Proliferation and the action of an anti-proliferative agent in Dupuytren's fibroblast cultures.

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1999

A thesis submitted to the University of London for the degree of Doctor of Medicine (M.D.)

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Acknowledgements

This thesis is dedicated to my father.

I wish to thank my supervisor Professor D A McGrouther for his invaluable supervision and contagious enthusiasm, Mr A O Grobbelaar for his unwavering support, Mr P J Smith for providing me with an abundance of patients, Mr R Grover for his ideas, Messrs D H Harrison, D Gault, P Cussons and N Reissis for their steady supply of material and Professor R Sanders for making it all possible.

I would also like to thank Drs C Linge for advice on cellular experiments, G Wilson for advice on immunohistochemical work and P Richman for advice on histology. Drs S Fischer and S Daugaard for supply of fibrosarcoma sections and Drs R Brown and M Eastwood for their help on the Culture Force Monitor.

I would furthermore like to thank Mr J Shelton for teaching me tissue-culture techniques, Mrs F Daley for immunohistochemical assistance, Mrs S Barnet, Mr K Ladhani and Ms J Archer for histological assistance.

I am very grateful to the staff in Plastic Surgery Theatres at Mount Vernon Hospital, for keeping material for me, everyone at R.A.F.T. for making it a pleasant stay and the Phoenix Institute and the Gray Laboratory for making me feel welcome.

I wish to thank the Trustees of the Restoration of Appearance and Function Trust, the Royal College of Surgeons of England and the British Society of Surgery of the Hand for their financial support.

At last I wish to thank my mother and father, my brother and Lynn for their support and constant encouragement.
Abstract

Dupuytren's disease (DD) is a common and disabling hand condition with proliferative and contractile properties. Treatment is surgical, but recurrence is common. Review of the literature suggested that Transforming Growth Factor β1 (TGFβ1) influenced myofibroblast differentiation, which was a cause of contraction and that DD shared certain characteristics with malignant tumours, which arise from an imbalance between proliferation and apoptosis.

The hypothesis was therefore proposed that co-expression of the c-myc oncogene (mediates control of cell-cycle progression) and the bcl-2 gene (protects against apoptosis) results in an imbalance between proliferation and apoptosis in palmar fascia, leading to DD. It was furthermore suggested that the proliferation, differentiation and contractile properties of Dupuytren's disease may be inhibited by 5-fluorouracil in vitro. This was investigated utilising immunohistochemical methods in DD, non-diseased palmar fascia (NDF) from DD hands, carpal ligament (CL) and fibrosarcoma specimens, 30 day cell culture studies in DD, NDF and CL and 24 hour gel-contraction studies in DD.

C-myc expression was elevated in primary DD and fibrosarcoma. Mib-1 antibody, measuring proliferation and bcl-2 were absent. C-myc expression in recurrent DD was found to be correlated to the total digital angle deformity. A single dose of 5-fluorouracil decreased proliferation for a longer time period in control DD and NDF fibroblasts than in CL. Also myofibroblast differentiation was continuously decreased in DD fibroblasts, incompletely in NDF, whilst CL was un-affected. TGFβ1 produced a general decrease in proliferation, amplified by 5-fluorouracil, and a sustained increase in myofibroblast differentiation in DD fibroblasts only, incompletely inhibited by 5-fluorouracil. 5-fluorouracil also decreased DD fibroblast contraction.

These experiments demonstrated that DD is not caused by an imbalance between proliferation and apoptosis. However, 5-fluorouracil inhibited proliferation, differentiation and contraction of DD fibroblasts in vitro. It is therefore suggested that 5-fluorouracil may reduce contracture and recurrence in DD in vivo.
Declaration of originality

I declare that the laboratory research for this thesis is original and that the ideas were developed in conjunction with my supervisor.

I performed the experiments myself with the guidance and technical assistance of the scientific staff at the Phoenix Laboratories, University College London Hospitals, the Restoration of Appearance and Function Trust and the Gray Laboratory, Mount Vernon Hospital.

Statistical advice was provided by the LREC statistician at King’s College, London.
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<tr>
<td>αSMA</td>
<td>Alpha Smooth Muscle Actin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CFM</td>
<td>Culture Force Monitor</td>
</tr>
<tr>
<td>CL</td>
<td>Carpal ligament</td>
</tr>
<tr>
<td>DD</td>
<td>Dupuytren's Disease</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Iso-Thio-Cyanate</td>
</tr>
<tr>
<td>NDF</td>
<td>Non-Diseased Fascia</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<td>TBS</td>
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Introduction

Dupuytren's contracture is a common and disabling disease of the hand. The available treatment consists of surgery, although high recurrence rates represent a major clinical problem. The clinical picture will be established by review of the relevant extant literature, including history, cell biology, pathology and the individual topics relevant for the following hypothesis. The disease shares in vitro and in vivo characteristics with malignant tumours, e.g. abnormal cell-cycle progression, proliferation and apoptosis. These properties are all governed by the C-myc proto-oncogene.

The hypothesis is therefore proposed that a high level of c-myc oncogene in conjunction with the anti-apoptotic bcl-2 gene results in an imbalance between proliferation and apoptosis in palmar fascia, leading to Dupuytren's contracture. It is furthermore proposed that the proliferation, differentiation and the contractile properties of Dupuytren's disease may be inhibited by 5-fluorouracil in vitro.

The following experiments to test the hypothesis will be presented with results and discussion.

- Measurement of c-myc oncogene levels in Dupuytren's contracture, non-diseased fascia, carpal ligament and fibrosarcoma specimens by immunohistochemical methods. **Chapter II**
- Measurement of proliferation, using the mib-1 antibody and levels of the bcl-2 gene, signifying protection against apoptosis, by immunohistochemical methods in Dupuytren's contracture, non-diseased fascia, carpal ligament and fibrosarcoma specimens. **Chapter III**
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Chapter I

Review of the literature
1. Dupuytren's disease in general

1.1. History

Dupuytren's disease is thought to have been described in Icelandic Sagas in the 12th and 13th century (Whaley and Elliot 1993). A possible case was described in the European literature by the Swiss doctor and anatomist Felix Plater in 1614: “Contraction of the fingers of the left hand into the palm. A certain well-known, stone mason rolling a large stone, caused the tendons to the ring and little fingers in the palm of the left hand to cease to function. They contracted and in so doing were loosed from the bonds by which they are held and became raised up, as two cords forming a ridge under the skin. These two fingers will remain contracted and drawn forever” (Elliot 1988).

Plater, according to a translation of the Latin text by Belusa at al (1995), had in his anatomic studies dissected the palmar fascia as “tendons” arising from palmaris longus, superficial to the flexor tendons and should therefore have described the palmar fascia, not flexor tendons, in the above text.

Descriptions of this disease have previously been ascribed to Sir Henry Cline (1777), Cooper (1822) and later Dupuytren (1832) (reviewed by Elliot 1988). Cline dissected two cadaveric hands with Dupuytren’s contracture in 1777, noted the effect of dividing the involved palmar fascia and proposed an operative cure in the form of fasciotomy. Cooper in 1822 in addition to fasciotomy described splintage as a potential post-operative treatment and Dupuytren described treatment by fasciotomy, open palm and splintage (Elliot 1988), whilst Goyrand (1834) advocated fasciectomy (McGrouther 1988). As recurrence became a recognised problem and the possibility of more extensive surgery a reality, more aggressive techniques were employed and today the discussion continues as to which is the superior method (McGrouther 1988).

Despite the optimal treatment of Dupuytren's disease having been discussed for centuries a further understanding and therefore better chance of optimising treatment should include the biological processes in addition to surgery.

1.2. Incidence, prevalence and aetiology

Few epidemiological studies have been performed with regards to Dupuytren’s Contracture. According to Bunnell (1944) and Enzinger and Weiss (1995) the disease has an incidence of 1-2 % in the general population, but affects 20 % in the over 65 year
old age group, although the source of this data is not reported. Mikkelsen (1972) examined over 15,000 Norwegians and found Dupuytren's disease in 10.5% of men and 3.1% of women, with the prevalence peaking in men at 70 and for women at 80 years. In a highly selected series the female to male ratio has been quoted as 1:4.9 and the age of onset for men: 54.4 and for women: 60.2 years by McFarlane (1990).


Hueston (1985) proposed two hypotheses to explain the role of the palmar aponeurosis in the production of Dupuytren's disease. The intrinsic theory is based upon the proposition that changes within the normal pre-existing palmar aponeurosis fibres lead to formation of hypercellular nodules and hypertrophic bands. The extrinsic theory states that tissues overlying the anterior aspects of the palmar aponeurotic structures, between the aponeurosis and the dermis, produce a shortening of the aponeurotic structures by secondary involvement of these structures and the development of bands is secondary to the tension produced within them. These concepts however do not incorporate cellular or molecular mechanisms or the means by which these may be modified by physical forces.

Hueston furthermore made the suggestion that the main geographical distribution was well matched to the extent of the Viking invasions and concluded therefore that the prevalence of the disease was far greater in the Scandinavian and North European countries than elsewhere, indicating a strong hereditary element (Enzinger and Weiss 1995).

Ling (1963) examined 832 relatives of 50 patients with Dupuytren's disease and concluded that a single autosomal dominant gene, was likely to be involved but that Dupuytren's disease probably was not a homogenous condition in a pathogenic sense. A cytogenetic study (Wurster-Hill et al 1988), performed on Dupuytren nodules, found chromosomal abnormalities and instability, also present in apparently non-diseased
palmar fascia from the same patients, indicating a widespread distribution in the local tissues.

Epidemiological studies are few in number in the modern literature and despite the disease having been described and treated for centuries the aetiology remains elusive.

1.3. Clinical picture

The typical picture of Dupuytren's disease has been described as consisting of a palmar nodule, cord and digital flexion contracture. If only presented with an isolated palmar nodule the diagnosis is made more secure by the presence of pitting over the mass, disease in the opposite hand, plantar nodules, knuckle pads and Peyronie's disease or a strong family history (reviewed by Smith 1991).

The nodule is considered the pathognomonic manifestation and is typically found just distal to the distal palmar crease in line with a digital ray or on the digit over the proximal phalanx, adherent to the overlying skin. It can sometimes be tender, especially in phases of rapid increase in size. A more intimate attachment develops to the skin and a distortion of the palmar flexion crease is thought to precede the development of the cord. The cord may or may not end in a nodule, in the direct axis of the ray and is presumed to cause contracture of the digits (McGrouther 1982).

Knuckle pads or Garrod's nodes are variable manifestations of Dupuytren's disease, the incidence quoted as ranging from 1-2% (Enzinger and Weiss 1995) to 44% (Skoog 1948) and 45% for men and 62% for women operated on for Dupuytren's disease (Mikkelsen 1977). Other associated diseases proposed are Plantar fibromatosis or Lederhose's disease (5-20%), and Peyronie's disease (2-4%), a contraction of the penile fascial envelope (Hueston 1984, Enzinger and Weiss 1995).

The disease has been quoted as bilateral in approximately 50% (Skoog 1948, Hauer and Wilhelm 1978, McFarlane et al 1990). Skoog (1948) and Mikkelsen (1972) reported the ring, little, middle, index and thumb to be affected in descending frequency, whilst McFarlane et al (1990) found the little finger to carry the highest incidence, though all indicate that the ulnar side of the hand is most commonly involved.

The lesions of Dupuytren's disease are not random but have a specific appearance in defined positions. Dupuytren's disease is still mistaken for other diseases of the hand and therefore does not receive appropriate medical attention. With a better general knowledge of the presentation of the disease this could be rectified.
2. Anatomy and Pathology

2.1. Macroscopic

In order to appreciate the distribution of disease in the hand of a patient with Dupuytren's disease, a basic knowledge of the anatomy is necessary. The pathognomonic lesion in Dupuytren's disease has been described as a nodular thickening of the palmar aponeurosis (Skoog 1948). According to Caughell et al (1988) both transverse and longitudinal layers of the palmar aponeurosis and the palmaris longus tendon are present and in continuity at 5 weeks of gestation, whilst vertical fibres (McGrouther 1982) become apparent at 12 weeks. The fascia in the palm and fingers is proposed to contain a 3-dimensional ligamentous interweaving of longitudinal, transverse and vertical fibres (McGrouther 1982, McGrouther 1990).

