems in processing have necessitated omission of results for FASVG (unloaded) and FASPM (loaded).

Nine of 14 Dupuytren samples show an increase in collagen synthesis on loading, and five show a decrease.

Figure 4.5b is derived from *Figure 4.5a* and shows total collagen synthesis in control and Dupuytren samples. Loading itself does not have a statistically significant effect on total collagen synthesis in either control or Dupuytren samples when calculated using the Mann-Whitney U test.

Figure 4.5a Total collagen synthesis in gel and media by palmar fascia and Dupuytren's fibroblasts over 24 hours as measured in counts per minute in 100 µl aliquots of supernatant following bacterial collagenase digestion(white bars-unloaded samples, black bars-loaded samples)

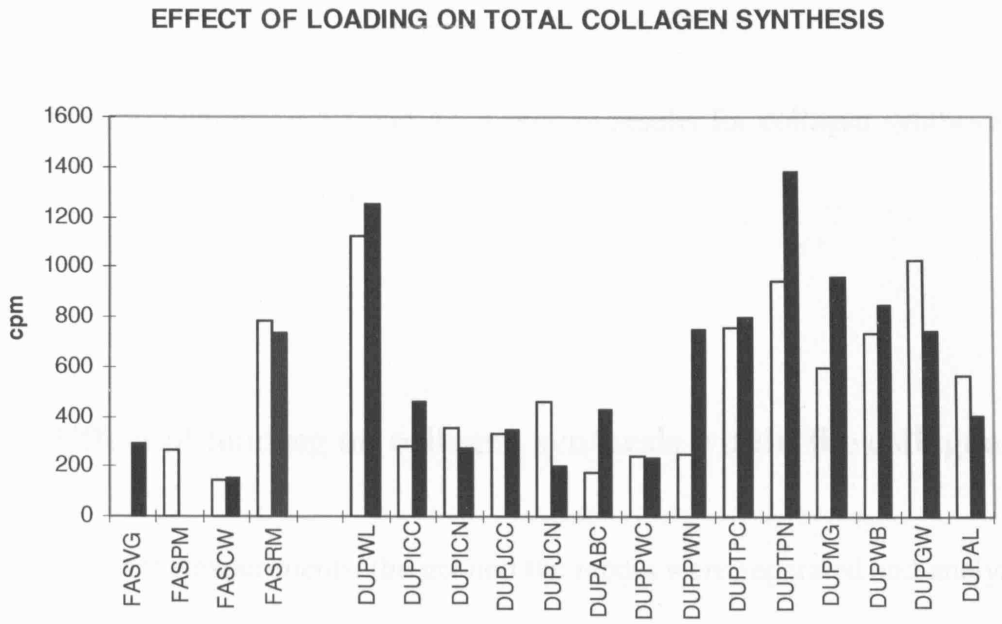
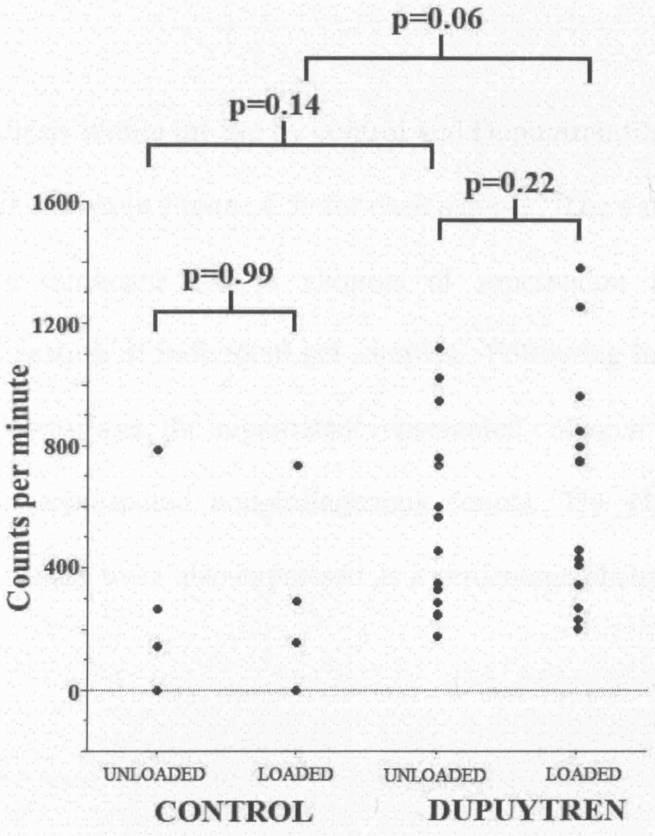


Figure 4.5b Plot of total collagen synthesis. (Mann-Whitney U test for p values)



In summary, there is a significant difference in total collagen synthesis when comparing loaded control samples with loaded Dupuytren samples.

The following sections, 4.5.2 and 4.5.3 present results for collagen synthesis in the gel and media individually.

4.5.2 Effect of loading on collagen synthesis within the collagen gel

At the end of the experiments, the gel and the media were separated and analyzed to measure collagen synthesis during the 24 hour experimental period. The results for both gel and media were added together to give *Figures 4.5a and b*. In this section, only the results for collagen synthesis within the gel are discussed.

Collagen synthesis within the gel by control and Dupuytren fibroblasts before and after loading is shown in *Figure 4.5c* for each sample. The values are cpm values obtained from duplicate 100 µl aliquots of supernatant following bacterial collagenase digestion of individual gel samples. Following bacterial collagenase digestion and centrifuge, the supernatant represented collagen soluble counts, and the precipitate represented non-collagenous counts. The changes in collagen synthesis on loading were also expressed as a percentage change in *Figure 4.5d.*

Three of the control samples showed an increase in collagen synthesis on loading, although the greatest percentage increase is 30% in FASVG. One control sample shows a slight decrease in collagen synthesis on loading (FASRM).

In contrast, 11 of 14 Dupuytren samples showed an increase in collagen synthesis on loading, one showed no change, and two showed a decrease. The percentage increase in collagen synthesis is substantial, and greater than 50% in seven samples.

Figure 4.5e shows values for collagen synthesis derived from from *Figure 4.5c.* There was a statistically significant increase in collagen synthesis in the gel by Dupuytren fibroblasts when loaded ($p=0.02$).

The results show that normal palmar fibroblasts do not respond to loading by increasing collagen synthesis, but Dupuytren fibroblasts do appear to increase collagen synthesis on loading. This is an original observation, and suggests that the fibroblasts derived from Dupuytren's tissue exhibit an abnormal response to mechanical loading. It is known that palmar fascia affected with Dupuytren's Disease contains excess Type I and Type III collagen, and is also under tension. The results of this series of experiments show that the increase in collagen synthesis may be a response to the tension within the tissue.

Figure 4.5c Collagen synthesis in gel by palmar fascia and Dupuytren fibroblasts over 24 hours as measured in counts per minute in duplicate 100 µl aliquots of supernatant following bacterial collagenase digestion (white bars-unloaded samples, black bars-loaded samples)

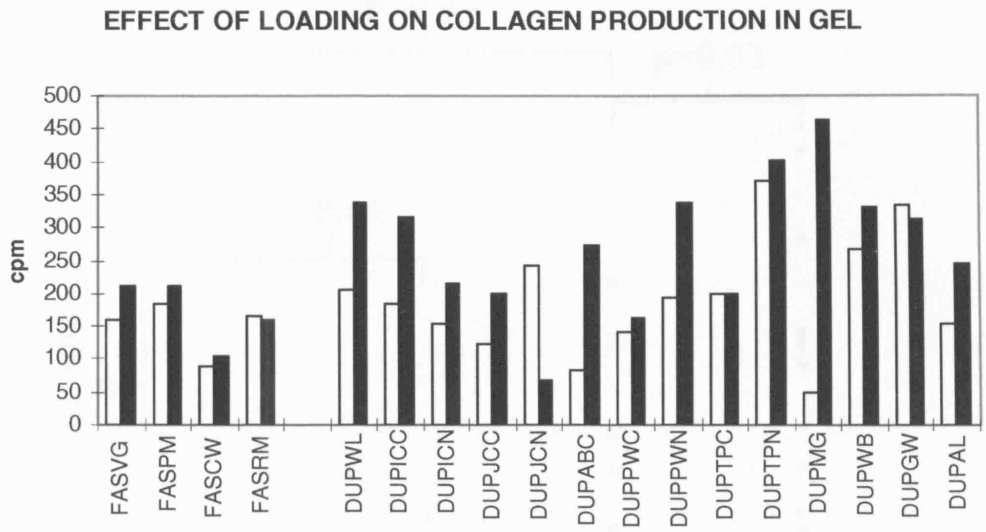


Figure 4.5d Collagen synthesis in gel expressed as percentage change from Figure 4.5c-samples displayed in same order as Figure 4.5c