According to McFarlane (1985) the normal structures which have been observed to become involved in Dupuytren's disease are: the palmar aponeurosis, the natatory ligament and the hypothenar fascia. The palmar aponeurosis is described as reaching each digit as pretendinous bands inserting into the palmar skin just beyond the level of the metacarpophalangeal joints. The natatory ligament is described as passing across the palm at the level of the web spaces, attaching to each fibrous tendon sheath as well as the lateral digital fascia (Tubiana and De Frenne 1976) and the hypothenar fascia as a condensation along the ulnar border of the hand (Barton 1984, White 1984). According to McFarlane (1974, 1985), who dissected 69 digits in 50 consecutive operations for Dupuytren's disease, the digital fascia is made up of longitudinal, oblique and transverse fibres, condensed into named bands. The pretendinous bands have been described with the palmar fascia. The lateral digital sheet is thought to arise from a continuation of fibres from the natatory ligament, Cleland's and Grayson's ligaments and the spiral bands of Gosset. Cleland's ligament originates from the lateral part of the interphalangeal joint region and passes obliquely to the skin posterior to the neurovascular bundle. Grayson's ligament passes transversely from the fibrous tendon sheet, over the proximal and middle phalanges, to the skin superficial to the neurovascular bundle. The deep extension of Gosset or spiral band are fibres from the pretendinous band passing on either side of the metacarpophalangeal joint, deep to the neurovascular bundle, to reach the side of the digit (McFarlane 1990).
Luck (1959) proposed that cords represented a functional hypertrophy of fascial bands which were defined as normal longitudinal coalescence of fibrous material organised into recognisable, pliable, noncontracted structures. According to McFarlane (1974) the main diseased cords are: the pretendinous, central, spiral, natatory and lateral. The pretendinous cord stems from the pretendinous band and due to its superficial and central course in relation to the neurovascular bundles, it is not though to distort the normal course of the latter, but may cause metacarpophalangeal contracture. According to McGrouther (1990) the metacarpophalangeal joint is not necessarily affected by the pretendinous cord, but may instead be affected by spiral cords or the deeply penetrating fibres lateral to the metacarpophalangeal joint. McFarlane (1974) found that the central cord arose from an extension of the pretendinous cord and inserted into the bone, fibrous tendon sheath or skin of the middle phalanx. Section of this cord over the proximal phalanx usually permitted release of a contracture of the proximal interphalangeal joint. In 1985 McFarlane described the spiral cord as consisting of diseased portions of the pretendinous and spiral band, the lateral digital sheet and Grayson’s ligament. The term ‘spiral cord’ was suggested due to the spiral course of these fibres in relation to the neurovascular bundle, displacing this structure. Contraction of the natatory cord was seen to cause obliteration of the webspaces, so the fingers could not be separated. Finally the lateral cord was observed to consist of the diseased lateral digital sheet and possibly to cause contraction of the proximal interphalangeal joint.

![Diagram](image)

**Figure 1: The structures in the web-space involved in Dupuytren's disease.**

(McGrouther 1990)
A summary is presented in the table below which divides diseased cords according to the normal bands they are thought to have arisen from (MacFarlane 1985):

<table>
<thead>
<tr>
<th>Normal bands</th>
<th>Diseased cords</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretendinuous bands</td>
<td>pretendinuous cords</td>
</tr>
<tr>
<td>vertical septae of Legeau and Juvara</td>
<td>not affected</td>
</tr>
<tr>
<td>central fibrofatty tissue</td>
<td>central cord</td>
</tr>
<tr>
<td>deep extension of Gosset</td>
<td>spiral cord</td>
</tr>
<tr>
<td>superficial transverse ligament*</td>
<td>not affected in central palm</td>
</tr>
<tr>
<td>natatory ligament of Grapow</td>
<td>natatory cord</td>
</tr>
<tr>
<td>lateral digital sheet of Gosset</td>
<td>lateral cord</td>
</tr>
<tr>
<td>Grayson’s ligament</td>
<td>parts of central, spiral and lateral cords</td>
</tr>
<tr>
<td>Cleland’s ligament and retrovascular band</td>
<td>retrovascular cord</td>
</tr>
<tr>
<td>Landsmeer’s ligament</td>
<td>not affected</td>
</tr>
</tbody>
</table>

Table 1: Normal bands and diseased cords in Dupuytren's disease

*transverse fibres of the palmar aponeurosis (Nomina Anatomica, 36th ed.)

The above table differs from the same table published in 1974 by the same author (McFarlane 1974) in that the diseased cords described as arising from Cleland’s ligament consist of the spiral and lateral cords.

A knowledge of the anatomical pathology of the palmar fascia is essential for the rational surgical treatment of Dupuytren's contracture, so that the neurovascular bundles are not damaged and maximum correction of the contracture is accomplished. Pharmacological treatments in the future may require precise delivery at defined sites within the palmar fascial fibres requiring a detailed anatomical knowledge.

2.2. Microscopic

In order to explore adjuvant non-surgical modalities of treatment, the cellular pathology of Dupuytren's disease will be reviewed first. The most numerous cell of Dupuytren's disease is the fibroblast. According to Luck (Luck 1959) Dupuytren's disease can be considered in three phases: the proliferative, the involutional and the fibrotic, though due to the diversity of the disease the stages co-exist in different areas of the diseased palm. Luck (1959) described the proliferative phase as being characterised by proliferating fibroblasts and scanty extracellular material, manifesting itself as a nodule. Furthermore
the cells in the involutional and fibrotic phase were described to align themselves with the major lines of stress that passed through the nodules. As the cells became more mature and seemed smaller, their number decreased and the proportion of collagen increased. With complete involution in the residual or fibrotic stage the nodule disappeared, leaving only a focus of dense adhesions and a reactive proximal fibrous cord, which was almost acellular and tendon-like.

Enzinger and Weiss (1995) have classified Dupuytren's disease amongst the fibromatoses, but the early stage of the disease has been mistaken for a fibrosarcoma in the hand (Enzinger and Weiss 1995, Erdmann et al 1995, Meis-Kindblom and Enzinger 1996), due to apparently high mitotic rates. Allen (1977) also quotes an incidence in which review of the histology of a plantar lesion originally described as a spindle cell sarcoma, revealed plantar fibromatosis. In a clinicopathological study involving 42 patients suffering from Dupuytren's disease Ushijima et al (1984) found that fibroblasts from some nodules exhibited a vertically upward growth, invading the overlying skin. On a morphological basis Martini and Puhl (1980) observed that the border between cutis and underlying tissue ceased to be distinct in Dupuytren's disease, the subcutaneous fat being replaced by collagen which retracted the cutis, whilst collagen bundles in the non-diseased fascia exhibited considerable variation in diameter and structure.

In vitro, Azzarone et al (1983) found Dupuytren's fibroblasts displayed in vitro biological properties that were intermediate between those expressed by normal fibroblasts and sarcoma cells or cells derived from the nodule and transformed with SV40 virus. The Dupuytren derived cells were observed to form colonies in soft agar, aggregate spontaneously in suspension and require less Foetal Calf Serum to grow than normal fibroblasts.

In Dupuytren's disease the collagen production has been quoted to change, decreasing type I collagen, with an overall increase in the type III/I collagen ratio (Notbohn et al 1995, Fitzgerald et al 1995). Other biochemical changes observed include a higher proteoglycan content, lysyl (a residue)-overmodification of collagen I, an increase in fibronectin content and reduced crosslinking of the tissue compared to normal aponeurosis tissue (Notbohn et al 1995). Ushijima et al (1984) found vascular features within Dupuytren nodules consisting of one or more layers of concentrically arranged fibroblasts with oval nuclei, surrounding the vessels. Neumüller et al (1988) found hyperplasia of the endothelium and multiplication of pericytes, in the vicinity of myofibroblasts and mast cells and Murrell et al (1989) observed completely or partially
occluded capillaries in the nodules. Chiu and McFarlane (1978) suggested that the cell dominating the early phase is the perivascular fibroblast.

Pasquah-Ronchetti et al (1993) compared aponeurotic tissue from seven normal subjects and biopsies from apparently unaffected aponeurosis, nodules and cords from 16 Dupuytren patients by optical and electron microscopy, immunoreactions for collagen type I and III, plasma fibronectin, biglycan, decorin, vitronectin, tenascin, elastin and α-smooth muscle cell actin. On the basis of these investigations, they suggested that there seemed to be no relation between clinical stage and structural alterations. Furthermore they proposed that the apparently non-diseased fascia was already affected by the pathological processes, because they found the cells exhibited a phenotype different from controls and similar to that found in nodules and fibrotic cords. 

*The cellular pathology of Dupuytren's contracture thus appears to be characterised in the early stages by fibroblast proliferation and progresses through biochemical and physical changes in the palmar fascia producing contraction.*

### 3. Regulation of the cell-cycle

#### 3.1. C-myc

The c-myc gene has been located on chromosome 8(q24) and encodes for two proteins of between 62 and 64 kDa molecular weight (Minks et al 1992). The protein is active within the nucleus and it functions as a sequence-specific transcription factor, specifically binding to nucleotide sequences in the DNA and as a heterodimer with other proteins regulates gene transcription (Shi et al 1992).

C-myc proto-oncogene is a cell cycle regulator, directly involved in oncogenesis according to Lewin (1991) and Müller-Ladner et al (1995) regarded it as the initial trigger for a variety of malignant transformations e.g. malignant myeloma, as one of the initiators of lung, cervical and squamous carcinoma of the head and neck, in synovium in rheumatoid arthritis and in Sjögren's disease. Grover et al (1997) showed it to be a prognostic survival marker for malignant melanoma.

Reed (1994) found that c-myc mediates control of cell-cycle progression, proliferation and apoptosis and according to Marcu et al (1992) its expression is transiently induced in the G1 phase by a variety of stimuli in quiescent fibroblasts. C-myc is believed to facilitate cell cycle progression by initiating the first step of the proliferative response, termed competence (Pledger et al 1977). In proliferating cells Hann et al (1985) and
Thompson et al (1985) found c-myc to be expressed at a constant rate throughout the cell-cycle, but Campisi et al (1984), Dean et al (1986) and Waters et al (1991) showed that in quiescent fibroblasts it was not detectable. Cells deprived of growth factors have not been found to express c-myc (Dean et al 1986, Waters et al 1991), but transformed cells are less dependent upon growth factors and cell cycle control is lost following chemical transformation of fibroblasts according to Campisi et al (1984).

Serum starvation in cells overexpressing active c-myc resulted in cell death by apoptosis (Wagner 1993 and 1994, Evan et al 1992), but in some cells this required the p53 tumour suppressor protein to be present (Lenahan and Ozer 1996). A study by Muller et al (1996) found no p53 protein in Dupuytren tissue.

Expression of an exogenous c-myc gene renders fibroblasts unable to exit from the cell cycle upon withdrawal of growth factors or serum. Instead, these cells continue cycling and concomitantly undergo apoptosis. These observations lead to the suggestion of a model in which proliferation and cell death were processes co-induced by c-myc (Evan et al 1992, Reed 1994) and modulated by cytokine action (Evan and Littlewood 1993, Harrington et al 1994). Waters et al (1991) do not mention apoptosis and imply that a fall in c-myc protein levels at any time of the cell-cycle, due to mitogen depletion or the action of anti-proliferative cytokines, merely causes the cell to arrest at the next available opportunity i.e. the G1 in the case of fibroblasts. The review by Evan and Littlewood (1993) suggested that the c-myc protein induces both proliferation and apoptosis in cells under different conditions. To accommodate these apparently contradictory attributes of c-myc, it was suggested that the proliferative and apoptotic pathways are coupled or overlapping processes. Once established, cell growth and cell death are then independently modulated by other genes, cytokines and external factors. C-myc is induced by mitogens and cell proliferation is inhibited either by a withdrawal of mitogens or by the direct effect of cytostatic factors. C-myc induced apoptosis is inhibited by the anti-apoptotic bcl-2 gene or specific cytokines (Harrington et al 1994).

3.1.1. Flowcytometry

C-myc is assayed by flowcytometry.

Flow cytometry quantifies substances in minute quantities, assays intracellular components and sorts cells (reviewed by Carter and Meyer 1994). The principle of flowcytometry is based on the measurement of fluorescence from labeled cells, proteins or other macromolecules. In flow cytometry a line of labeled particles, usually cells or
nuclei, are delivered into a beam of laser light, one at the time, by virtue of "hydrodynamic focusing" within a fluid system. Fluorochrome markers, incorporated into a cell or protein, are excited by the laser and emit light of a specific wavelength. Therefore, the amount of substance, or the number of cells are expressed as a function of their fluorescence. Measurements are taken of each individual particle within the suspension and not as an average of the whole population.

As all particles are accounted for in the flowcytometer and contribute to the overall picture it is important, that the sample should be well prepared to minimise contamination. The target protein or cell requires a highly specific label, usually a monoclonal antibody or dye, which is labeled with a fluorochrome tag, such as Fluorescein Isothiocyanate (FITC). Several parameters can be simultaneous assayed, using different fluorochromes, but emission spectra must be different to avoid spectral overlap.

Particles are orientated within a flow chamber to intersect with the laser by hydrodynamic focusing, most commonly using laminar flow with viscous drag (Carter and Meyer 1994); fluid at the centre of a moving channel flows more rapidly than that in contact with the walls of the chamber, which is slowed by viscous drag. The flow at the front therefore assumes the form of a parabola. As a result of this velocity gradient particles are drawn towards the centre by hydrodynamic focusing to form a stable parabola, before entry into the detection point.

3.1.2. Detection systems.

Fluorochromes emit light of specific wavelength in response to intense light stimulation (reviewed by Ormerød 1994). Emitted light is scattered and focused prior to collection on photodetectors that convert light energy into electrical signals. In order to distinguish the output of several combined fluorochromes, light is optically filtered into separate bands of differing wavelengths. This is achieved by dichroic mirrors, interference phenomena and absorption filters, which selectively deflect or allow passage of various pulses of light, depending on their wavelength. Finally, separated signals are processed and digitally converted prior to graphic display on a screen. The output is analysed via computers.