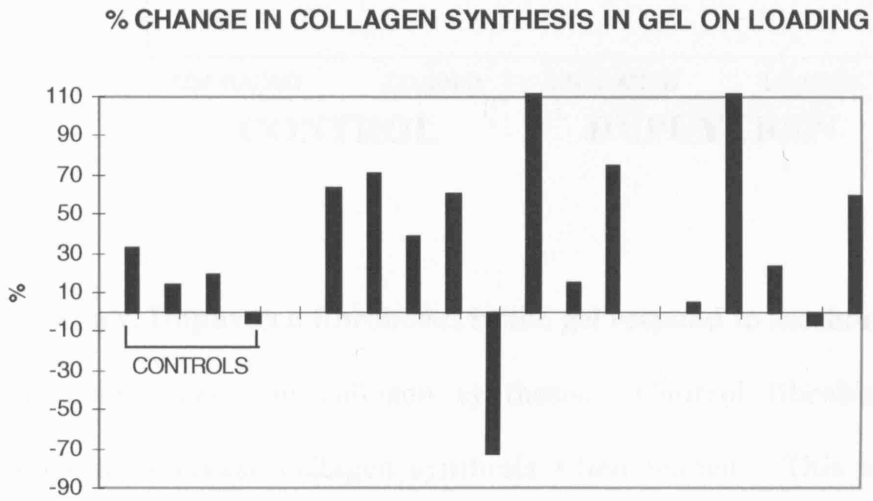
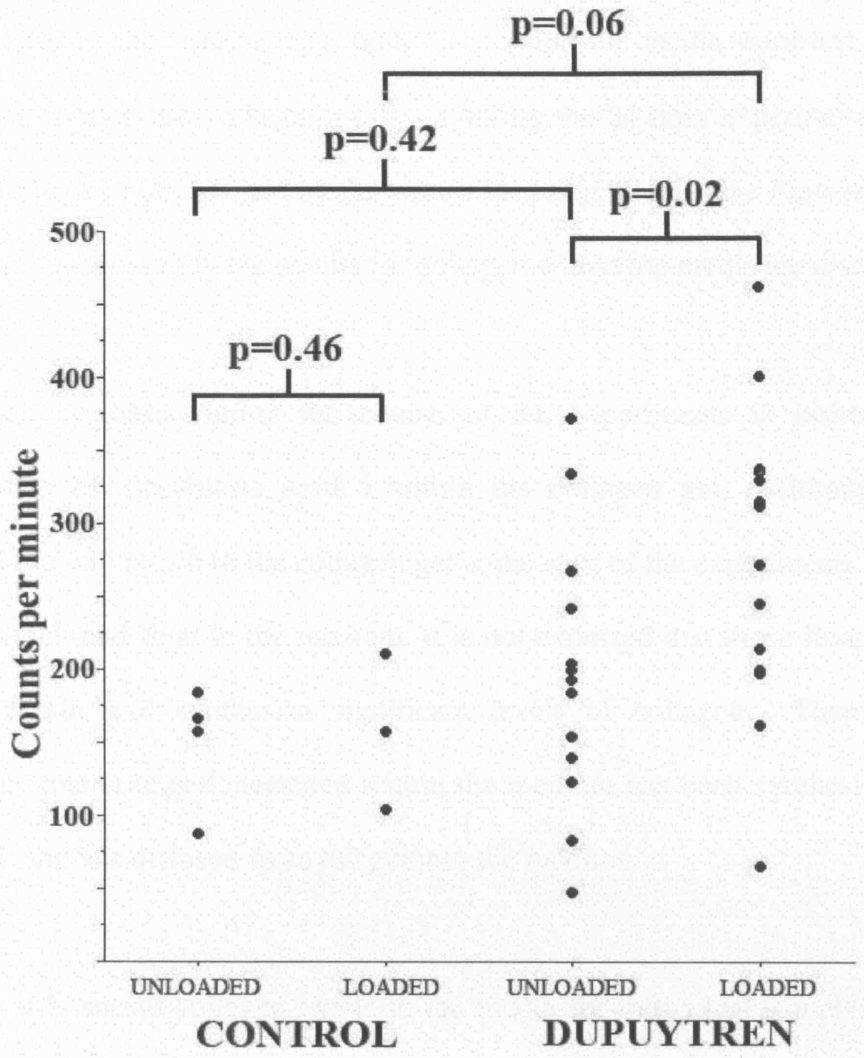


Figure 4.5e Plot of collagen synthesis in gel (derived from Figure 4.5c-p values from Mann-Whitney U test)



In summary, Dupuytren fibroblasts in the gel respond to mechanical load by significantly increasing collagen synthesis. Control fibroblasts do not significantly increase collagen synthesis when loaded. This may help to explain the underlying reasons for deposition of excess collagen in fascia affected with Dupuytren's Disease.

4.5.3 Effect of loading on collagen within media

At the end of the experiments, both the gel and the media were separated and analyzed to measure collagen synthesis during the 24 hour experimental period. The results for both gel and media were added together to give *Figures 4.5a and b*. In this section, only the results for collagen within the media are discussed.

Collagen synthesis during the course of the experiments is assumed to be performed by fibroblasts seeded within the collagen gel. Although not all fibroblasts will attach to the collagen gel at the start of the experiments, and a few may detach and float in the medium, it is not expected that those floating within the medium will synthesize significant levels of collagen. Therefore it is assumed that collagen measured within the medium has been synthesised within the gel, and has diffused from the gel into the medium.

Figure 4.5f shows collagen levels in the media for individual samples. Two of the controls show a decrease in collagen levels on loading. Seven Dupuytren samples showed an increase, and seven showed a decrease in collagen levels on loading. There was no significant difference between control and Dupuytren samples when unloaded or loaded.

Figure 4.5f Collagen levels in media (white bars-unloaded samples, black bars-loaded samples)

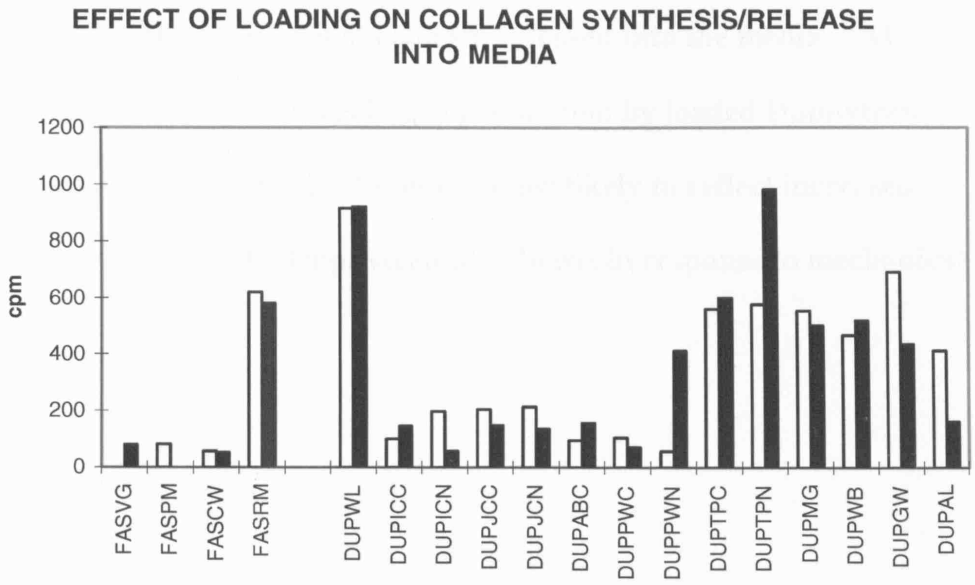
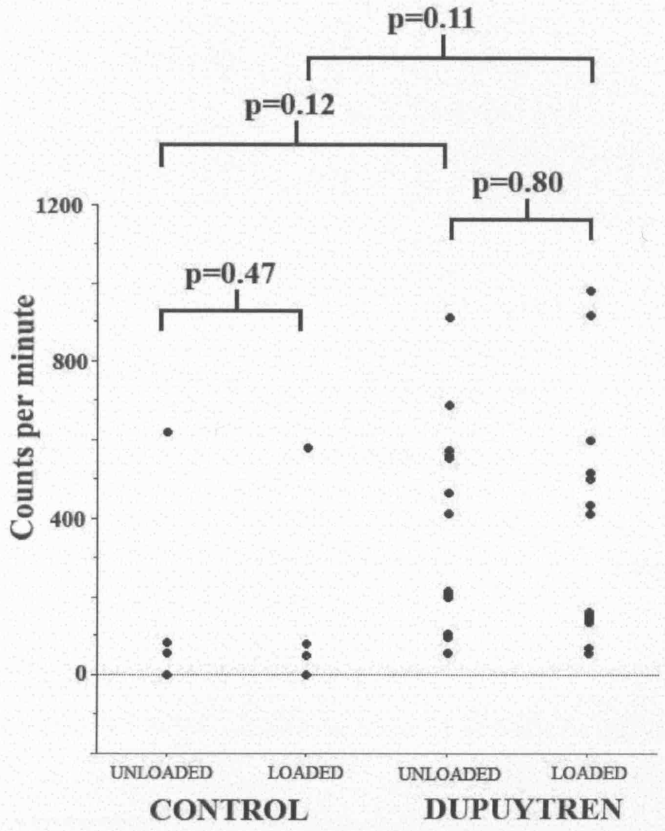


Figure 4.5g Plot of collagen levels in media. (Mann-Whitney U test for p values)



In summary, there was no significant difference in collagen released into the media by control or Dupuytren fibroblasts. Neither does loading alone significantly affect the level of collagen released into the media. The significant increase in total collagen production by loaded Dupuytren fibroblasts (*Figure 4.5b)* is, therefore, most likely to reflect increased synthesis of collagen by Dupuytren fibroblasts in response to mechanical load.

4.5.4 Relative collagen levels in gel and media

The distribution of newly synthesized collagen in the gel and media was examined by comparing mean values from *Figure 4.5e* and *Figure 4.5g*. The results are summarised in *Figure 4.5h*.

Figure 4.5h Mean values (cpm) of collagen in gel and media

	GEL	*MEDIA*	% IN GEL
FASCIA			
Unloaded	149	253	37
Loaded	171	237	42
DUPUYTREN			
Unloaded	192	367	34
Loaded	275	374	42

Table 4.5h shows the distribution of newly synthesized collagen between the gel and the media after 24 hours. Both fascia and Dupuytren samples show a similar pattern of percentage of collagen within the gel. Collagen levels in the media samples are very similar before and after loading in fascia and Dupuytren samples. Total collagen levels are higher in Dupuytren samples.

In summary, as shown in Figures 4.5c-g, there is greater total collagen synthesis in the gel by Dupuytren fibroblasts on loading, and so the total collagen levels increase, but the collagen levels in the media are the same.

4.6.1 Effect of loading on non-collagen protein synthesis

The results discussed in section **4.5** relate to collagen synthesized during the experiments by normal palmar fascia and Dupuytren fibroblasts under unloaded and loaded conditions. Collagen synthesis was assessed by measuring radioactive counts per minute in supernatant following bacterial collagenase digestion and centrifuge of gel and media samples. This section presents results obtained in counts per minute from precipitate following bacterial collagenase digestion and centrifuge, which are considered to represent non-collagen protein synthesised during the experiments. The identification of these proteins was without the scope of this thesis. The results in *Figures 4.6a and b* are total results of both gel and media combined. The results for gel and media are considered separately in **4.6.2** and **4.6.3.**

Figure 4.6a shows individual changes in non-collagen protein (NCP) synthesis for normal palmar fascia and Dupuytren samples before and after loading. Technical problems in processing have necessitated omission of results for FASVG (unloaded) and FASPM (loaded). *Figure 4.6b* is derived from *Figure 4.6a.*

The normal palmar fascia results are within a close range of each other (as seen with total collagen synthesis in *Figures 4.5a and b*). 10 Dupuytren samples showed an increase in NCP synthesis when loaded, and four showed a decrease,

although *Figure 4.5b* shows this increase in NCP synthesis by Dupuytren fibroblasts was not statistically significant.

When unloaded fascia samples are compared to unloaded Dupuytren samples, no significant difference in NCP synthesis was found, whereas loaded fascia and loaded Dupuytren samples did differ significantly in NCP synthesis ($P<0.01$). This is of note and bears comparison to *Figure 4.5b* which showed that Dupuytren fibroblasts synthesize significantly more collagen than control fascia when a mechanical load is applied.

Figure 4.6a Total non-collagen protein synthesis in gel and media by normal palmar fascia and Dupuytren fibroblasts over 24 hours as measured in counts per minute in duplicate aliquots of precipitate solubilised in 0.8M NaOH following bacterial collagenase digestion (white bars-unloaded samples, black bars-loaded samples)

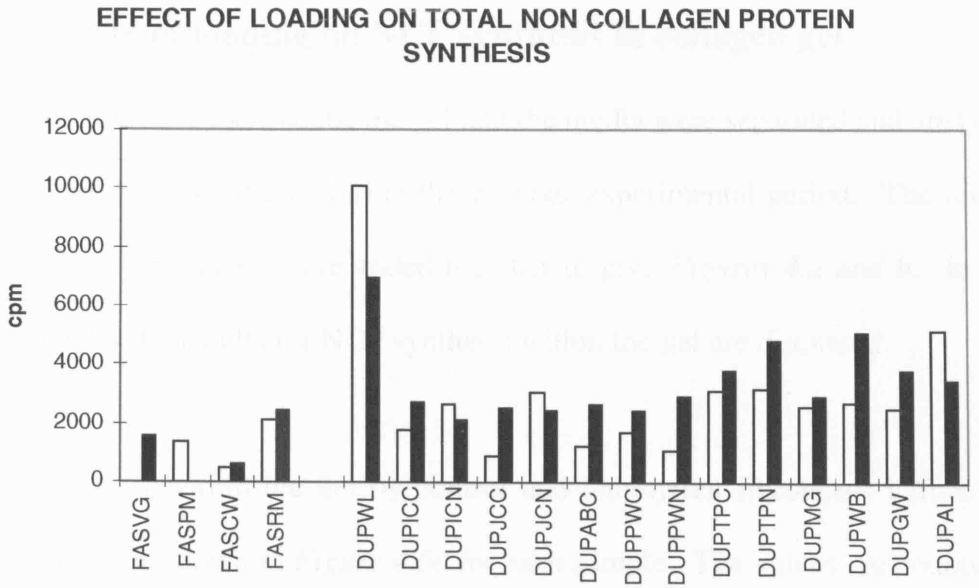
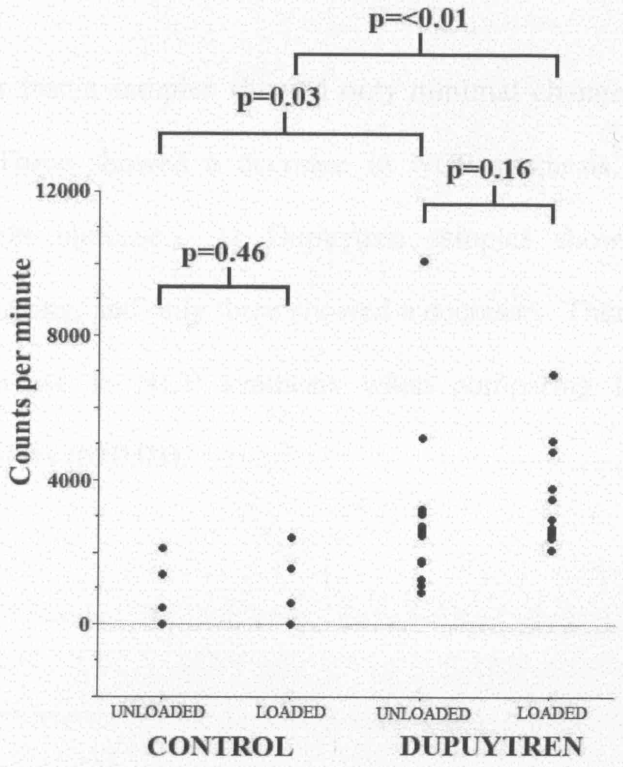


Figure 4.6b Plot of total NCP synthesis derived from Figure 4.6a . (Mann-Whitney U test for p values)



The following sections, **4.6.2** and **4.6.3** examine the gel and media contributions to NCP synthesis.

4.6.2 Effect of loading on NCP synthesis in collagen gel

At the end of the experiments, the gel and the media were separated and analyzed to measure NCP synthesis during the 24 hour experimental period. The results for both gel and media were added together to give *Figures 4.a and b*. In this section, only the results for NCP synthesis within the gel are discussed.

NCP synthesis within the gel by control and Dupuytren fibroblasts before and after loading is shown in *Figure 4.6c* for each sample. The values are counts per minute obtained from duplicate 100 µl aliquots following addition of 250 µl 0.8M NaOH to the precipitate resulting following bacterial collagenase digestion and centrifuge of individual gel samples.

Normal palmar fascia samples showed only minimal changes in NCP synthesis on loading. Three showed a decrease in NCP synthesis, and only FASVG showed a slight increase. 11 Dupuytren samples showed increased NCP synthesis on loading, and only three showed a decrease. There was a statistically significant increase in NCP synthesis when comparing loaded control and Dupuytren samples ($p=0.03$).

Figure 4.6c NCP synthesis in gel by normal palmar fascia and Dupuytren fibroblasts over 24 hours (white bars-unloaded samples, black bars-loaded samples)

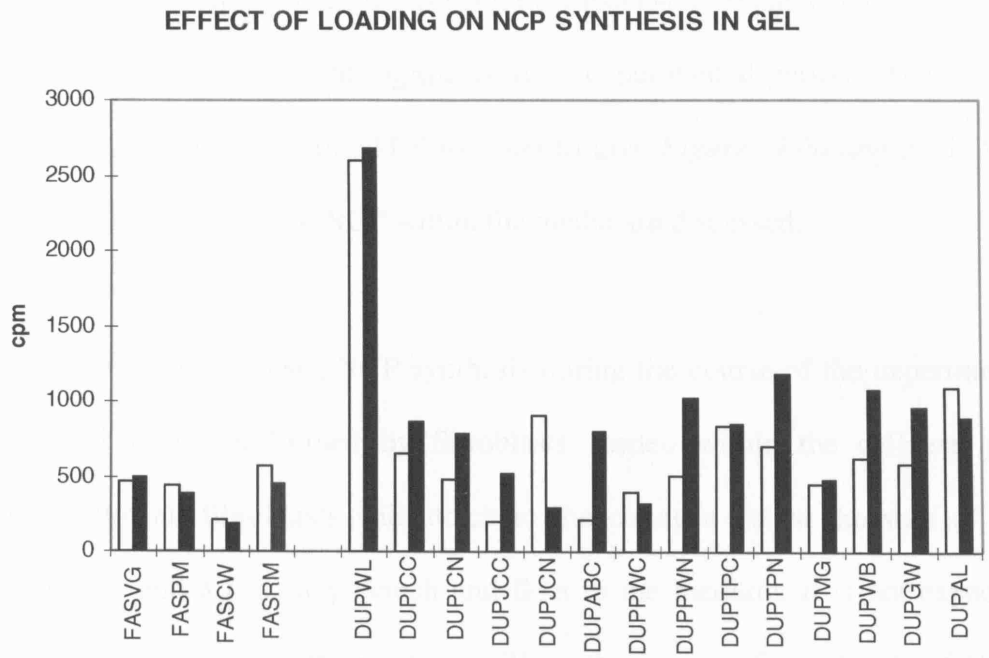
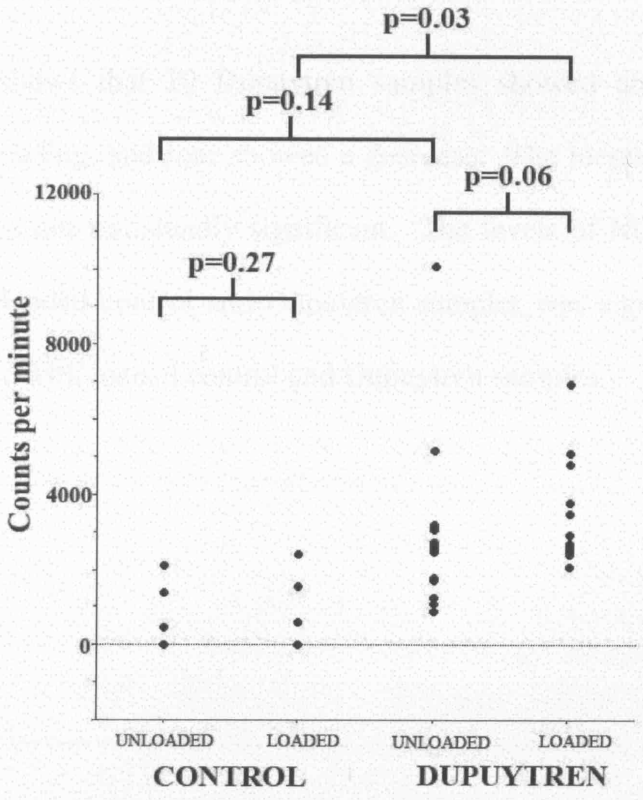