C-myc stimulates mitogenesis and apoptosis and concomitant activation of bcl-2 can nullify the apoptotic influence of c-myc, thus unleashing the proliferative effects of c-myc, leading to a further selective growth advantage (Reed 1994). This was
3.2. Proliferation

C-myc induces proliferation, which is defined as the increase in cell number resulting from completion of the cell cycle, as opposed to growth, which is the increase in cell mass (reviewed by Pardee 1989). The G1 phase (Gap1) represents the start of the cycle. Once stimuli are received, quiescent cells may move from a G0 phase into G1, though this distinction is arbitrary. Once the cell passes the "start" point it is committed to divide and proceed to the S phase (synthesis phase). During this time the necessary metabolites for DNA and chromosomal synthesis are produced and the genome replicated. The S phase is followed by a second period of relative inactivity, denoted the G2 (Gap 2) phase in which the cell contains a tetraploid number of chromosomes. The cell then undergoes mitosis, producing two cells and restores the normal diploid number of chromosomes. The progeny can either re-enter the cycle at the G0/G1 phase, proceed to differentiate or apoptose. Extracellular factors will determine whether a quiescent cell will begin to proliferate and also whether a normal proliferating cell in G1 will continue to cycle or revert to quiescence according to Pardee (1989).

![Cell Cycle Diagram](image)

Figure 2: cell cycle (Ham and Cormack 1979)

3.2.1. Mib-1 antibody

The monoclonal antibody Mib-1 is regarded as a marker of proliferation (Lopez et al 1994) and reacts with the Ki-67 antigen, a nuclear protein expressed in all phases of the cell cycle except G0 according to Diaz-Cascajo et al (1995) and early G1 according to...
Gee et al (1995). DAKO Rabbit Anti-Human Ki-67 Antigen is an affinity isolated antibody, the affinity isolation being performed using immobilized Ki-67 peptide, deduced from a 62 base pair region of the human Ki-67 gene, which is presumed to encode for the Ki-67 epitope (Gerdes et al 1991). The antibody is supposed to work on cryostat sections and formalin fixed, cytopsins and paraffin embedded tissue sections. In a study comparing Ki-67 measurement of paraffin sections and frozen (Ki-Froz) sections of breast cancer, paraffin sections were found to stain stronger than frozen sections (Gee et al 1995) and therefore subsequent staining for Mib-1 was performed on paraffin sections.

Oshiro et al (1995) showed that the Mib-1 labeling index (percentage of positive cells of more than 500 cells) for atypical fibroxanthoma, regarded as a fibrohistiocytic tumour with intermediate potential, to be lower than the frankly malignant fibrous histiocytoma and higher than the benign fibrous histiocytoma thereby linking proliferation with malignancy. Diaz-Cascajo et al (1995) utilised Mib-1 to compare the malignant counterpart of a benign tumour (dermatofibrosarcoma protuberans and dermatofibroma) but did not find any difference in proliferation rate between the tumours. Geisler et al (1995) however showed a change in mib-1 positivity between primary and recurrent epithelial ovarian cancer, with a proliferation index of 4.1% for the primary cancer and 38.73 % for the recurrent in one case. This proliferation index was also proven useful as a prognostic marker in soft tissue sarcomas (Takafumi et al 1989, Choong et al 1995) and Mib-1 positivity in pre-treatment biopsies in high grade soft tissue sarcoma has been shown to correlate with post-treatment response and is significantly reduced following neoadjuvant therapy (Randolph et al 1995).

The pathognomonic lesion of Dupuytren's disease is considered to consist of the nodule (McGrouther 1982) which contains proliferating fibroblasts (Luck 1959) as reviewed previously. It is therefore proposed that proliferation of the palmar fascia fibroblasts is the disease process which ultimately leads to Dupuytren's disease. The Mib-antibody was utilised in experiments assessing the proliferative index in Dupuytren's disease in the following chapters.

3.3. Apoptosis and protection against apoptosis (Bcl-2)

Apoptosis is a form of cell death with a different morphology to necrosis (Wyllie et al 1980) and is an intrinsic death programme operating to eliminate unwanted cells during normal development (Kohlhuber et al 1995). Wyllie (1998) has suggested an apopto-
specific pathway initiated by caspase cleavage, which terminates in activation of a
nuclease responsible for internucleosomal digestion of DNA. This endonuclease is
restricted to the process of apoptosis but not to any particular target sequence, which
results in the effective destruction of DNA.

The active death of many mammalian cell types occurs by apoptosis and most authors
use “programmed cell death” and “apoptosis” as synonymous (Lenahan and Ozer 1996,
1991), but some authors are of the opinion that this is incorrect (Williams et al 1992).
Apoptosis apparently does not elicit an inflammatory response like necrosis in which cells
die as a result of injury (Williams et al 1992, Kohlhuber et al 1995).

The size of any particular population of cells can be controlled by changes in the rate of
cell death, as well as the rates of cell differentiation and cell division. The biological
activity specifically associated with the population can therefore also be influenced by
modulation of cell death. In some cells, expression of the proto-oncogene bcl-2 inhibits
cell death and this is exploited in physiological regulation according to Pezzella and

It has been suggested that inappropriate expression of bcl-2 is an important factor in
oncogenesis (Williams et al 1992, Reed 1994) and according to Hockenbery et al (1990)
produces extension of cell survival when overexpressed. Expression of transfected bcl-2
in myeloid pre-cursor cells has been shown to promote cell survival on removal of
cytokine, which normally leads to apoptosis (Williams et al 1992). Bcl-2 has been found
in cell lineages and tissues (Hockenberry et al 1991), characterised by apoptotic cell
turnover and restricted to the long-lived progenitor cells that renew these lineages and
select post-mitotic cells requiring an extended life-span, but a number of tissues have
been found to lack bcl-2 reactivity: liver, lung, heart, kidney, cervix, myometrium, testis,
ovary and bladder.

3.3.1. Bcl-2 antibody

The expression of the bcl-2 gene, signifying apoptotic inhibition was measured utilising
the bcl-2 antibody. The bcl-2 protein is associated with mitochondria and plays a central
role in the inhibition of apoptosis (Hockenbery et al 1990, Pezzela and Gatter 1995, Liu
1991, Korsmeyer 1992). The bcl-2 gene is encoded for by a gene involved in the t(14;18)
chromosomal translocation (Pezzella et al 1990). This cytogenetic abnormality, which is
frequently found in human follicular lymphoma, brings the bcl-2 gene into juxtaposition
with the immunoglobulin heavy chain gene and causes overexpression of bcl-2 (Hockenberry et al 1990, Pezzella and Gatter 1995). The 14;18 chromosomal translocation is not a prerequisite for bcl-2 protein expression since this occurs in many cases without this rearrangement (Pezzella et al 1990). The bcl-2 protein (molecular mass 25kDa) is an integral membrane protein which lies within the cell rather than on the cell surface (Hockenberry et al 1990, Liu et al 1991). The monoclonal mouse antibody is raised against a synthetic peptide comprising amino acids 41-45 of human bcl-2 protein (Pezzella and Gatter 1995) and delivered as dialysed tissue culture supernatant.

If considered in conjunction with c-myc, which influences proliferation and apoptosis, reviewed previously, activation of the bcl-2 gene can counteract the apoptotic influence of c-myc. This unleashes the proliferative effects of c-myc, which leads to a selective growth advantage. This theory was the basis of the hypothesis proposed and investigated for Dupuytren's disease in this thesis. The Bcl-2 antibody was utilised in assessing the level of protection against apoptosis in Dupuytren, non-diseased fascia, carpal ligament and Fibrosarcoma specimens in the following chapters.

### 3.4. Growth factors

As described earlier the model suggested for c-myc regulation of proliferation and apoptosis (Evan et al 1992, Reed 1994) was shown to be modulated by cytokine action (Evan and Littlewood 1993, Harrington et al 1994). Therefore the major growth factor implicated in Dupuytren's disease, Transforming Growth Factor beta (TGFβ), is reviewed below.

Growth factors are naturally occurring peptides which influence cellular proliferation and differentiation in vitro and in some models in vivo. The major cytokine released by platelets, macrophages and T lymphocytes in wound healing is TGFβ, which influences fibroblast proliferation, chemotaxis, collagen metabolism, indirect angiogenesis and action of other growth factors (Sporn and Roberts 1992, Diegelman 1997).

Kloen et al (1995) and Alioto et al (1994) have linked TGFβ to disease processes which are characterised by excessive fibrosis, especially Dupuytren's disease. Roberts et al (1985 and 1986) have suggested that the increase in extra cellular matrix deposition is caused by TGFβ. Staining for TGFβ has been shown to be increased in the early stages of Dupuytren's disease, i.e. in nodules but not in cords (Zamora et al 1994), though Badalamente et al (1996) found TGFβ₁ staining in fibroblasts, myofibroblasts and...
capillary endothelium in Dupuytren’s disease regardless of the disease stage. Berndt et al (1995) found that synthesis of TGFβ was restricted to the proliferative foci, but that TGFβ proteins could be detected in the surrounding apparently non-diseased fascia, perhaps an indication of recruitment of neighbouring quiescent fibroblasts in the fibromatous tissue formation. Kloen et al (1995) furthermore found two types of TGFβ receptors in Dupuytren fibroblast cell cultures obtained by explant from patients with a minimum of 30° of metacarpophalangeal joint contracture. Type I receptor was expressed less or had a lower affinity for TGFβ₁ than type II receptor in Dupuytren cell cultures, whilst there was no difference in binding to these two receptor types in normal fibroblasts.

In Diegelman’s (1997) review TGFβ is cited to have a number of effects on extracellular matrix deposition: increasing the transcription of genes for collagen, proteoglycans and fibronectin, decreasing the production of proteases responsible for break down of the matrix and stimulating the protease inhibitor, tissue inhibitor of metallo proteinase (TIMP). More specifically, intracellular TGFβ₁ staining has been shown to be associated with fibroblasts, myofibroblasts and capillary endothelium in Dupuytren samples, regardless of the disease stage. The latter observation is in contrast with previous findings (Zamora et al 1994, Berndt et al 1995). TGFβ₁ has been shown to induce myofibroblast differentiation in vitro, especially in experiments with a high cell plating density (Badalamente et al 1996) and to stimulate collagen production (Alioto et al 1994, Häkkinen et al 1996) and cell proliferation (Badalamente et al 1992). It has been suggested that the mitogenic effect of some interleukins (IL-2) are potentiated by TGFβ and other growth factors and that a combined expression of several such factors (Fibroblast Growth Factor, TGFβ, Platelet Derived Growth Factor α, Platelet Derived Growth Factor β) is necessary to sustain the chronic fibroblast proliferation and matrix production seen in Dupuytren’s disease (Baird et al 1993). Indeed TGFβ has been shown to activate connective tissue growth factor, suggesting a cascade process controlling tissue repair, especially in fibrotic conditions (Igarashi et al 1996). It has been recognised that the mitogenic effect of TGFβ₁ involves an indirect loop involving the production of PDGF (Kloen et al 1995).

Other growth factors have been suggested in Dupuytren's disease, such as platelet derived growth factor (Badalamente et al 1992, Terek et al 1995), basic fibroblast growth factor (Baird et al 1993, Lappi et al 1992, Gonzalez et al 1992), epidermal
growth factor (Kloen et al 1995) and connective tissue growth factor, the latter being produced by fibroblasts after activation by TGFβ (Igarashi et al 1996). Pietenpol et al (1990) showed that TGFβ1 suppressed growth in skin keratinocytes, by reducing c-myc expression and thereby reducing proliferation (Pietenpol et al 1990). TGFβ1 has been shown to be an important factor in woundhealing and Dupuytren's disease, influencing myofibroblast differentiation and extracellular matrix deposition.

3.5. Cellular differentiation

As discussed it has been suggested that the action of TGFβ1 influences the proliferation and differentiation of fibroblasts, the most numerous cell in Dupuytren's disease and therefore the effect of TGFβ1 on these properties was investigated in vitro in the following chapters.

The origin of the fibroblast responsible for Dupuytren's disease has been heavily disputed. In a review Murrell (1992) suggested that the Dupuytren fibroblast was identical to those native to the hand in the palmar fascia and that the major phenomenon in Dupuytren's disease was an increase in proliferating fibroblasts rather than an alteration in the type of fibroblasts. Fitzgerald et al (1995) stained Dupuytren and dermatofasciectomy specimens with Herovici's picropolychrome stain, which differentiates between collagen type I and III and found that in Dupuytren's disease the proportion of collagen type III diminished from the papillary dermis through the reticular dermis and subcutaneous tissues down to the nodules and finally the cords. Therefore Fitzgerald et al (1995) suggested that the fibroblasts producing collagen type III were migrating from the dermis to the palmar fascia and that possibly these fibroblasts in some undefined way influenced the palmar fascia, as suggested by Hueston in his extrinsic theory (1984). Ushijima et al (1984) have implied that the vertically upward invasive growth directly from the main nodular lesion, as previously reviewed under the microscopic description of Dupuytren's disease, cause fixation of the overlying skin in the early stage of the clinical course, though this could potentially also be interpreted as vertical growth downwards from the dermis, resulting in a nodule. McCann et al (1993) have speculated whether the cells which lie in the palmar dermis of dermatofasciectomy specimens are myofibroblasts. This would provide an explanation for the low recurrence rate after this operation (Brotherson et al 1994), especially since the presence of
myofibroblasts has been linked to recurrence (Gelberman et al 1980, Salamon et al 1997).