Figure 4.6d Plot of NCP synthesis in gel (derived from Figure 4.3c-(Mann-Whitney U test for p values)



4.6.3 Effect of loading on NCP levels in media

At the end of the experiments, the gel and the media were separated and analyzed to measure NCP synthesis during the 24 hour experimental period. The results for both gel and media were added together to give *Figures 4.6a and b*. In this section, only the results for NCP within the media are discussed.

As with collagen synthesis, NCP synthesis during the course of the experiments is assumed to be performed by fibroblasts seeded within the collagen gel. Although not all fibroblasts will attach to the collagen gel at the start of the experiments, and a few may detach and float in the medium, it is not expected that those floating within the medium will synthesize significant levels of NCP. Therefore it is assumed that NCP measured within the medium has been synthesised within the gel, and has diffused from the gel into the medium.

Figure 4.6e shows that 10 Dupuytren samples showed an increase in NCP synthesis on loading, and four showed a decrease. The increased NCP synthesis on loading was not statistically significant. The levels of NCP synthesis, when comparing unloaded control and Dupuytren samples was significantly different, as was the case with loaded control and Dupuytren samples.

Figure 4.6e NCP synthesis in media (white bars-unloaded samples, black bars-loaded samples)

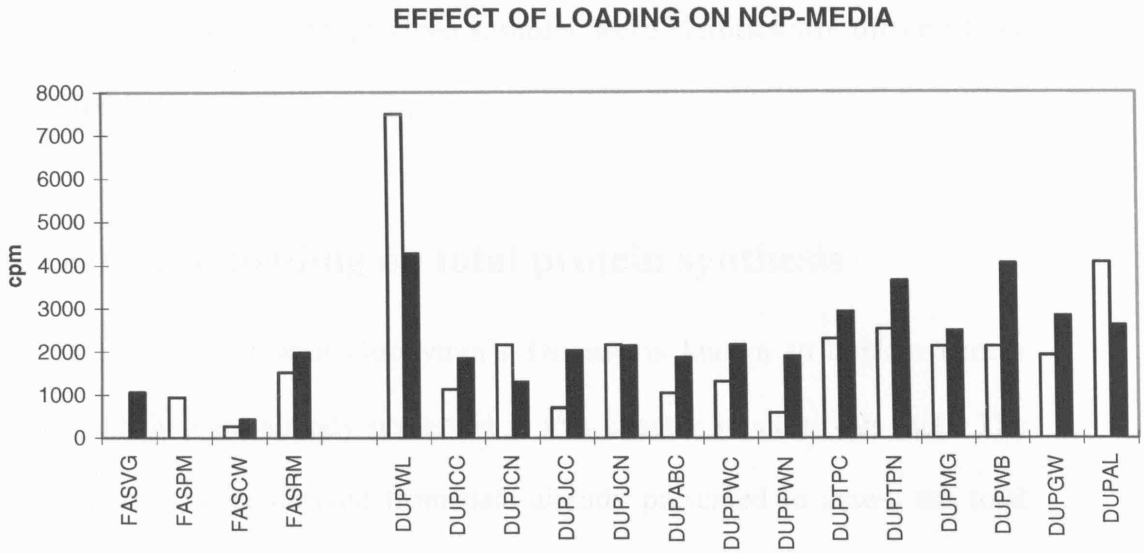
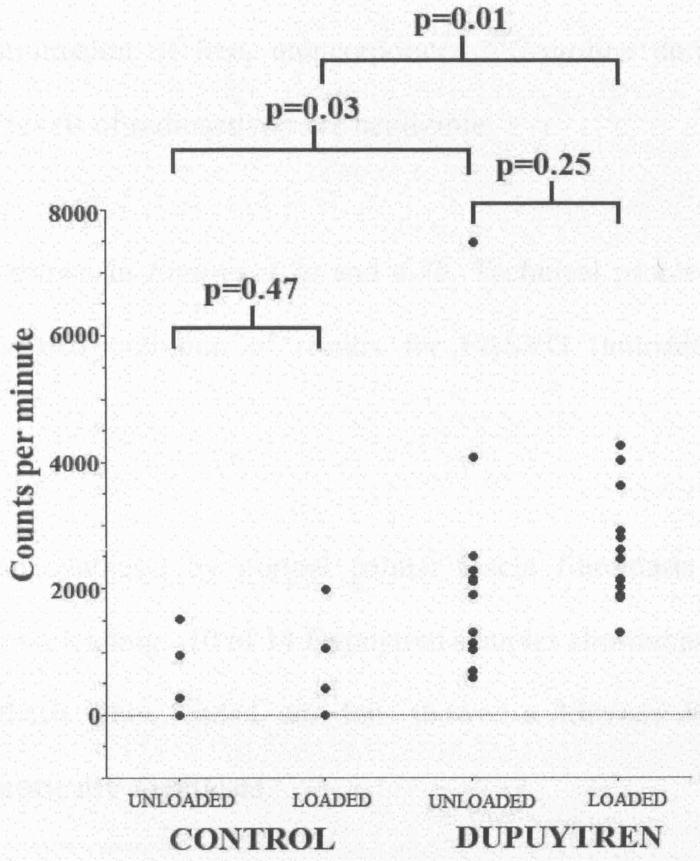


Figure 4.6f Plot of NCP synthesis in media (derived from Figure 4.6e) (Mann Whitney U test for p values)



In summary, although loading itself did not have a significant effect on NCP levels in the media in control or Dupuytren samples, levels of NCP synthesis by unloaded control and Dupuytren samples were significantly different, as were loaded levels.

4.7.1 Effect of loading on total protein synthesis

Palmar fascia affected with Dupuytren's Disease is known to contain excess collagen, and the data already presented in this chapter supports this fact. The following graphs were derived from data already presented to assess the total protein synthesised during the experiments by adding collagen and NCP totals in gel and media (in counts per minute) together. The resulting figures represented total protein synthesis (ie. which incorporated ^{14}C-proline) during the 24 hour experiments. It has been established from autoradiographs that there was complete elimination of free, unincorporated ^{14}C-proline during dialysis, and background levels of radioactivity are negligible.

Results are shown in *Figures 4.7a* and *4.7b*. Technical problems in processing have necessitated omission of results for FASVG (unloaded) and FASPM (loaded).

Total protein synthesis by normal palmar fascia fibroblasts did not change significantly on loading. 10 of 14 Dupuytren samples showed an increase in total protein synthesis when loaded, and four showed a decrease, but these changes were not statistically significant.

DUPWL showed a decrease in total protein synthesis from 11216 cpm when unloaded, to 8210 cpm when loaded. The unloaded result of 11216 cpm has been treated as an outlier as it is more than five standard deviations from the mean of the Dupuytren unloaded samples-mean=2962, SD 1313.

Two Dupuytren samples showing a decrease in total protein synthesis were cultured from nodular tissue (DUPICN and DUPJCN). The respective cord samples from these patients show an increase in total protein synthesis when loaded. DUPTP is the only other sample in the study in which it was possible to isolate both cord and nodule explants, and both these samples show an increase in total protein synthesis on loading.

Figure 4.7b shows that Dupuytren samples showed a statistically significant difference between unloaded control and Dupuytren samples, and loaded control and Dupuytren samples.