To date no consensus has been reached as to the origin of the Dupuytren fibroblast or whether it is the myofibroblast which initiates or causes Dupuytren's contracture.

3.6. Myofibroblasts

Gabbiani and Majno (1972) described fibroblasts acquiring certain of the morphological and biochemical features of smooth muscle cells and named them myofibroblasts. Later (1995) they described a specific antibody test based on the expression of alpha smooth muscle actin, the actin isoform typical of vascular smooth muscle cells. The distribution of myofibroblasts is widespread (Murrell 1992, reviewed by Schürch et al 1992): in the spleen, lymphnodes and Wharton's jelly, in granulation tissue and in pathological tissue, such as liver cirrhosis, burn contractures, fibromatosis (including Dupuytren's disease) and in carcinomas characterised by desmoplastic stromal reaction, such as invasive ductal mammary carcinomas and annular stenosing colon carcinomas. Myofibroblasts share morphologic features with both fibroblasts and smooth muscle cells (reviewed by Schürch et al 1992). Fibroblasts have fusiform nuclei, well developed Golgi apparatus, dilated rough endoplasmatic reticulum cisternae, scattered mitochondriae and microfilaments. Fibroblasts are smooth and have few cytoplasmic extensions. Plasmalemma attachment plaques, basal lamina, pinocytic vesicles, intercellular junctions and cell-to-stroma attachment sites are absent.

In contrast, smooth muscle cells are enveloped by a continuous basal lamina, have numerous plasmalemmal attachment plaques, pinocytic vesicles, intercellular gap and adherent junctions. There is an abundance of parallel orientated microfilaments along the length of the smooth muscle cells, interspersed with dense bodies and bands, comparable to Z-lines of striated muscle. In both of these structures alpha smooth muscle actin has been demonstrated (Schollmeyer et al 1973, Schollmeyer et al 1976). According to Gabella (1984) force transmission within the smooth muscle cells, from the contractile apparatus to the cell membrane, occurs via the insertion of bundles of actin filaments into the dense bands and transmitted from cell to cell by adherent or intermediate junctions.

Myofibroblasts have long cytoplasmic extensions, are connected by intermediate, adherens or gap junctions, are partly enveloped in a basal lamina with plasmalemmal attachment plaques and pinocytic vesicles (Singer 1979, Singer et al 1984) in contrast to fibroblasts, whilst the fibronexus, transmembrane microfilaments, connect the cell with
extracellular fibronectin fibres. These cell-to-stroma attachments are numerous in the myofibroblast, which also contain a high amount of microfilament bundles, so called stress fibers, orientated along the long axis of the cell and in continuity with plasmalemmal attachment plaques. Franke and Shinko (1969) found that the myofibroblast, possesses a well developed rough endoplasmatic reticulum and Golgi apparatus in common with fibroblasts, but the nucleus of the myofibroblast exhibits numerous indentations, indicating cellular contraction. Morphologically myofibroblasts posses many long cytoplasmic extensions and are usually larger than fibroblasts.

Myofibroblasts have been shown to produce a wide range of extracellular matrix proteins like fibronectin, tenascein, collagen type IV and laminin. Laminin was previously thought to be unique to smooth muscle cells (Berndt et al 1994). Myofibroblasts also co-express TGFα and epidermal growth factor receptor in the proliferative and involutional phase suggesting an autocrine growth stimulation (Magro et al 1997). Of special interst is the suggestion that myofibroblasts produce collagen type I, III and V (Gabbiani et al 1976).

In granulation tissue myofibroblasts are numerous and the collagen type ratio (type III>type I) is similar to that found in early Dupuytren's disease. As granulation tissue disappears so do the myofibroblasts and the predominant collagen becomes type I (Rudolph et al 1977), as in late Dupuytren's disease (Schürch et al 1992). Fitzgerald et al (1995) demonstrated an increased ratio of type III to type I collagen within the palmar fascia even before clinical symptoms and signs appeared. The myofibroblast is prominent at different stages of Dupuytren's disease as divided by Luck's classification (Luck 1959): it is very prominent in the proliferative (stage I), less so in the involutional (stage II) and almost if not completely absent in the residual (stage III). The cells also change with regards to gap junctions, which in the residual phase are completely absent (Schürch et al 1992). Myofibroblasts have been found in the apparently non-diseased fascia of Dupuytren patients together with high levels of collagen type III (Bazin et al 1980), indicating that the disease is more widespread within the palmar aponeurosis than just the visible involved areas.

In experiments cloning fibroblast cultures, by trypsinising cultures and plating individual cells separately, from fetal rat tissue, adult rat dermis, mouse embryo (3T3), human embryo lung (ICIG-7), Dupuytren's nodule and mammary gland and assessing these cultures for α-smooth muscle actin, Desmoulière et al (1992) found that cloning fibroblast cultures in this manner always resulted in populations containing a proportion
of α-smooth muscle actin positive cells. When fibroblasts metamorphose into myofibroblasts, they synthesise, like stimulated macrophages, very high amounts of plasminogen activators, which could be taken as a biochemical sign of evolution of contracture (Merlo et al 1987). Myofibroblast phenotype can be determined by the measurement of intracellular actin content and fibroblast:globular actin ratios (Foo et al 1992) and four different main phenotypes of myofibroblasts have been described using cytoskeletal markers (Desmoulière and Gabbiani 1995).

Gabbiani et al (1971) showed that granuloma pouches in male Wistar rats stained with double-layer immunological methods utilising Human anti-smooth muscle sera and fluorescent sheep anti-human IgG exhibited cytoplasmic labeling in a few cells at day 7, to reach a maximum at day 20-30, when fluorescent cells were widely distributed through the whole wall, with the exception of the innermost layer, which contained mainly polymorphs and macrophages. Later, as the granulation tissue became replaced by dense collagen, the outermost layer of the wall lost its fluorescence.

According to a review by Desmoulière et al (1995) myofibroblasts are poorly developed in early granulation tissue, most numerous during the phase of wound contraction and progressively disappear in the scar. Desmoulière et al (1995) therefore suggested that the myofibroblastic phenotype either reverted to a quiescent form when the wound was closed or that myofibroblasts disappeared selectively through apoptosis. Because myofibroblasts have been found in excessive scarring (hypertrophic scars) and under fibrotic conditions (Schürch et al 1992) it could be hypothesised that the persistent presence of myofibroblasts is necessary for pathological scarring to occur. Examining skin and underlying cord obtained at dermofasciectomy for Dupuytren's disease McCann et al (1993) found α-smooth muscle actin in areas of hypercellular Dupuytren tissue extending into the overlying dermis and even reaching the dermis, apart from diffusely distributed α-smooth muscle actin positive cells separate from the Dupuytren foci. This lead to the suggestion that myofibroblasts within the dermis could explain the high recurrence rate after fasciectomy.

Myofibroblasts have been implicated as leading to contracture (Gabbiani and Majno 1972, Gabbiani et al 1972, Gabbiani 1994) after granulation tissue was observed to contain myofibroblasts and react similar to smooth muscle tissue when tested pharmacologically in vitro. Tomasek and Rayan (1995) showed, by determining the proportion of cells expressing α-smooth muscle actin in Dupuytren fibroblasts cultures
and assessing their capacity for contraction of free-floating collagen lattice contraction, that the acquisition of myofibroblast phenotypes correlated with increased contractility in Dupuytren's disease.  

*It has been suggested that myofibroblasts differentiate from fibroblasts and cause contraction in Dupuytren's disease.*

### 3.7. Collagen gel

Tissue contraction can be measured by the superfusion isometric recording system (Naylor et al 1995) using the whole tissue specimen to be examined. However this presents problems of tissue viability (Irwin et al 1997). Bell et al (1979) developed an in vitro model for investigating contractile properties using three dimensional collagen gels and showed that the shrinkage of fibroblast populated collagen lattices was directly proportional to the cell number and required protein synthesis, intact microfilaments and microtubules. It was apparently also dependant on which collagen was used to produce the lattice as it was found that type III contracted more than type I, which in turn contracted more than type II (i.e. III>I>II) (Ehrlich 1988).

Ever since this model for contraction was developed it has been speculated whether the lattices contract by cell locomotion or cell contraction. ‘Tractional’ forces have been suggested in the reorganisation and alignment of collagen substrates, as cells move during development or repair (Stopak 1982). Ehrlich and Rajaratnam (1990) analysed free floating collagen lattices and found that fibroblasts remained fibroblasts at the center of the gel, whilst they underwent metamorphosis to myofibroblasts at the periphery. Comparing the contraction of the center with the periphery they found no difference in rate of contraction after 48 hours. Therefore they proposed the theory that contraction in a collagen lattice was due to cell locomotion: Fibroblasts reorganise the collagen and therefore contract the lattice. They also found that fibroblasts in the center of the gel were disorganised with very few cell contacts, as opposed to the myofibroblasts at the periphery. Rittenberg and Ehrlich (1992) found that contraction was enhanced by the availability of fatty acid, without altering cell numbers or the microtubular contraction and concluded that since the fatty acid composition of the fibroblast plasma membrane influences the membrane fluidity, this supported the theory that the mechanism for lattice contraction is by cell locomotion, rather than cell contraction. According to Guidry and Grinnel (1986) the collagen fibrils are initially reorganised mechanically by the cells and the continued presence of the cells is required.
to maintain this arrangement, but with time the fibrils become stabilised by non-covalent bonding independent of the cells. The cell is supposed to interact with the surrounding collagen via the actin cytoskeleton (Tomasek and Hay 1984).

In contrast to these findings Tomasek and Rayan (1995) showed that the contraction of collagen gels was proportional to the number of myofibroblasts present in the gel, i.e. more myofibroblasts resulted in more contraction. It can perhaps be hypothesised that the process of lattice contraction is a combination of cell locomotion and cell contraction, rather than a mutual exclusion.

Exposing fibroblasts on collagen coated membranes to cyclic strain has been shown to increase the expression of platelet derived growth factor-A and perhaps thereby increase the proliferation (Alman et al. 1996) and therefore contraction. By video recording floating gels Nishiyama et al (1988) showed that the contraction has three phases: initial lag, rapid and slow contraction. Factors affecting gel contraction were found to be an increase in cell number, in culture medium or in serum concentration and for phase one: the collagen concentration.

The system of cells embedded in 3-dimensional gels provides a adjustable model, mimicking the in situ environment of cells according to Farsi (1984). It has however been shown that the distinct environment of a contractile collagen-matrix determines the susceptibility of normal primary fibroblasts to apoptosis, with a peak at day 2-4 (Fluck et al 1998). Greco and Erlich (1992) found that fibroblasts from primates were not seen to proliferate for three days in collagen lattices, despite an increase in DNA content and would therefore seem to have arrested in the G2 phase of the cell cycle. The total number of primate fibroblasts seeded into a collagen lattice decreased from 100,000 to 80,000 over two days. If released from the collagen, the primate fibroblasts doubled in number despite the presence of hydroxyurea (an inhibitor of DNA synthesis) and thus seemed to have completed the cell cycle previously arrested.

The effect of fibroblast contraction on collagen gels has been studied extensively, as a model for wound contraction (Guidry 1986, Ehrlich 1990, Finesmith 1990, Garana 1992, Tranquilo 1992) and forces generated by non muscle cells have been examined in a number of in vitro experimental models ranging from wrinkling of silicone membranes by fibroblasts (Stopak 1982), to optical measurement of collagen gel shrinkage (Nishiyama 1988, Khan 1997). The techniques are limited in that force generated by the cells is not measured directly, is highly substrate dependant and is often only semi-quantitative.

Machines have been developed to measure the cellular contractile forces accurately and
continuously in vitro, able to measure minute forces over protracted periods of time (Kolodney and Wysolmerski 1992, Eastwood et al 1993). The Culture Force Monitor developed by Eastwood et al (1994) allows precise quantitative values to be derived for the forces generated by the cells alone in culture and was used for experiments regarding Dupuytren fibroblast contraction in this thesis.

Measuring the effect of a potential treatment on the contraction of cells in vitro may suggest the potential clinical benefit.

4. Treatment

4.1. Current Treatment

The main thrust of treatment has centered on surgical intervention, though recurrence rates at the operated site remain high. Published series report recurrence rate with the range of 34-71% (Mayer et al 1986, Norotte et al 1988, Hoet F et al 1988, Romboutts et al 1989, Adam and Loynes 1992, Vigroux and Valentin 1992, Brotherson et al 1994, Tropet et al 1994, Foucher et al 1995, De Maglio et al 1996, Duthie and Chesney 1997). The options available for surgical treatment today are numerous, distinguishing themselves by different incisions, level of dissection and closure or skincover. This has been reviewed in detail by other authors (McGrouther 1990) and only a schematic overview will be given here.

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**Figure 3: Shematic representation of Dupuytren surgery**

Examples: In dermofasciectomy $\rightarrow$ an extensive dissection is performed and the wound is closed by a skin graft. In regional fasciectomy $\rightarrow$, the wound can be left open, closed directly, with z-plasties, a local flap if on the digit or grafted.