Figure 4.7a Total protein synthesis (gel and media) by control and Dupuytren fibroblasts over 24 hours as measured in cpm in supernatant and precipitate following bacterial collagenase (white bars-unloaded samples, black bars-loaded samples)

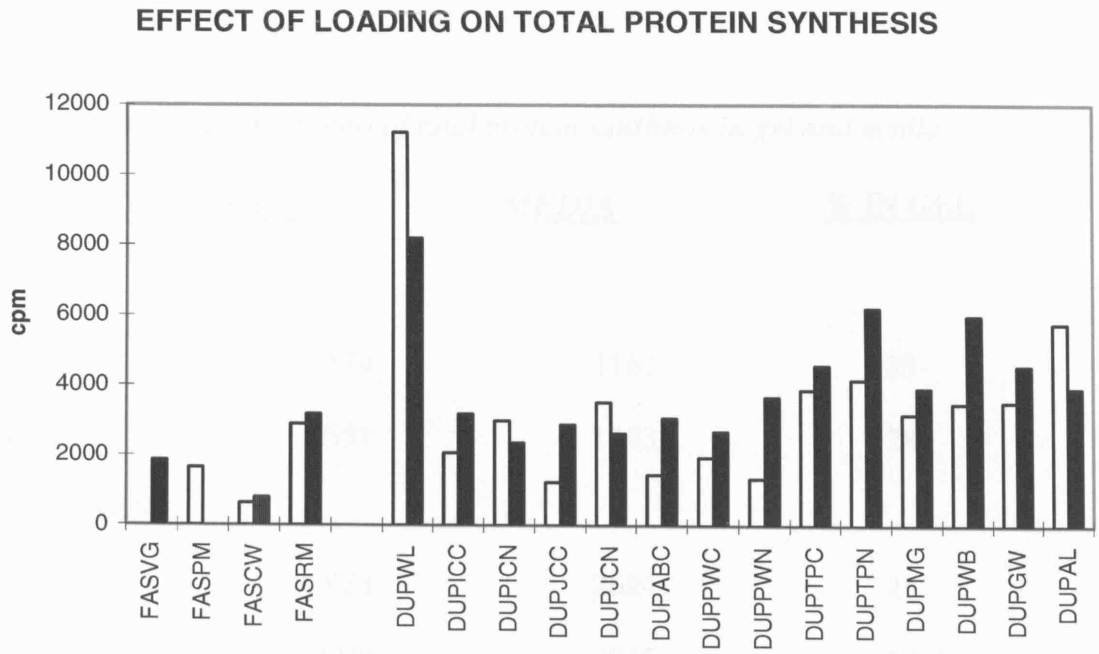
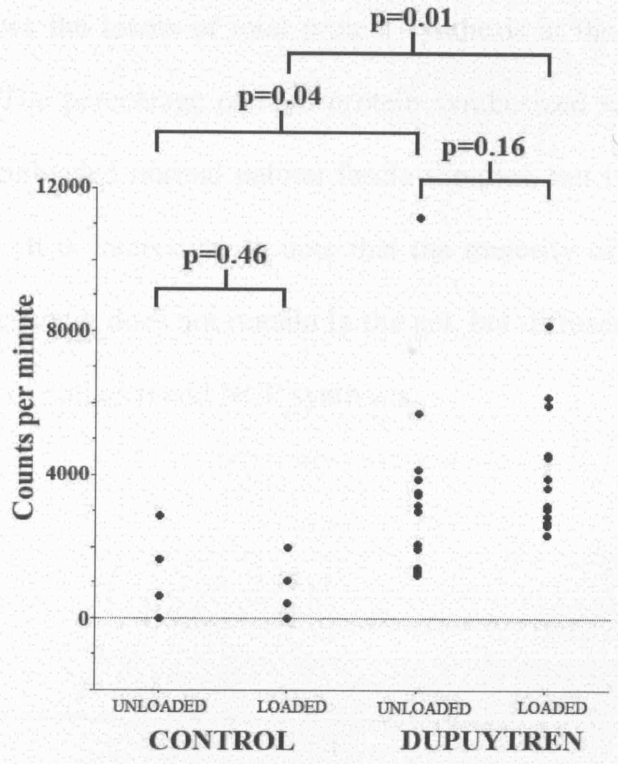


Figure 4.7b Plot of total protein synthesis derived from Figure 4.7a (Mann Whitney U for p values)



4.7.2 Total protein levels in gel and media

Figure 4.7.2 was constructed to take account of all new protein synthesized which incorporated ^{14}C-proline during the experiment.

Table 4.7.2 Mean values (cpm) of total protein synthesis in gel and media

	GEL	*MEDIA*	% IN GEL
FASCIA			
Unloaded	574	1162	33
Loaded	551	1393	28
DUPUYTREN			
Unloaded	928	2624	26
Loaded	1186	2945	28

This table shows the levels of total protein synthesis at the end of the 24 hour experiments. The percentage of total protein synthesized which remains in the gel is 33% in unloaded normal palmar fascia samples, but is very similar in the other samples. It is interesting to note that the majority of protein synthesised during the experiment does not remain in the gel, but diffuses out into the media. Relative levels of collagen and NCP synthesis.

4.7.3 Relative levels of collagen and non collagen protein

Table 4.7.3 displays data from the previous section to show the relative levels of collagen and NCP synthesized by fibroblasts.

Table 4.7.3 Collagen synthesis expressed as percentage of total protein (cpm)

	COLLAGEN	*NCP*	% COLLAGEN
FASCIA			
Unloaded	399	1320	23
Loaded	395	1535	20
DUPUYTREN			
Unloaded	560	2991	15
Loaded	650	3431	15

Table 4.7.3 shows that 23% of protein produced by normal palmar fascia fibroblasts when not loaded is collagen, and this figure is slightly less (20%) when the fibroblasts are loaded.

Dupuytren fibroblasts synthesize proportionally less collagen (15%) in unloaded and loaded experiments than control fascia, although the absolute levels of both collagen and NCP were significantly greater than the control samples.

In summary, Dupuytren fibroblasts synthesize more total protein and collagen than controls.

4.8 Effect of loading on collagenase levels

Collagenase is an important member of the family of matrix metalloproteinases (MMP). It has a central role in remodelling of the extracellular matrix, both in health and disease. There has been significant recent interest in its levels in the fields of aortic aneurysm research, and also in metastasis of malignant cells.

Collagenase levels have not been studied in Dupuytren's Disease. The disease involves extensive changes in the extracellular matrix, so it would seem logical that collagenase will have a role in this process.

Collagenase was measured using an ELISA which was commercially available, and had therefore been validated. Collagenase analysis was performed on media samples only, because gel samples were completely processed for collagen and NCP analysis.

Figure 4.8a shows the levels of collagenase in individual samples. The normal palmar fascia samples show low levels of collagenase, apart from FASCW after loading.

The Dupuytren samples, in general, have much higher levels of collagenase, although this is not true in each individual sample (DUPMG and DUPWB). Eight Dupuytren samples show an increase in collagenase activity on loading, and five show a decrease. The differences are only significant when comparing unloaded control and Dupuytren samples, although a general trend of higher values in Dupuytren's samples is apparent.

When interpreting these results, it must be remembered that these results are only from the media samples because the gel was consumed during analysis for collagen and non collagen protein. It would, however, not be surprising to find that Dupuytren fibroblasts synthesise collagenase at higher levels than normal palmar fascia on order that they might remodel the extracellular matrix to lay down excess collagen and other matrix proteins. This idea would be supported by this incomplete data, although no firm conclusions may be drawn.

Figure 4.8a Collagenase in media samples synthesized by normal palmar fibroblasts and Dupuytren fibroblasts over 24 hours measured by ELISA (white bars-unloaded samples, black bars-loaded samples)

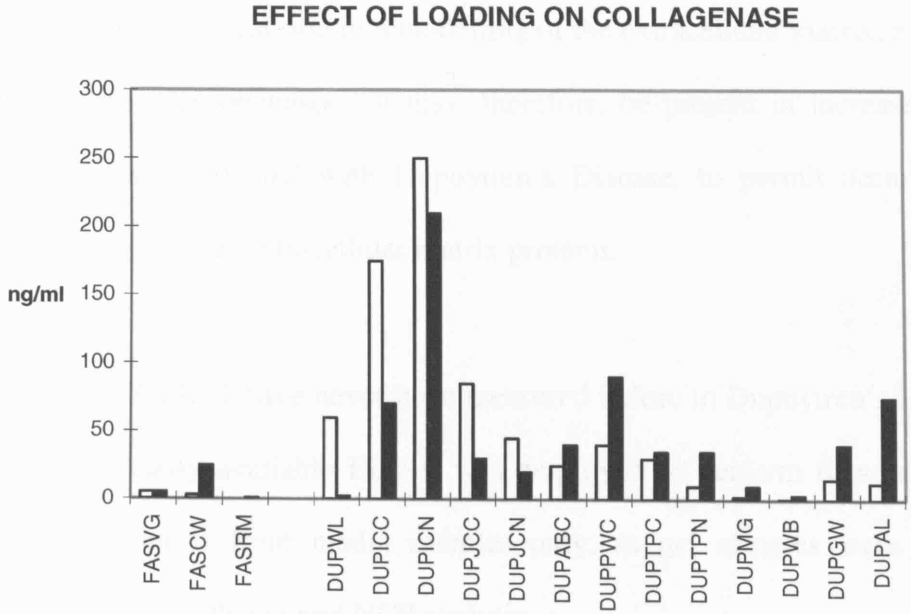
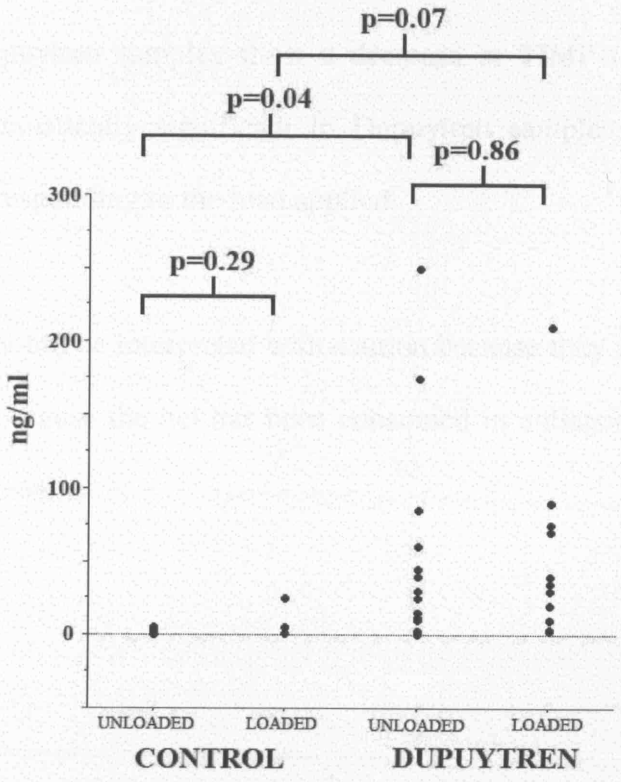


Figure 4.8b Plot of collagenase synthesis (derived from Figure 4.6d) (Mann Whitney U for p values)



4.9 Effect of loading on TIMP-1 levels

TIMP-1 has a central role in remodelling of the extracellular matrix, by inhibiting matrix metalloproteinases. It may, therefore, be present in increased levels in palmar fascia affected with Dupuytren's Disease, to permit accumulation of collagen and other extracellular matrix proteins.

Levels of TIMP-1 have never been measured before in Dupuytren's Disease, and a commercially available ELISA was employed to perform these experiments. TIMP-1 data is from media samples only, as gel samples were completely processed for collagen and NCP analysis.

The results show that almost all samples, both normal palmar fascia and Dupuytren's fibroblasts, show an increase in TIMP-1 as a response to loading. Only three Dupuytren samples show a decrease in TIMP-1 on loading. The difference is statistically significant in Dupuytren samples, showing that the fibroblasts are responding to the load applied.

These results should be interpreted with caution because they represent the media samples only, because the gel has been consumed in collagen and non collagen protein measurement.

Figure 4.9a TIMP in media samples synthesized by normal palmar fibroblasts and Dupuytren fibroblasts over 24 hours measured by ELISA(white bars-unloaded samples, black bars-loaded samples)

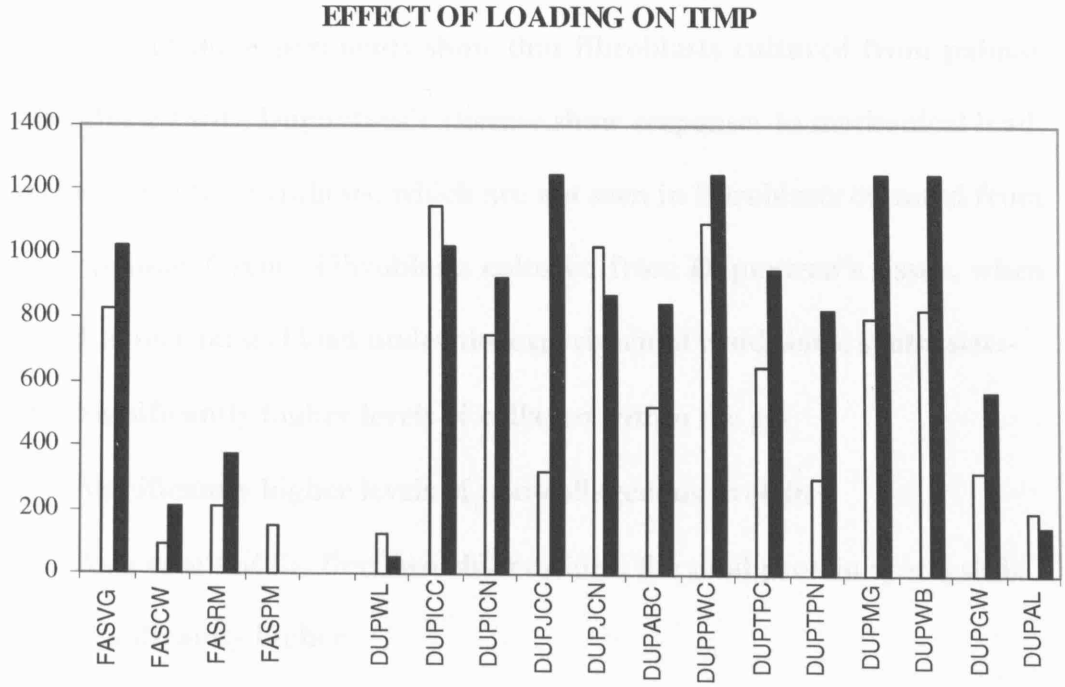
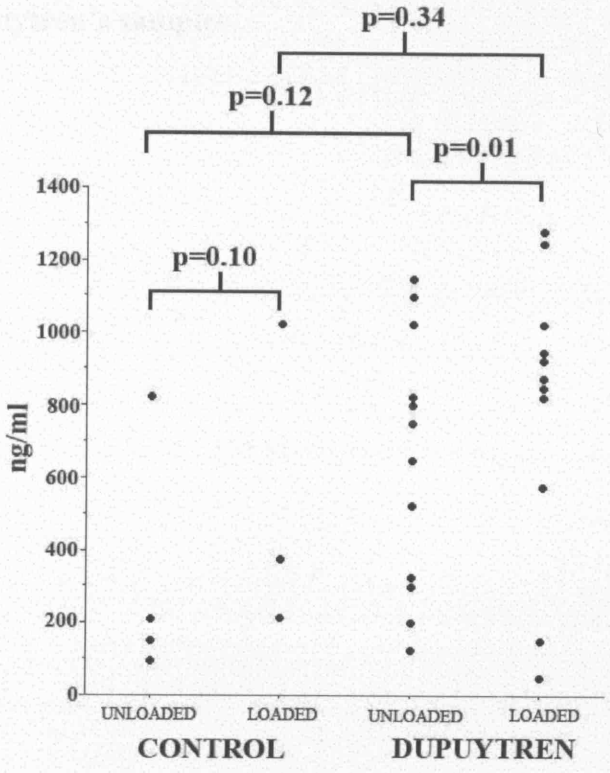


Figure 4.9b Plot of TIMP-1 synthesis (derived from Figure 4.9a-Mann Whitney U for p values)



4.11 Summary of Results

The results of the experiments show that fibroblasts cultured from palmar fascia affected with Dupuytren's Disease show responses to mechanical load, in terms of protein synthesis, which are not seen in fibroblasts cultured from normal palmar fascia. Fibroblasts cultured from Dupuytren's tissue, when exposed to mechanical load under the experimental conditions, synthesize:-

- **Significantly higher levels of collagen within the gel**
- **Significantly higher levels of non-collagenous protein**
- **As a result of the first two observations, the total protein synthesis is significantly higher**
- **These responses are not observed in controls**
- **This is an original finding**
- **Collagenase and TIMP levels have been quantitated for the first time in Dupuytren's samples.**

DISCUSSION

5.1 Introduction

Dupuytren's Disease is common and causes significant morbidity. Surgical intervention is frequently necessary to maintain useful hand function. In many cases, surgery does not cure the disease, and further operations are necessary to treat recurrence. The underlying mechanism of the disease is unknown. Palmar fascia affected with Dupuytren's Disease contains an excess of collagen, and is under tension.

The relationship between the excess collagen synthesis observed in diseased tissue, and the tension which develops, has not been examined. Despite a large number of papers in the literature, there is no scientific evidence which examines the response of fibroblasts to mechanical loading in collagen gels. No previous experimental work has specifically addressed this question, so the results obtained are original, and contribute novel data to the literature by investigating a fundamental aspect of the disease.

The aim of this thesis was, therefore, to investigate collagen metabolism of normal palmar fascia fibroblasts and Dupuytren fibroblasts *in vitro*, specifically changes in response to mechanical load. These data give an insight into

underlying mechanisms of the disease, and give targets for further directed research.

5.2 Experimental design and methodology discussion

Previous *in vitro* studies of Dupuytren's Disease using cultured fibroblasts have used forearm dermal or foreskin fibroblasts as control samples. Although these fibroblasts are of the same mesenchymal origin, they are not derived from palmar fascia, and have therefore not been subjected to the same extracellular matrix environment. The use of control fibroblasts from the same tissue of origin as the diseased tissue was intended to give as close a comparison as possible.

Addition of ^{14}C-proline at the start of the experiment permitted specific labelling of proteins incorporating ^{14}C-proline which were synthesized during the course of the experiment. At the end of the experiment, digestion of the gel and media with bacterial collagenase permitted differentiation of the collagen and non-collagenous proteins which had been synthesised. Although non-collagenous protein synthesis was measured, these proteins were not specifically identified.

Both free floating gel and loaded gel experiments were run simultaneously for each cell line in the same incubator, using the same number of cells with the same collagen gel constituents and media. The pairing of these experiments in identical conditions increased the probability that observed differences between the unloaded and loaded samples are valid. Due to the large number of fibroblasts required to seed each gel, it was not possible to run duplicate samples.