As recurrence has become a recognised problem, more extensive surgery and more aggressive techniques continue to be discussed.
4.2. Nonsurgical Treatment

The continuous extension of Dupuytren's disease prior to aponeurectomy was introduced by Messina (1989) (Messina and Messina 1991, Hodkinson 1994, Borchardt and Lanz 1995, Citron and Messina 1998). Bailey et al (1994) observed a change in the cross-link profile of the collagen, indicating an increase in newly synthesised collagen due to increased turnover. This was confirmed by gel electrophoresis which demonstrated an increase in the levels of degradative enzymes. This in turn led to the suggestion that the increase in enzyme activity was generated by tension on the fibroblasts of this metabolically active tissue produced during the continuous extension of the retracted fingers. Brandes et al (1994) examined the palmar fascia of patients after complete elongation of their Dupuytren's contracture using the continuous elongation technique with light and electron microscopy and compared these with non-elongated samples from patients at the same stage. The nodules in the non-extended palmar fascia predominantly contained myofibroblasts, some with a prominent cytoskeleton others with extensive rough endoplasmatic reticulum and Golgi apparatus. These cells were surrounded by a tight mesh of fine filaments, presumed to be proteoglycans and collagen fibrils, whilst away from the cells larger collagen fibres with an irregular outline in cross section. The cords in the non-extended palmar fascia were composed of large bundles of thick fibres, with collagen fibrils of varying diameter were densely packed in random directions. In contrast the palmar fascia after continuous extension was seen to consist bundles of large collagen fibres with cells, orientated according to the stretching forces, between. Electron microscopy revealed these cells to be fibroblasts and myofibroblasts with an extensive rough endoplasmatic reticulum and a large Golgi apparatus, suggesting increased biosynthetic activity. Since there was no histological evidence of either haemorrhage or interruption of collagen fibres, the increase in length of the fascia was suggested to be a dynamic biological process. The predominance of small unimodal fibres suggested their new synthesis and degradation of the thicker fibres with varying diameter. Therefore the hypothesis that the contracted palmar fascia reacted to external forces, such as the elongation technique, with neoformation and reorientation of all tissue components by myofibroblasts was suggested.

Nonsurgical treatment of polyfibromatosis include topical, intralosomal and systemic steroids, potassium para-aminobenzoate, penicillamine, methotrexate, minoxidil and
isotretinoin (reviewed by Lee et al 1996). Currently the injection of collagenase is being investigated by Dr M Badalamente et al at Stony Brooks Hospital, Long Island. Histamine is a mediator in the early phase of acute inflammation and has been found to increases the synthesis of collagen in wound-healing (Sandberg 1962). Fibroblasts cultures in the presence of histamirincreased the collagen production as shown by the increase in labeled hydroxyproline, but if H1/ H2 blockers, like chlorpheniramine and cimetidine, were added to the same cultures this increase was not seen (Hatamochi 1985). Boucek (1973) showed that serotonin, bradykinin and nor-epinephrine consistently enhanced fibroblast growth, which could potentially also be blocked by pharmacological means. Keilholz et al (1996) treated early Dupuytren's disease with orthovoltage radiotherapy, consisting of two courses of daily fractionation of 5 x 3 Gy, giving a total dose of 30 Gy) separated by a six week interval. Out of fifty-seven patients with a minimum follow-up of 5 years 77% experienced no disease progression, whereas 23% progressed (14% outside and 9% inside the irradiated field). In 1968 Aron found that chemotherapeutic treatment of patients with lymphoma resulted in regression of Dupuytren nodules, whilst established fibrotic bands persisted in patients who concomitantly suffered from Dupuytren's disease.

On the basis of Aron’s findings (1968) and due to the current use of 5-fluorouracil in Ophthalmic surgery, as discussed below, 5-fluorouracil was investigated in vitro as a potential adjuvant treatment for Dupuytren's disease.

There is a multitude of non-surgical treatments available, but none have replaced surgery as the main treatment and few have been tried in conjunction with surgery.

4.3. Proposed treatment (5-fluorouracil)

Fluorouracil is a pyrimidine antimetabolite that when converted to the active nucleotide, inhibits the enzyme thymidylate synthetase and thereby blocks DNA synthesis (reviewed by Skeel 1991).
5-FLUOROURACIL THYMINE

(Diasio and Harris 1989).

Figure 4: 5-fluorouracil

The primary indications (reviewed by Skeel 1991) for the use of systemic 5-fluorouracil are cancer of the: breast, colorectal, stomach, pancreas, oesophagus, liver, bladder and head and neck. Topical 5-fluorouracil is used for basal and squamous carcinomas of the skin (Skeel 1991). The systemic doses used are 500 mg/m² intravenously on day 1-5 every 4 weeks or 450-600 mg/m² intravenously weekly, intracavitary 500-1000 mg for pericardial effusion, 2000-3000 mg for pleural or peritoneal effusions and intraarterial (liver): 800-1200 mg/m² as a continuous infusion on day 1-4, followed by 600 mg/m² as a continuous infusion on days 5-21 (Skeel 1991). The main toxic effects at these dosages are myelosuppression, nausea and vomiting, mucocutaneous effects (including stomatitis, partial alopecia, hyperpigmentation, maculopapular rash, “hand-foot” syndrome with painful erythematous desquamation and fissures in the palms and soles) increased by sun-exposure and other miscellaneous effects such as neurotoxicity and increased lacrimation (Skeel 1991).

A study concerning the tissue distribution of 5-fluorouracil after intramural, intraluminal and intravenous administration for gastrointestinal cancer found that the best overall
concentration was found after intramural administration, intraluminal gave the lowest overall concentrations and intravenous gave levels between the two (Shukla et al 1977). This could indicate that intralesional application of 5-fluorouracil may be required for efficacy.

Scarring in the eye prompted the search for a pharmacological agent capable of selectively inhibiting the growth of rapidly proliferative cells without unacceptable toxic effects on normal cell populations. Blumenkranz et al (1984) investigated the effect of 5-fluorouracil and other antiproliferative drugs in vitro and found that the therapeutic range of 5-fluorouracil was broader and the resulting therapeutic index safer, whilst 5-fluorouracil still exhibited a strongly dose-dependant inhibition of fibroblast proliferation (Blumenkranz 1984). The application of drug data derived from cell culture to clinical disease has many limitations according to Andreotti et al (1995). Variables such as bioavailability, diffusional barriers, metabolic inactivation, excretion, drug resistance and enzyme induction prohibit simple extrapolation of cell culture data to in vivo experimental models of disease. Nevertheless, this basic approach to drug selection is appropriate in several regards. There is ample evidence to suggest that in certain instances there is excellent correlation between drug efficacy in cell culture and clinical effect in vivo. It has been shown by a variety of assays that there is a direct correlation between the ability of synthetic steroids to inhibit murine fibroblastic proliferation in cell culture and to suppress dermal inflammation (Ruhman and Berliner 1967). A similar relationship has been demonstrated for mouse myeloma cell culture lines, in which response to chemotherapeutic agents in vitro is strongly predictive of clinical response and survival time in vivo (Ogawa et al 1973). Andreotti et al (1995) correlated clinical response with the 50% inhibitory concentration (IC_{50}) and ATP luminescence measurements for cell viability and found a >90% accuracy for cisplatin resistance of ovarian carcinoma.

Elion and Hitchings (1965) proposed that 5-fluorouracil is converted enzymatically to the nucleotide 5-fluoro-2-deoxyuridine phosphate which is a potent inhibitor of thymidylate synthetase. This effectively inhibits the conversion of uridylylate to thymidylate. In the absence of adequate levels of phosphorylated deoxyribonucleotides of thymidine for incorporation in DNA, mitotic activity is reduced and cellular proliferation ceases. This block, at the level of thymidylate synthetase, can be overcome by the addition of exogenous thymidine or folic acid. However, Blumenkranz et al (1984) showed that the presence of these substrates did not notably alter the inhibitory effect of
5-fluorouracil, indicating that this agent probably has a mechanism other than just the inhibition of thymidylate synthesis. It was also Blumenkranz et al (1984) who suggested that 5-fluorouracil is converted to its corresponding ribophosphate, which is then incorporated into RNA. This results in the production of abnormal ribosomes, altered translation from messenger RNA and abnormal protein synthesis. In experiments with haematopoietic stemcells, Randall and Weissman (1997) found that treatment with 5-fluorouracil decreased the expression of one of the major growth factor receptors (c-kit) and induced expression of an adhesion molecules of the integrin family (Mac-1), probably due to activation of the surviving population due to the loss of cycling haematopoietic progenitors. Some chemotherapeutic agents have been suggested to result in vascular toxicity and as it is proposed that 5-fluorouracil be used in an open wound this might be of some consequence, as a systemic effect is not desirable. A study by Watts et al (1997) showed that 5-fluorouracil did not alter the permeability of human umbilical cord vein endothelial cell monolayers and that there was no change in the F-actin cytoskeleton of the cells after treatment. Mohay and McLaughlin (1995) however found that normal and 5-fluorouracil inhibited bovine corneal endothelial cells after wound healing differed in cell geometry (exhibiting polymegathism) and actin and vinculin redistribution after wound closure.

5-fluorouracil is used to inhibit fibroblast proliferation in the eye causing a high recurrence rate after glaucoma filtration surgery. Initially 5-fluorouracil was utilised for postoperative injections of 5-fluorouracil into the eye (The fluorouracil filtering surgery study group 1989), later for a peri-operative treatment (Smith et al 1992, Lanigan et al 1994). The perioperative treatment protocol followed by Smith et al (1992) consists of a standard trabeculectomy, followed by a 5 minute application of a sponge soaked in 5-fluorouracil (50 mg/ml) both surrounding the scleral flap and subconjunctivally, before the site is rinsed and the paracentesis tract performed. Lanigan et al (1994) uses a concentration of 25 mg/ml 5-fluorouracil and places the sponge between the sclera and conjunctival flap, before rinsing, raising a partial thickness scleral flap, performing a paracentesis and peripheral iridectomy.

Khaw et al (1992) had illustrated through in vitro experiments, that even short exposures to 5-fluorouracil have long-term effects on cell culture proliferation. This was followed by in vivo experiments (Khaw et al 1992, Khaw et al 1993), exposing rabbits undergoing glaucoma filtration surgery to topical 5-minute intraoperative treatments with 50 mg/ml
5-fluorouracil as described above. This showed that the effect was well localised in the eye and affected both proliferation and motility as measured by fibroblast outgrowth from biopsies taken after treatment. Further clinical applications have been investigated in the use of 5-fluorouracil in the management of scarring in the injured tendon, based on in vitro experiments using endotenon and synovial sheet fibroblasts (Khan et al 1995). From the above 5-fluorouracil seems capable of inhibiting proliferation in Dupuytren's disease and the treatment proposed in this thesis follows the principles of use of 5-fluorouracil in ophthalmic surgery.

The exact mechanism of 5-fluorouracil is not known, but the agent inhibits proliferation and may via interaction with DNA and RNA inhibit protein synthesis and contraction.

5. Conclusion

Much attention has been paid to the surgical treatment of Dupuytren's disease, but in the hope of improving treatment outcome the cellular and molecular basis of the disease require attention. Extracellular matrix, collagen fibres and ground substance, mechanical forces, growth factors and integrins are being investigated in an attempt to elucidate the underlying biological processes responsible. Many aetiologies have been suggested, but it could be hypothesised that the clinical features designated Dupuytren's disease are a combination of several conditions which in concert or as a cascade influence the anatomy of the hand.

The aim of this thesis is to investigate the hypothesis that Dupuytren's disease is due to an imbalance between proliferation and apoptosis in the palmar fascia as a result of the combination of high levels of c-myc oncogene and anti-apoptotic bcl-2 gene. It will furthermore be investigated whether the proliferation and differentiation in Dupuytren's fibroblasts, with and without exposure to TGFβ1, as well as the contractile properties of Dupuytren's disease can be inhibited by 5-fluorouracil in vitro.
Chapter II

C-myc expression in Dupuytren's tissue, non-diseased fascia, carpal ligament and fibrosarcoma.
1. **Introduction**

As reviewed in Chapter 1 fibroblasts from Dupuytren's show abnormal cell-cycle progression, proliferation and apoptosis; properties governed by the C-myc oncogene.

1.1. **Aims**

The aims of this chapter were 1. To measure the expression of the c-myc protooncogene in histological specimens from Dupuytren's tissue and compare this with non-diseased fascia, carpal ligament and fibrosarcoma. 2. To investigate c-myc as a potential prognostic factor for recurrence in Dupuytren's disease by correlating c-myc levels with clinical factors.

2. **Material and Methods**

2.1. **Patients and histology**

Specimens from Dupuytren patients and patients undergoing Carpal Tunnel release were collected from Mount Vernon Hospital, Northwood operating theatre and embedded in paraffin after 10 % formal saline fixation. Archival paraffin embedded Fibrosarcoma specimens were obtained from Bispebjerg Hospital and Rigs Hospital, (the Royal National hospital) Copenhagen, Denmark.