The use of the FPCL is an accepted model of the cellular environment in the extracellular matrix (Stopack and Harris (1982), Grinnell and Lamke (1984), Tomasek et al (1992), and mimics the *in vivo* environment to a much greater degree than monolayer cell culture on a bacteriological plate or culture flask. The collagen gel permits cellular attachment by various membrane receptors, and provides a mobile substrate through which forces transmitted by the tCFM may influence fibroblast behaviour. The gel is, by definition, mainly composed of water, and collagen fibres in the gel are therefore in a less dense arrangement surrounding the fibroblast than the *in vivo* situation.

Results from electrophoresis and autoradiographic studies show that dialysis was effective in removing free unincorporated ^{14}C proline. This was an essential part of the methodology because the inclusion of free unincorporated ^{14}C proline in the results would lead to falsely high values. The radioactive background count was negligible.

A further essential step in the design of the experiment was to confirm that collagen was actually synthesized in the FPCL within the 24 hour period of the experiment. The autoradiographs confirm that ^{14}C proline was incorporated into Type I collagen.

It is accepted that some ^{14}C proline is incorporated into proteins other than collagen. These include fibronectin, proteoglycans (large and small-versecan and aggrecan respectively), elastin and cytoskeletal proteins. The proportion of ^{14}C proline incorporated into these individual proteins is considered to be small, and

has not been quantitated in the methodology (in common with all other studies in the literature using the radiolabelling incorporation method).

There are several limitations to the experimental design. The main limitation is the time over which the experiments could be run (24 hours). This was due to sterility problems in the gel and media after this length of time. The incubator provided a disinfected, but not sterile environment. All experiments were run at 37^0C and although the media and gel did not appear discoloured or tainted to the naked eye after 24 hours, no experiments were run for longer than this period. The time limitation is significant because Dupuytren's Disease is seen in clinical practice as a chronic disease running a course of many years. Therefore, based on this clinical observation, differences in metabolic cellular activity between control and Dupuytren fibroblasts over 24 hours may not be expected. The fact that significant differences in synthetic abilities can be shown between control and Dupuytren fibroblasts within 24 hours strongly suggests that Dupuytren fibroblasts are metabolically distinct, and maintain these metabolic differences *in vitro*.

Histological examination of the Dupuytren samples was not performed prior to explant culture in order to classify their stage as proliferative, involutional or residual (Luck 1959). It is possible, therefore, that a bias may be introduced if samples in the proliferative phase produced more vigorous explant cultures with higher protein synthesis potential.

5.3 Collagen synthesis by Dupuytren's fibroblasts

The most important observation from the data presented in this thesis is that fibroblasts derived from palmar fascia affected with Dupuytren's Disease synthesize significantly more collagen than fibroblasts derived from normal palmar fascia when subjected to mechanical loads (*Figure 4.5b-e).* Collagen synthesis by Dupuytren fibroblasts within the gel significantly increases with the application of a cyclical load. This response is not seen in control fibroblasts, and has not previously been described.

The data published on collagen metabolism by Dupuytren's fibroblasts is limited, which is somewhat surprising because it is universally accepted that Dupuytren's Disease is a pathological manifestation of excess collagen. Bailey et al (1977) were the first group to report changes in collagen in tissue affected with Dupuytren's Disease, particularly with respect to increased proportions of Type III collagen. Their study was based on analysis of fresh tissue, and did not involve cell culture.

Murrell et al (1991) examined collagen synthesis by Dupuyren's fibroblasts in monolayer culture using a radiolabelling method (preincubation with ^{3}H proline) and subsequent quantitation with electrophoresis. They found that control forearm dermal fibroblasts, control palmar fascia fibroblasts, and Dupuytren's fibroblasts showed no significant difference in collagen production. They conclude that the fibroblast in Dupuytren's Disease is not abnormal, in contrast to this study.

There is no experimental data examining collagen synthesis in Dupuytren's fibroblasts under the influence of a mechanical load. Furthermore, there is no data on synthesis of collagen by Dupuytren's fibroblasts within an FPCL under the influence of mechanical loading.

Gupta et al (1998) examined intracellular calcium changes in normal palmar fibroblasts and Dupuytren fibroblasts cultured on a plate in a specially designed flow chamber which allowed fluid shear stress to be applied in a controlled, reproducible manner. This study showed that Dupuytren fibroblasts respond to fluid shear stress with a significantly smaller change in intracellular calcium levels than control normal palmar fascia fibroblasts. They suggest that the fibroblasts are therefore responding to a purely mechanical cue, and that this response to mechanical stress may be the underlying abnormality in the Dupuytren fibroblast.

Tarpila et al (1996) examined contraction of free floating FPCL by fibroblasts cultured from nodules of Dupuytren's tissue and found that the FPCL were contracted to a greater degree by control tissue (forearm dermal fibroblasts) than by Dupuytren's fibroblasts over 36 hours (35% vs. 19% area, $P<0.01$). This result may in part be due to the nature of the control tissue which was not normal palmar fascia, but dermal fibroblasts.

Rayan et al (1996) examined the effect of pharmacological agents on Dupuytren's fibroblasts cast in an FPCL. These included promoters of smooth

muscle contraction such as angiotensin II, serotonin, prostaglandin $F_{2\alpha}$, and lysophosphatidic acid. Based on the results they proposed a mechanism for an intracellular signalling cascade regulating Dupuytren fibroblast contraction via myosin light-chain kinase.

Quaglino et al (1997) cast fibroblasts into free floating FPCL and the retraction of these gels was measured over a 72 hours. They found that normal forearm fibroblasts contracted the free floating FPCL to the greatest extent when compared to three other populations (control fibroblasts from unaffected palmar aponeurosis, fibroblasts from clinically unaffected zones of palmar aponeurosis in a patient with Dupuytren's, or fibroblasts from the fibrous cord of Dupuytren's). These results concur with those of Tarpila et al (1996).

Therefore although Dupuytren's fibroblasts have been cast in FPCL, their ability to synthesize collagen within the lattice has not been examined. Furthermore, the only published study on application of mechanical stress to Dupuytren fibroblasts has been performed in a flow chamber, and in this study the cells were in a monolayer on a quartz slide, not in a FPCL (Gupta et al 1998).

Collagenase and TIMP-1 results are of interest because there is no data on these enzymes in the Dupuytren's literature. The data are limited by the absence of levels from the gel samples which were consumed in analysis of collagen and non collagen synthesis, so only media samples were used. The collagenase levels were highly variable and therefore did not reach levels of statistically significant difference, although there appeared to be a clear trend of higher levels in the

Dupuytren tissue. TIMP-1 levels were significantly increased by loading in both control and Dupuytren samples.

The results of the experiments show that fibroblasts cultured from palmar fascia affected with Dupuytren's Disease *show responses to mechanical load, in terms of protein synthesis*, which are not seen in fibroblasts cultured from normal palmar fascia. Fibroblasts cultured from Dupuytren's tissue, when exposed to mechanical load under the experimental conditions, synthesize:-

- **Significantly higher levels of collagen within the gel**
- **Significantly higher levels of non-collagenous protein**
- **As a result of the first two observations, the total protein synthesis is significantly higher**
- **These responses are not observed in controls**
- **This is an original finding**
- **Collagenase and TIMP levels have been quantitated for the first time in Dupuytren's samples.**

5.4 Effect of load on the extracellular matrix

Lambert et al (1992) examined collagen synthesis, total protein synthesis, collagenase activity and mRNA levels in human dermal fibroblasts (cultured from a single individual) in collagen lattices which were either fixed to the edge of a bacteriological plate, or left free to contract. They observed that both collagen and total protein synthesis were significantly greater in the bound lattice when compared to the free lattice. Collagenase activity reached barely

measurable levels within the first 24 hours, but thereafter rose rapidly from day 2 to day 5, and was significantly higher in the bound lattice when compared to the free lattice. Despite significant differences in the methodology, the broad pattern of results in the first 24 hours is similar to the responses seen in experiments with normal palmar fibroblasts and those of Dupuytren's Disease origin. Lambert et al suggest that regulation of extracellular matrix components is determined by mechanical information transmitted to the cells (via the cytoskeleton), which directly affects modulation of protein synthesis at the pretranslational level.

The influence of mechanical forces on fibronectin fibril assembly and the fibronexus has been studied by Halliday and Tomasek (1995) using human palmar fibroblasts in free floating and mechanically stressed collagen gels. In synthetic terms there was no difference in fibronectin levels, as measured by protein and mRNA levels, between free floating and stressed gels. Fibroblasts in mechanically relaxed gels did not develop stress fibres and did not assemble fibronectin into fibrils, in contrast to those in stressed gels. It was noted that, despite addition of inhibitors of fibronectin fibril assembly, cells continued to generate contractile force, suggesting links to the matrix through extracellular matrix proteins other than fibronectin.

Procollagen synthesis (both protein and mRNA) in response to mechanical load was studied in foetal rat cardiac fibroblasts (Butt and Bishop 1997). Their studies on loading were performed in the absence and presence of foetal calf serum, and a synergistic interaction between mechanical load and serum presence was

observed. They suggested that cardiovascular cells may need to be 'primed' by the presence of growth factors before they are able to respond to mechanical load.

Several previous studies have examined the link between mechanical load and its effect on the synthesis of extracellular matrix molecules. The general pattern that emerges is that application of mechanical load appears to increase the synthesis of collagen. The mechanisms for this increase have not been elucidated. These studies have all used a single cell type, rather than a control and pathological cell line.

5.5 Potential mechanisms of increased protein synthesis in response to load

The extracellular matrix is generally considered to have two distinct functions, although they are interconnected (Chiquet et al 1996). The first is mechanical and gives support to tissues to maintain body shape eg. cartilage and bone. The second is to act as a substrate for cell adhesion, migration and differentiation. Therefore the extracellular matrix is a source of information for cells which provides cues for their behaviour.