The clinical data collected included sex and age, for the Fibrosarcoma specimens grade according to Myhre Jensen (1991) and for the Dupuytren specimens stage (Luck 1959), severity of disease (as measured by total joint-angle deformity) and whether the lesion was primary or recurrent.
Dupuytren's disease:

<table>
<thead>
<tr>
<th>Type</th>
<th>Patients</th>
<th>Gender</th>
<th>Age (years ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>21</td>
<td>3F, 18M</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>Recurrent</td>
<td>9</td>
<td>2F, 7M</td>
<td>64 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>All grade III</td>
</tr>
</tbody>
</table>

| Fibrosarcoma | 11 | 5F, 6M | 68 ± 10 | Three grade I, two grade II and 6 grade III |
| Non-diseased fascia | 6 | 2F, 4M | 71 ± 14 |
| Carpal ligament | 12 | 10F, 2M | 53 ± 17 |

Table 1: Patient data for c-myc studies on Dupuytren, non-diseased fascia, carpal ligament and fibrosarcoma specimens.

2.2. Dewaxing of specimens

Four 35 um thick sections were cut from each paraffin embedded block and dewaxed by bathing in 5 mL xylene (BDH, Poole UK) for 10 minutes. After repeating the process the specimens were rehydrated through a series of ethanol (Hayman Ltd, UK) commencing with 100%, 90%, 70% and finally 50% before nuclear extraction was performed.

2.3. Nuclear extraction

Nuclear extraction was performed by incubation with pepsin solution (4mg/mL in 0.1 M hydrochloric acid) for 45 minutes at 37°C. After filtering through a 35 um mesh in a Swinnex holder the suspension was aliquoted into two samples (one for c-myc staining and one to act as an antibody control) and the concentration adjusted to 10^6/mL with phosphate buffered saline.

2.4. C-myc antibody

Expression of the c-myc oncogene leads to production of a nuclear oncoprotein. This was assayed by dual parameter flow cytometry following extraction of intact nuclei from paraffin embedded material using an adaptation of the method described by Hedley et al (1983). C-myc protein was identified using rabbit polyclonal antibody raised against myc oncoproteins (Genosys Biotechnologie (Europe) Ltd, UK) raised against a synthetic peptide: Ala-Pro-Ser-Glu-Asp-Ile-Trp-Lys-Lys-Phe-Glu-Leu-Cys (Ralston and Bishop 1982). The IgG was isolated directly from rabbit sera and
affinity purified prior to stabilisation and lyophilisation. C-myc was identified using a rabbit polyclonal antibody to human myc oncoprotein (Rabbit Polyclonal Antibody to myc family proteins, Genosys Biotechnologies (Europe) Ltd).

A nuclear pellet of the test sample was incubated with the myc antibody at a dilution of 1:25 in 100 ul of phosphate buffered saline (PBS) containing 0.5% normal goat serum (NGS) and 0.5% detergent (Tween 20/ Polyoxyethylenesorbitan monolaureate, Sigma Chemical Co.) for 1 hour at room temperature. The control sample was incubated in the absence of the myc antibody, but with rabbit immunoglobulin fraction (Sigma Ltd, UK), in order to act as a baseline for subsequent flow cytometry analysis. After washing with PBS the suspension was incubated with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (DAKO) for 45 minutes at room temperature. The secondary antibody was added at a dilution of 1:20 in phosphate buffered saline containing 0.5% NGS and 0.5% Tween 20. After washing, the mixture was resuspended in 1 mL of PBS and DNA staining performed by the addition of 20 μl propidium iodide (Sigma, Poole UK) to enable analysis by flow cytometry.

2.5. FACscan

Stained samples were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The FACscan system is an automated cell analyser developed for both research and clinical application. It consists of a bench top sensor module coupled with a computer module which controls both acquisition and analysis of data. Cells or nuclei enter the flow chamber in a single file and are irradiated by a 15mW, 488nm air-cooled argon-ion laser. Dichroic mirrors spectrally filter emitted light, separating and deflecting longer wavelengths whilst transmitting shorter wavelengths. Longer wavelengths are detected on two light scatter channels (front and side). These shorter wavelengths are further separated by other mirrors into the path of one of three photomultiplier detectors, FL1, FL2 and FL3, following which the signal is digitised and processed by the computer.

At 530 nm the FL1 detector is optimised for FITC detection whilst the FL3 detector transmits wavelengths in excess of 650 nm, suitable for the detection of red light emitted by propidium iodide. The FL2 channel detects the intermediate wavelengths (585nm), emitted by phycoerythrin in the red/orange band (this channel was not used in the present investigations). Dual parameter collection of data on FL1 and FL3 allows bivariate dot
plots to be formulated, recording expression of FITC-labeled data on the FL1 channel against DNA content on the FL3 channel.

2.6. **Data analysis and measurement of onco-protein.**

Data was analysed using a computer acquisition/analysis programme, Lysys II (Becton Dickinson, San Jose California). Events were quantified by the imposition of computer generated windows (CGW) to define specific regions on the histogram or two-dimensional dot plot.

2.7. **Use of computer generated windows.**

CGWs allow definition of specific populations of nuclei or cells and of the required phases of the cell cycle. Regions can be set around populations to omit extraneous interference from debris or from populations of cells whose data is not required. These regions are retained and superimposed on the control sample, to allow comparison of the number of nuclei and fluorescence in identical regions.

2.8. **Calculations of oncoprotein levels.**

Using the computer programme Lysys II, oncoprotein positivity was calculated from comparison of the number of events within the regions applied to the dot plots of both control and test sample. Initially a region is set around the central dot plot to demarcate and differentiate labeled from unlabelled nuclei. However the secondary antibody (FITC) adheres non-specifically to a small portion of nuclei and cellular debris, estimated to be less than 2% of the whole population of labeled material. To exclude this fluorescence, a region is set around those 2% of nuclei exhibiting the highest fluorescence values (R1). This region is automatically superimposed on the antibody labeled dot plot of the same specimen and subtraction of the event count of the antibody sample from the control gives the overall number of nuclei showing specific labeling due to oncoprotein expression. This is represented as a percentage of all nuclei, to give the % positivity.

Further regions can be set around populations of nuclei that lie within the different phases of the cell cycle, to allow analysis of oncoprotein expression in each phase. The mean fluorescence of the FITC labeled-nuclei gives an estimate of the relative amount of protein contained within each cell cycle phase and can be represented numerically as a ratio of mean green fluorescence of positive cells compared to control (unlabelled) cells.
Below an example of a dot plot of c-myc expression in a diploid primary Dupuytren’s sample (Figure 1). The R1 area represents approximately 2% of the control sample (black). This region is automatically superimposed on the antibody labeled dot plot of the same specimen (red) and subtraction of the event count of the antibody sample from the control gives the overall number of nuclei showing specific labeling due to oncoprotein expression.

Figure 5: Dot-plot of c-myc expression

Bivariate dot plot of diploid primary Dupuytren's disease including the G1, S and G2 phase. The x-axis represents DNA content and the y-axis represents the logarithmic expression of FITC-labelled data.

2.9. Statistical analysis

Correlation of c-myc expression and clinical data was investigated using a linear fit model, analysing variance and the difference between c-myc levels were analysed utilising the Mann Whitney-U test.
3. **Review of the histology**

All samples were stained with Haematoxylin and Eosin and representative micrographs will be presented in figures 2-5. The different stages of Dupuytren's disease according to Luck (1959) was described in chapter I.

![Histology of carpal ligament](image)

**Figure 1: Histology of carpal ligament**

*Longitudinal section of Haematoxylin and eosin stained carpal ligament at x 10 magnification.*

Carpal ligament is described as uniform fibrotic tissue, with elongated spindle shaped cells surrounded by ample collagenous stroma.
Figure 2: Histology of non-diseased fascia

*Haematoxylin and eosin staining of non-diseased palmar fascia from a Dupuytren hand at x 10 magnification.*

Non-diseased fascia exhibits some of the same features as carpal ligament, such as elongated cells surrounded by collagen. The 3-dimensional arrangement of the fibrils becomes apparent as the section transverses them at different angles.
Figure 3: Histology of Dupuytren's disease

*Haematoxylin and eosin staining of Dupuytren's disease at x 10 magnification.*

Dupuytren tissue is described as collagenised fibrous tissue containing spindle shaped fibroblasts. Fibroblasts producing collagen are larger and reside in high cell density areas, whilst the smaller and inactive fibroblasts reside in mature collagen. The irregularity of the tissue illustrates how the different histological stages (Luck 1959) can coincide. Very cellular specimens can suggest neoplasia and mimic the histological pattern seen in fibrosarcoma specimens.
Figure 4: Histology of fibrosarcoma.

*Haematoxylin and eosin staining of fibrosarcoma specimen at x 10 magnification.*

The highly cellular section of Fibrosarcoma exhibits a characteristic herring bone pattern, numerous mitotic figures and scanty stroma.
4. Results

C-myc levels were measured in Dupuytren's disease, both primary and recurrent, fibrosarcoma specimens, non-diseased fascia and carpal ligament (Figure 5). Levels in primary Dupuytren (68% +/- SE 4%) were not significantly different from those found in fibrosarcoma specimens (77% +/- SE 4%) (p>0.14), but significantly (p= 0.048) higher than those found in recurrent Dupuytren's disease (50% +/- SE 8%), non-diseased fascia (38% +/- SE 2%) and carpal ligament (20% +/- SE 5%). C-myc expression in non-diseased fascia was not significantly different from recurrent Dupuytren's disease (p>0.46), but significantly higher than carpal ligament (p=0.014).

Figure 5: C-myc expression in Dupuytren's disease, non-diseased fascia, carpal ligament and fibrosarcoma specimens.

*The columns along the x-axis illustrate the tissue types investigated: carpal ligament (n=12), non-diseased fascia (6), Fibrosarcoma (n=11), primary (n=21) and recurrent (n=9) Dupuytren's disease. The y-axis represents the oncoprotein estimate found by subtraction of the event count of the antibody sample from the control, giving the overall number of nuclei showing specific labeling due to oncoprotein expression as a percentage.*
To evaluate c-myc expression as a predictor of disease severity total joint angle deformity in degrees was plotted against c-myc levels. There was no significant relationship between total joint deformity and c-myc expression (Figure 6) for primary Dupuytren’s disease, but this was highly significant (p>0.01, $R^2 = 0.95$) for recurrent disease (figure 7).

**Figure 6:** C-myc expression in primary Dupuytren’s disease related to severity.

The x-axis represents the c-myc activity as a percentage as described in figure 5 whilst total joint deformity (metacarpophalangeal, proximal and distal interphalangeal joints) as measured in degrees on the Dupuytren affected hands (n=21) is depicted on the y-axis.
Figure 7: C-myc expression in recurrent Dupuytren's disease related to severity.

The x-axis represents the c-myc activity as a percentage as described in figure 5 whilst total joint deformity (metacarpophalangeal, proximal and distal interphalangeal joints) as measured in degrees on the Dupuytren affected hands (n=9) is depicted on the y-axis.

C-myc levels for Dupuytren's disease were not significant using the paired t-test if plotted against patients age or number of operations in recurrent disease.
5. Discussion

It has been suggested that cellular c-myc expression is dependant on the presence of mitogens (reviewed by Evan and Littlewood 1993), except in transformed cells which do not require mitogenic stimulation for c-myc expression (Campisi et al 1984). C-myc is also thought to be expressed at a constant rate throughout the cell-cycle in proliferating cells (Hann et al 1985, Thompson et al 1985), but not in quiescent fibroblasts (Campisi et al 1984, Dean et al 1986, Waters et al 1991). C-myc is thought to mediate control of cell-cycle progression, proliferation and apoptosis (Reed 1994) and once these processes were established, Evan and Littlewood (1993) suggested that the cell-growth and cell-death could be speculated to be independently modulated by other genes, cytokines and other external factors.

Levels of c-myc expression could therefore hypothetically be interpreted as a measure of cellular activity (high cell turn-over) and Fibrosarcoma specimens would accordingly be expected to exhibit very high levels of c-myc which were confirmed. By contrast carpal ligament representing tissue with a low level of cellular turnover, exhibited very low levels of c-myc. Primary Dupuytren’s disease tissue exhibited a high level of c-myc expression not significantly different from Fibrosarcoma specimens and would therefore be expected to possess a similar degree of cellular turnover as the malignant tumour. According to the theories discussed above, the high c-myc expression would indicate that Dupuytren fibroblasts were either under the influence of mitogens, represented transformed cells or proliferating.

Recurrent Dupuytren's disease and non-diseased fascia showed intermediate levels of c-myc which could indicate a lower availability of or lower sensitivity to mitogens, represented by a higher ratio of quiescent to proliferating cells. Considering the disease progresses from non-diseased fascia to primary Dupuytren's disease and then reappears as recurrent Dupuytren's disease, it could be hypothesised that these intermediate levels could be interpreted in two ways.

If expression of c-myc is dependant on the availability of mitogens, the intermediate level of c-myc in non-diseased fascia may rise with increased availability of cytokines. If this is the case therefore, non-diseased fascia would exhibit c-myc expression characteristic for primary Dupuytren's disease if exposed to an increased amount of
mitogens. This may in turn lead to disease progression from non-diseased fascia to primary Dupuytren's disease.

In contrast it could be hypothesised that the intermediate level of c-myc expression in recurrent Dupuytren's disease is due to the reduced sensitivity to cytokines. Recurrent Dupuytren's disease may represent a different tissue, such as scar tissue, which due to its fibrotic nature may be less sensitive to available cytokines.

If the theory of disease progressing from non-diseased fascia to primary Dupuytren's disease and then reappearing as recurrent Dupuytren's disease is accepted, then c-myc expression has been shown to fluctuate at different times of the disease process. Individual measurements of c-myc expression from nodules and cords were not performed and may therefore account for some difference in these results.