Components of the extracellular matrix are recognised by cell surface receptors, the most important being integrins. Integrins are thought to act as two-way intermediaries between the extracellular matrix and cellular signal transduction pathways. Cells are known to react to altered strain patterns within the extracellular matrix and react by attempting to restore the force balance eg. smooth muscle cell hypertrophy in blood vessel walls in response to chronic arterial hypertension (Schwartz et al. 1986), cartilage matrix biosynthesis increases in response to cyclical loading and decreases in static compression (Buschmann et al 1995). The external cues a cell receives from the matrix therefore may upregulate or downregulate synthetic and degradative pathways.

Several hypotheses have been suggested to explain mechanochemical transduction:-

- Strain on the cell membrane, to which the extracellular matrix is connected, activates stretch induced ion channels (Harter et al. 1995)

- In cartilage, compression causes a decrease in extracellular matrix cell volume and cell volume as ions stream out of the cell which may trigger signal transduction at the cell membrane (Buschmann et al 1995)
- Strain on the extracellular matrix and thus the cell membrane may alter the response of the cell to growth factors and hormones by changing the distribution of cell surface receptors (Adams and Watt 1993)
- Cytoskeletal restructuring in response in response to changes in the extracellular matrix (Ingber 1994)
- Integrins themselves acting as mechanochemical transducers (Ingber 1991)

The *in vivo* situation may be a combination of the above factors, but integrins are likely to play a central role in transduction. Integrins, as the name implies, are cell surface receptors considered to be central to interaction between the cell and the extracellular matrix (Giancotti and Ruoslahti 1999). They comprise two subunits, α and β, and bind various extracellular matrix proteins depending on the type of subunit.

Integrins relay signals from either direction, to or from the cell. As integrins bind to the extracellular matrix they become clustered in the cell membrane and associate with actin microfilaments which form stress fibres and cause further integrin clustering (positive feedback). The clusters of matrix proteins, integrins, and cytoskeletal proteins can be detected by immunofluorescence microscopy.

The cytoplasmic tails of the β subunit are linked not only to the actin cytoskeleton (Hynes 1992), but also to protein kinases eg. $pp125^{FAK}$ (focal adhesion kinase-Schaller and Parsons 1994). $pp125^{FAK}$ is activated by most integrins, and is thought to be coupled to the assembly of focal adhesions. Autophosphorylation of $pp125^{FAK}$ which results from binding of an extracellular matrix ligand to integrin then results in activation of tyrosine kinases (eg. src-tyrosine kinase), which in turn phosphorylates cytoskeletal proteins such as paxillin and talin. Integrin receptors have also been shown to associated with specific growth factor receptors eg. $\alpha_5\beta_1$ integrin is associated with the EGF receptor (Miyamoto et al 1996) and it has been suggested by the same group that the aggregation of the integrin and growth factor-receptor complex allows a certain threshold to be reached resulting in partial activation. Integrins are also thought to be important in allowing the cell to continue through the cell cycle.

The only study on integrins in Dupuytren's Disease was undertaken by Magro et al (1995) using light microscopy and immunohistochemical techniques to examine the distribution of fibronectin and $\alpha_5\beta_1$ integrin in 23 samples of Dupuytren's tissue, but no controls. They demonstrated that both were expressed in areas of high cellularity, but not in areas of low cellularity.

Genes for certain extracellular matrix components may be controlled by mechanical signals. Harter et al (1995) demonstrated that a human osteosarcoma cell line may increase mRNA synthesis for collagen α1 (I) when mechanically loaded on flexible silicone membranes (compared with the same cells under non-loaded conditions).

Tenascin is a large extracellular matrix protein which is deposited in the embryo in regular, restricted patterns in areas which develop into high load bearing structures, such as ligaments, tendons and bone. Tenascin synthesis has been found to be increased in collagen gels which are under tension when compared to free floating gels (Chiquet et al 1996).

Multiple mechanisms have been proposed to explain how loading affects metabolism of connective tissue cells. The pace of understanding of intracellular pathways and their effect on DNA is rapidly advancing. The triggering of the intracellular pathways is thought to involve several routes at the membrane level, but integrins are probably central to the triggering process. No specific results exist in the Dupuytren's literature at this stage in regard to specific differences in fibroblast metabolism at this level.

5.6 How does this information help us understand Dupuytren's Diesease?

The only straightforward aspect of Dupuytren's Disease is the diagnosis. Understanding of the aetiology, pathogenesis and treatment are far from complete. The literature contains many epidemiological articles and extensive operative series of surgical results. These data are debated amongst surgeons who develop their treatments of Dupuytren's Disease based on practical surgical training under different superiors, published series in peer reviewed journals, and

(possibly most importantly) those techniques which seem to work best in their own hands. At a basic scientific level the literature contains a range of investigations based on collagen investigations and microscopic studies in the 1970s and 1980s, through to more molecular work examining growth factors and receptors in the 1990s, but no unifying theory prevails.

Luck (1959) suggests that "...the cord of Dupuytren's contracture forms, for the most part, proximal to the nodule and represents *reactive functional hypertrophy in response to the repeated tension stresses on the band of fascia* from which the nodule took its origin."

MacFarlane (1974) performed anatomical studies of Dupuytren's Disease and described the pattern of abnormal tissue in the digits. He states that the disease follows anatomical pathways and does not occur in a random fashion.

McGrouther (1982) meticulously described the anatomy of the palmar aponeurotic fibres, particularly in the distal palm, and revealed a precise three-dimensional arrangement allowing a small degree of relative motion between fibre systems. He also concludes that the disease follows anatomical pathways in the distal palm, supporting MacFarlane's ideas.

Combining the ideas of a precise, dynamic, three-dimensional anatomical structure and the concept of work hypertrophy proposed by Luck (1959), McGrouther suggests that any disordered transmission of forces within the intricate system of aponeurotic fibres will give rise to areas of stress

concentration, where hypertrophy (a biological reaction, rather than failure/rupture, a mechanical reaction) will occur.

An area of stress concentration in the palmar fascia may then lead to hypertrophy, and the origins of a Dupuytren's nodule. The fundamental question is what is the precursor to the stress focus? Is it the inevitable fate of the fibroblast based on abnormal genetic information contained within the fibroblasts in the palmar fascia in the affected individual? Individuals may therefore be born with genetically abnormal palmar fascia fibroblasts destined to develop Dupuytren's Disease. Alternatively does an external event, possibly traumatic, profoundly affect the aponeurotic fibres and/or fibroblasts, triggering a profound change in metabolic activity of the fibroblast, leading ultimately to excess deposition of extracellular matrix proteins and the clinical phenomenon of fibrosis and digital deformity?

The evidence presented in this thesis demonstrates that fibroblasts in Dupuytren's Disease are indeed different to normal palmar fibroblasts in terms of their synthetic abilities under conditions of mechanical loading. They produce significantly more protein, both collagenous and non-collagenous, under loaded conditions. This therefore lends weight to the concept of work hypertrophy occurring in the palmar fascia, the hypertrophy resulting from an increased synthesis of extracellular matrix proteins, particularly collagen, when mechanical loads are applied.

The difference in response to mechanical load between normal palmar fascia fibroblasts and Dupuytren's fibroblasts is an inherent quality of the fibroblast ie. it has been maintained in culture, and is therefore a phenotypic, and possibly genetic, difference with respect to normal palmar fascia fibroblasts. Whether this difference is congenital or acquired is uncertain. There is certainly a strong hereditary element to the disease in many cases, demonstrated in many epidemiological studies.

The ability to respond to mechanical cues may be under tight control in the normal fascia fibroblast, but this tight control is lost in fibroblasts affected by Dupuytren's Disease. The response to mechanical cues may be switched off in normal palmar fascia cells, but, due to an unknown stimulus, the fibroblasts in Dupuytren's Disease cease to be "deaf" to mechanical cues, and they respond with protein synthesis.

Alternatively this ability to respond to mechanical cues may represent a regression of the cells behaviour to that approaching an embryonic cell which relies on mechanical cues, as well as chemical gradients, to develop. The excess production of Type III collagen by fibroblasts in Dupuytren's Disease is well documented and may be thought of as a step back to a more foetal type of cell.

The work presented in this thesis shows that fibroblasts cultured from individuals with Dupuytren's Disease have a different response to mechanical load than normal palmar fascia fibroblasts. The increase in collagen synthesis when loaded supports the hypothesis of 'work

hypertrophy' suggested by Luck in 1959 using techniques unavailable to him. This adds another piece of data to the 'enigma' of Dupuytren's Disease, and further investigations, particularly into the genetic control of collagen metabolism are likely to be rewarding.

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ACKNOWLEDGEMENTS

I would like to thank Professor McGrouther for giving me this opportunity to undertake research, and for his support during the project. It has been a fascinating time, and I have greatly enjoyed researching this enigmatic disease.

I am also very grateful to the many other members of the research team at University College London who helped with the experiments, particularly Dr. Robert Brown, Dr. Mark Eastwood, and Dr. Vivek Mudera. Thanks also to Zulfi, Kam, Rita and Kirsty.

Dr. Graham Reilly was extremely supportive and generous with his time and equipment in the Rheumatology Research Unit in Cambridge, and I am very grateful.

Thank you also to Mr. Smellie for his help in the write up of this work.

I would like to record my thanks to my wife and my parents for their continuous belief and support.