In recurrent Dupuytren's disease correlation between total joint angle deformity and c-myc levels was highly significant, i.e. the more severe the disease the higher the c-myc expression. The level of contraction encountered may however depend on patient neglect, at which stage the patient seeks medical attention and whether the disease process is recognised as Dupuytren's disease, though the latter was excluded in this study. Joint contracture remains the basis of Tubiana's (1974) classification of Dupuytren's disease and forms part of the clinical decision to operate. The correlation between total angle deformity and level of c-myc is however consistent with c-myc expression representing disease activity in Dupuytren's disease, as measured by joint contraction.

C-myc levels were not found to be correlated to total joint deformity in primary disease and may indicate that alternative criteria for disease severity are applicable in primary disease.

Because c-myc levels were not comparable with the number of recurrences in recurrent Dupuytren's disease, it will only become clear whether c-myc expression in primary disease can be used as a predictor of recurrence after long-term follow-up of primary cases.

The immunochemical methods employed in this chapter are well established and practiced routinely at the Gray Laboratory at Mount Vernon Hospital, though this does not exclude possible artifacts. Each sample posed as the corresponding internal control in the absence of the myc antibody, but was incubated with rabbit immunoglobulin fraction (Sigma Ltd, UK), in order to act as a baseline for subsequent flow cytometry analysis.
The large amount of collagen in the Dupuytren, non-diseased fascia and carpal ligament samples resulted in an intermittent slow flow through the FACscan, which required frequent flushing of the machine and caused a substantial amount of fluorescent debris. The use of CGWs however allowed the definition of specific populations of nuclei and regions were set around populations to omit extraneous interference from debris. These regions were retained and superimposed on the control sample, which allowed comparison of the number of nuclei and fluorescence in identical regions.

C-myc levels have been shown to be of comparable magnitude in Fibrosarcoma and primary Dupuytren specimens, with decreasing levels in recurrent Dupuytren's disease, non-diseased fascia and carpal ligament. C-myc expression in Dupuytren's disease may therefore be taken to fluctuate throughout the disease process, either due to reduced availability of or reduced sensitivity to cytokines. It is also proposed that primary Dupuytren's disease may represent transformed fibroblasts, which do not require mitogenic stimulation for c-myc expression.

*To establish the value of c-myc as a prognostic marker of recurrence in Dupuytren's disease long-term follow-up of primary Dupuytren's disease patients would be necessary.*

*Measuring c-myc levels on recurrent Dupuytren's contracture was however shown to predict the severity of the recurrent disease and may be used to facilitate the decision to operate.*
Chapter III

Markers of proliferation and anti-apoptotic genes in Dupuytren’s tissue, non-diseased fascia, carpal ligament and fibrosarcoma
1. **Introduction**

C-myc influences both proliferation and apoptosis as reviewed in chapter 1. The Bcl-2 gene protects against apoptosis and a high expression of this gene. Part of the hypothesis forming this thesis is based on Dupuytren's disease being due to an imbalance between proliferation and apoptosis and will be investigated in the following experiments.

2. **Aims**

The aim was to establish the level of proliferation and protection against apoptosis in histological specimens of Dupuytren tissue, non-diseased fascia, carpal ligament and fibrosarcoma specimens by immunohistochemical methods.

3. **Materials and Methods**

3.1. **Patients**

Tissue specimens from Dupuytren patients and patients undergoing Carpal Tunnel release were collected from Mount Vernon Hospital, Northwood operating theatre and embedded in paraffin after 10 % formal saline fixation. Archival paraffin embedded Fibrosarcoma specimens were obtained from Bispebjerg Hospital and Rigs Hospital, (the Royal National hospital) Copenhagen, Denmark.

The clinical data collected included sex and age, for the Fibrosarcoma specimens grade according to Myhre Jensen (1991) and for the Dupuytren specimens stage (Luck 1959), severity of disease (as measured by total joint-angle deformity) and whether the lesion was primary or recurrent.

<table>
<thead>
<tr>
<th>Dupuytren's disease:</th>
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<tbody>
<tr>
<td>primary</td>
<td>32 patients (four female and 28 male, age:63 +/- SD 12 years)</td>
</tr>
<tr>
<td>recurrent</td>
<td>11 patients (two female and 9 male, age:64 +/- SD 10 years)</td>
</tr>
<tr>
<td></td>
<td>All grade III</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>11 patients (five female and 6 male, mean age:68 +/- SD 10 years. Three grade I, two grade II and 6 grade III.</td>
</tr>
<tr>
<td>Non-diseased fascia</td>
<td>10 patients (two female and four male, age:71 +/- SD 14 years)</td>
</tr>
<tr>
<td>Carpal ligament</td>
<td>12 patients (10 female and two male, age:53 +/- SD 17 years)</td>
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**Table 1: Patient data for mib-1 and bcl-2 specimens**
3.2. Tissue collection for paraffin sections

Tissue from Dupuytren patients and normal controls were collected from theatre, wrapped in a moist sterile swab in a sterile container. The specimens were immediately fixed in 4% formaldehyde and processed within 2 weeks.

3.3. Preparation of paraffin sections for immunohistochemistry

The specimens were embedded in paraffin, four µm sections cut and fixed on superfrost slides (BDH, Merck Ltd, Dorset) for 24 hours at 56°C.

3.4. Processing of the paraffin sections for immunohistochemistry

3.4.1. Mib-1

Four µm paraffin sections were dewaxed by bathing in 5 mL xylene for 10 minutes. After repeating the process the specimens were rehydrated through a series of alcohols commencing with 100%, 90%, 70%, 50% through to destilled water. The endogenous peroxidase was blocked with 1% methanol/ hydrogen peroxide solution for 20 minutes at room temperature. The sections were then microwaved four times for four minutes in 10mM Citric Acid, titrated to pH 6 with 2M NaOH (Shi et al 1991), washed in water and flooded with Normal Swine serum (Swine Serum (Normal), DAKO, Denmark) diluted 1:5 with Tris Buffered saline (TBS) for 10 minutes. The excess water was shaken off and the sections were incubated overnight at 4°C with Mib-1 antibody (Rabbit Anti-Human, Ki-67 Antigen, DAKO, Denmark), diluted 1:200 with TBS, then washed in three changes of TBS and Biotinylated Swine-anti rabbit antibody (Biotinylated Swine Anti-Rabbit Immunoglobulins, DAKO, Denmark) diluted 1:300 with TBS added and left for 1 hour. The sections were then washed in three changes of TBS and incubated with 1% Avidin-Biotin Complex (ABComplex/HRP, DAKO, Denmark) for 1 hour and rinsed in Tris Buffer (TB). Diamino Benzidine 75 ug/10 mL in TB and 3 drops of 3% hydrogen peroxide was added for 10 minutes, before the sections were rinsed in TB, then water, counterstained in Mayer’s Haematoxylin, washed, dehydrated through graded alcohols into xylene and mounted with DPX.

The positive cells were counted under the light microscope by one observer and percent positivity was calculated from the total number of cells as an average of 10 randomly selected fields at x 40 magnification or the average of a minimum of 3 x 300 cells as per
standard immunohistochemical methods. Histological slides from a tonsil was used as a positive control due to the presence of germinal centres in the cortex, which should stain for Mib-1. In addition the satisfactory staining of samples was assessed by Dr P Richman, consultant histopathologist and Dr G Wilson, senior scientist at the Gray Laboratory, Mount Vernon Hospital.

3.4.2. Bcl-2

Four um paraffin sections were dewaxed by bathing in 5 mL xylene for 10 minutes. After repeating the process the specimens were rehydrated through a series of alcohols commencing with 100%, 90%, 70%, 50% through to destilled water. The sections were then microwaved two times for four minutes in 10mM Citric Acid, titrated to pH 6 with 2M NaOH, washed in water and rinsed with Tris Buffered saline (TBS). The excess TBS was shaken off and the sections were incubated overnight at 4°C with Bcl-2 antibody (Monoclonal Mouse Anti-Human bcl-2 Oncoprotein, DAKO, Denmark), diluted 1:40 with TBS, then washed in three changes of TBS and Biotinylated Rabbit-anti mouse bridge antibody (Biotinylated Rabbit Anti-Mouse Immunoglobulins, DAKO, Denmark) diluted 1:350 with TBS added and left for 1 hour. The sections were then washed in three changes of TBS and incubated with 1% Avidin-Biotin Complex (ABComplex/HRP, DAKO, Denmark) for 1 hour and rinsed twice in TBS. Diamino Benzidine 75 ug/10 mL in TB and 3 drops of 3% hydrogen peroxide was added for 10 minutes, before the sections were rinsed in TB, then water, counterstained in Mayer’s Haematoxylin, washed, dehydrated through graded alcohols into xylene and mounted with DPX.

The positive cells were counted under the light microscope by one observer and percent positivity was calculated from the total number of cells as an average of 10 randomly selected fields at x 40 magnification or the average of a minimum of 3 x 300 cells as per standard immunohistochemical methods. Histological slides from a tonsil was used as a positive control due to the known expression of bcl-2 on B-cells not in the germinal centres (Liu et al 1991). In addition the satisfactory staining of samples was assessed by Dr P Richman, consultant histopathologist and Dr G Wilson, senior scientist at the Gray Laboratory, Mount Vernon Hospital.
4. Results

4.1. Expression of Mib-1 in Dupuytren's tissue, non-diseased fascia, carpal ligament and fibrosarcoma.

Dupuytren's tissue, non-diseased fascia and carpal ligament did not express any mib-1 positivity, at three separate staining procedures overlooked by Mrs F Daley, senior assistant at the Gray Laboratory and verified by Drs Richman and Wilson. Due to the large amount of collagen in these specimens, the sections were found to adhere poorly to the slides after microwaving, despite previous exposure to 56°C for 24 hours, producing artifacts in the form of wrinkling or shrinkage.

Fibrosarcoma specimens exhibited a mean positivity of 16% +/- SE 6 (range 10-30%).

Tonsillar controls stained satisfactorily, showing marked staining of cells in the germinal centres in accordance with Gerdes et al (1991).

Representative micrographs of histological specimens stained for mib-1 are presented in Figures 1-5.
Figure 1: Tonsil stained for Mib-1

*Tonsillar section at x 40 magnification stained by immunohistochemical methods for Mib-1 and counterstained with Haematoxylin and Eosin.*

Mib-1 positive cells stained dark brown, whilst negative cells stained blue. Control in the form of tonsil showed mib-1 positive cells staining brown in the germinal center.
Figure 2: Carpal ligament stained for Mib-1

Section of carpal ligament at x 40 magnification stained by immunohistochemical methods for Mib-1 and counterstained with Haematoxylin and Eosin.

Carpal ligament sections showed a uniform blue staining of cells, indicating no positivity for mib-1.
Figure 3: Non-diseased fascia stained for Mib-1

Section of non-diseased fascia at x 40 magnification stained by immunohistochemical methods for Mib-1 and counterstained with Haematoxylin and Eosin.

Non-diseased fascia showed the same uniform blue staining of cells, indicating no positivity for mib-1 as carpal ligament specimens.
Figure 4: Dupuytren's disease stained for Mib-1

Section of Dupuytren's disease at x 40 magnification stained by immunohistochemical methods for Mib-1 and counterstained with Haematoxylin and Eosin.

Dupuytren's disease showed the same uniform blue staining of cells, indicating no positivity for mib-1 as non-diseased fascia and carpal ligament specimens.
Figure 5: Fibrosarcoma stained for Mib-1

Section of Fibrosarcoma at x 40 magnification stained by immunohistochemical methods for Mib-1 and counterstained with Haematoxylin and Eosin.

In contrast Fibrosarcoma specimens showed scattered dark grey mib-1 positive cells, occasionally exhibiting visible mitotic configurations.
4.2. **Expression of Bcl-2 in Dupuytren's tissue, non-diseased fascia, carpal ligament and fibrosarcoma.**

Dupuytren's tissue, non-diseased fascia and carpal ligament did not express any bcl-2 positivity at three separate staining procedures overlooked by Mrs F Daley, senior assistant at the Gray Laboratory and verified by Drs Richman and Wilson. Due to the large amount of collagen in these specimens, the sections were found to adhere poorly to the slides after microwaving, despite previous exposure to 56°C for 24 hours, again producing artifacts in the form of wrinkling or shrinkage.

Fibrosarcoma specimens exhibited a mean positivity of 10 % +/- SE 4% (range 5-25%). Tonsillar controls stained satisfactorily, showing marked staining of lymphoid cells in the T zone and follicular mantle, as described by Liu et al (1991).

Representative micrographs of histological specimens stained for bcl-2 are presented in Figures 6-10 on the next pages.
Figure 6: Tonsil stained for Bcl-2

*Tonsil section at x 40 magnification stained by immunohistochemical methods for bcl-2 and counterstained with Haematoxylin and Eosin.*

Bcl-2 positive cells stained dark brown, whilst negative cells stained blue. Control in the form of tonsil showed bcl-2 positive cells staining dark gray in the T zone.
Figure 7: Carpal ligament stained for Bcl-2

Section of carpal ligament at x 40 magnification stained by immunohistochemical methods for bcl-2 and counterstained with Haematoxylin and Eosin.

Carpal ligament sections exhibited a uniform blue staining of cells, indicating no positivity for bcl-2.
Figure 8: Non-diseased fascia stained for Bcl-2

Section of non-diseased palmar fascia from a Dupuytren patient at x 40 magnification stained by immunohistochemical methods for bcl-2 and counterstained with Haematoxylin and Eosin.

Non-diseased fascia sections also showed a uniform blue staining of cells, indicating no positivity for bcl-2.
Figure 9: Dupuytren's disease stained for Bcl-2

Section of Dupuytren's disease at x 40 magnification stained by immunohistochemical methods for bcl-2 and counterstained with Haematoxylin and Eosin.

Dupuytren's disease sections also showed a uniform blue staining of cells, indicating no positivity for bcl-2.
Figure 10: Fibrosarcoma stained for Bcl-2

Section of Fibrosarcoma specimens at x 40 magnification stained by immunohistochemical methods for bcl-2 and counterstained with Haematoxylin and Eosin.

Fibrosarcoma specimens exhibited bcl-2 positive brown staining cells scattered throughout the specimen.
5. Discussion

An imbalance between proliferation and apoptosis leads to uncontrolled growth as reviewed in chapter 1. In this chapter proliferation and anti-apoptotic genes were not found in histological sections of Dupuytren's disease, non-diseased fascia and carpal ligament using immunohistochemical methods. Fibrosarcoma specimens however exhibited mean mib-1 and bcl-2 levels of respectively 16% and 10%.

Considering proliferation it could be hypothesised that the relatively high levels of C-myc found in primary Dupuytren tissue without immunohistochemically proven proliferation may be due to the Dupuytren cell being comparable to transformed cells, which exhibit a high level of C-myc whether quiescent or proliferative (Campisi et al 1984, Martel et al 1995). Transformed cells have been shown to be less dependent upon growth factors and cell cycle control is apparently lost following chemical transformation of fibroblasts (Campisi et al 1984). The Ki-67 antigen is expressed in G1, S, G2 and mitosis but not in Go (Gerdes et al 1991) which also makes it possible that Dupuytren fibroblasts are in the Go phase of the cell cycle and therefore do not stain positively for Mib-1. Oshiro et al (1995) showed that the Mib-1 labeling index (percentage of positive cells of more than 500 cells) for atypical fibroxanthoma, regarded as a fibrohistiocytic tumour with intermediate potential, to be lower than the frankly malignant fibrous histiocytoma and higher than the benign fibrous histiocytoma, thereby linking proliferation with tumour potential. In the experiments described in this chapter a parallel was not found in Dupuytren's disease in relation to fibrosarcoma and carpal ligament.

Steady state C-myc expression in proliferating fibroblasts is continuously dependant on mitogenic stimulation and withdrawal of mitogens lead to the rapid and synchronous disappearance of c-myc mRNA and protein (reviewed by Evan and Littlewood 1993). The high expression of c-myc in Dupuytren's disease would therefore indicate that the fibroblasts were under constant stimulation from example growth factors as mentioned in chapter 2. The final step to mitosis is however also dependant on other factors in the cellular environment (Pardee 1989). Due to the fibrotic nature of the disease, vascularisation is poor and therefore the nutrient delivery is suboptimal. This may inhibit proliferation despite a high level of c-myc expression.

Consistent with the bcl-2 findings in fibrotic phase specimens, Wilutzky et al (1998) also showed that bcl-2 is absent in Dupuytren tissue in the proliferative and involutional
phase. Fibrosarcoma specimens were found to be positive for bcl-2 and as reviewed in chapter 1, c-myc combined with bcl-2 expression is a powerful promoter of malignant growth (Wagner et al 1993, Reed 1994). In these experiments this theory was consistent with the findings in fibrosarcoma specimens, but not in Dupuytren tissue.

Wilutzky et al (1998) also detected apoptosis related DNA fragmentation in numerous cells in the nodules of Dupuytren's disease, in the involutional phase, which was not examined in these experiments. Histological review of the samples however (by Drs G Wilson and P Richman) did not reveal apparent apoptosis. Studies reviewed by Evan and Littlewood (1993) have shown that c-myc induces apoptosis only under conditions of suboptimal or restrained cell proliferation as in reduced serum levels (Dhanaraj et al 1996). The apparently contradictory attributes of c-myc, capable of inducing both proliferation and apoptosis, lead Evans and Littlewood (1993) to hypothesise that the cell-proliferation and cell-death pathways were tightly coupled processes. Once established the cell-growth and cell-death were speculated to be independently modulated by other genes, cytokines and other external factors. The biological system suggested by this model was that proliferating cells, constituting a potential carcinogenic hazard, were primed for death should the cell fail to receive an appropriate survival signal.

The Dupuytren specimens examined in chapters 2 and 3 were not characterised by high cell density,- rather a certain paucity of cells, corresponding to Luck's histological stage III (Luck 1959) i.e. the fibrotic phase. It may therefore be hypothesised that the cells have retained their high C-myc expression from an earlier histological stage, Luck's stage I, characterised by proliferation, but have ceased to undergo mitosis due to reduced availability of growth factors.

These experiments may therefore imply that Dupuytren fibroblasts possess proliferative potential if exposed to mitogens, which would result in a high mitotic rate as found in wound-healing and early Dupuytren's disease.
Chapter IV

Proliferation of Dupuytren’s tissue, non-diseased fascia and carpal ligament in vitro and the effect of 5-fluorouracil on proliferation
1. **Introduction**

The local use of 5-fluorouracil, capable of selectively inhibiting the growth of rapidly dividing cells without unacceptable side effects on local cell-populations is already widely used by Ophthalmic surgeons, to reduce recurrence after glaucoma filtration surgery. Post surgery one component of the normal woundhealing response is an increase in cellular proliferation and it is possible that recurrence in Dupuytren's disease is triggered by this normal proliferative response. A similar response is seen in vitro when cells are plated at a sub-confluent degree. This model was therefore studied to emulate the post-surgical environment.

2. **Aim**

The aim of these experiments was to establish a cellular model for adjuvant treatment with 5-fluorouracil post-operatively.

3. **Materials and Methods**

3.1. **Patients**

The tissues used in these experiments were Dupuytren’s tissue, non-diseased fascia from the same hand and carpal ligament from carpal tunnel decompressions. For the latter ethical committee approval was sought and obtained.

The Dupuytren’s tissue was excised at operation for Dupuytren’s contracture and the non-diseased fascia was taken as a biopsy from a macroscopically uninvolved area of the hand at the edge of the primary incision. Carpal ligament was taken as a biopsy from the carpal ligament taken at carpal tunnel release in non Dupuytren hands.

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<table>
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<tbody>
<tr>
<td>Dupuytren's disease</td>
<td>6 patients, four primary, two recurrent</td>
</tr>
<tr>
<td></td>
<td>(all male, mean age: 65 +/- SD 8 years)</td>
</tr>
<tr>
<td>Non-diseased fascia</td>
<td>3 patients (all male, mean age: 65 +/- SD 16 years)</td>
</tr>
<tr>
<td>Carpal ligament</td>
<td>3 patients (two female and one male, age: 52 +/- SD 9 years)</td>
</tr>
</tbody>
</table>

**Table 1:** Patient data for in vitro proliferation studies in Dupuytren's disease, non-diseased fascia and carpal ligament.
3.2. Establishment of cell cultures

An explant method was used to obtain primary cell cultures from the harvested material. The tissue was collected from plastic surgery theatres, either wrapped in a moist swab or in human transport medium. Minced on a petri dish with a scalpel in a dedicated cell culture room, plated on 3.5 cm petri dishes and covered by a coverslip, sterilised in 70% alcohol. The cell culture was then incubated at 37°C, 5% CO₂ and covered with standard medium (Dulbecco’s Modified Eagle Medium (DMEM), 10% Foetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine (all Gibco Brl)). The media was changed twice a week thereafter.

3.3. Routine propagation of cell cultures

When cultures had reached confluency, they were propagated according to the following guidelines. After washing with Versene (Sigma) the cells were harvested using 1:10 Trypsin:Versene solution. This was neutralised with standard medium and the cells were recovered by centrifugation at 2000 rpm for 5 minutes, before the supernatant was discarded and the cells resuspended and plated in T25 flasks(p1). Again the media was changed twice a week and when confluency was reached, the cells were plated as above into a T75 flask(p2), then into four T75 flasks for a further passage (p3). All cell lines used were below passage 4.

3.4. Addition of 5-fluorouracil to cell cultures

Cells were plated at a density of 40.000 cells/ well in 6 well plates. Each experiment consisted of a single concentration of 5-fluorouracil (David Bull Laboratories, Warwick) and was conducted in triplicate. At day 0 the cells were plated and allowed to settle for 24 hours, before being treated for 5 minutes with 5-fu in serial dilution, ranging from 0-10 mg/mL at day 1. 5-fu was diluted using Phosphate Buffered Saline (PBS) (Sigma) according to the concentration needed. All cultures were then washed with PBS before being covered with media as normal.

3.5. Enumeration and staining

Cultures were enumerated at day 2, 7, 14, 21 and 30 after treatment with 5-fluorouracil. The cells were harvested, after washing with Versene, using 0.25 mL 1:10 Trypsin:Versene and neutralised with 0.25 mL medium. 10 µl of this cell suspension was
transferred to an Eppendorf tube and 10μl Trypan Blue (Sigma Chemical Company, Poole, Dorset) added. A haemocytometer was used to evaluate cell viability. Trypan blue is pumped out of live cell in normal conditions, thus any dead cell will appear blue in contrast to the living cells.

According to standard cell culture methods the average of three grids on the haemocytometer was taken as the nearest accurate number of cells in the specimen.

3.6. Freezing cells for storage

All surplus cells were stored at -80°C in a designated freezer or in liquid nitrogen at -70°C. The cells were harvested, as above, enumerated and frozen in 1 x 10^6 aliquots each in 1 mL of 1:10 DMSO (Dimethylsulfoxide, Sigma Chemical Company, Poole, Dorset):foetal calf serum in cryovials.

3.7. Thawing cells from frozen storage

The cryovials containing the cells were defrosted in 37°C waterbath and immediately washed with medium, centrifuged at 2000 rpm for 5 minutes, before the supernatant was removed and the cells plated.

3.8. Statistical analysis

The Wilcoxon paired rank test was used to analyse the effect of 5-fluorouracil exposure on fibroblast proliferation.

4. Results

A preliminary experiment (Figure 1) was used to determine the optimal concentration of 5-fluorouracil which would inhibit cellular proliferation in a fibroblast cell line, but not promote cell death. No significant difference in either proliferation or cell death was seen at day 2 between non-treated control cultures and 5-fluorouracil treated cells (p>0.1), regardless of 5-fluorouracil concentration used. At day 7 it became apparent that proliferation was inhibited more with rising concentrations of 5-fluorouracil and that 5-fluorouracil concentrations above 2.5 mg/mL increased the cell mortality (p<0.05), as shown by Trypan Blue exclusion. The serial range of dilutions was concentrated around 2.5 mg/mL after these initial results.
Figure 1: Growth curves for dermal fibroblasts (I) after exposure to 5-fluorouracil in serial dilution (0-25 mg/mL) for 5 minutes.

Growth curves were plotted for one normal dermal fibroblast cell line, derived by explant, treated with 5-fluorouracil for 5 minutes in serial dilution from 0-25 mg/mL. All experiments were performed in triplicate and mean values and standard error bars were calculated. N = 1 (male 34 years).

The total number of cells (in tens of thousands) is represented on the y-axis against time (days) on the x-axis and the concentration of 5-fluorouracil used is colour coded in the legend.
The experiment was repeated over a longer time course (30 days) using a range of concentrations of 5-fluorouracil of less than 10 mg/mL (Figure 2). Proliferation was again inhibited more with rising concentrations of 5-fluorouracil. Cultures not treated with 5-fluorouracil showed a slight decline in cell number after day 21, possibly because the cells had reached confluency at this stage. Cultures treated with 0.625 mg/mL 5-fluorouracil were inhibited for approximately 7 days, whilst 5-fluorouracil concentrations of 1.25 and 2.5 mg/mL inhibited cell division for approximately 14 days. 5-fluorouracil concentrations of 5 and 10 mg/mL inhibited cultures for 30 days.

Figure 2: Growth curves for dermal fibroblasts (II) after exposure to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.

Growth curves were plotted for one normal dermal fibroblast cell line, derived by explant, treated with 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. All experiments were performed in triplicate and mean values and standard error bars were calculated. N = 1 (male 34 years).

The total number of cells (in tens of thousands) is represented on the y-axis against time (days) on the x-axis and the concentration of 5-fluorouracil used is colour coded in the legend.
The same experimental procedure was then applied to 6 Dupuytren fibroblast cell lines and the mean cell proliferation at each time point calculated (Figure 3). Proliferation was seen to be inhibited more with rising concentrations of 5-fluorouracil. Cultures rinsed with PBS for 5 minutes acted as control and exhibited an almost linear growth throughout the experiment after day 2. The proliferation in cultures treated with 0.625, 1.25 and 2.5 mg/mL of 5-fluorouracil was delayed until day 7 though the subsequent rise in cell number was smaller with increasing 5-fluorouracil concentration. Cultures treated with 5-fluorouracil concentrations of 5 and 10 mg/mL only commenced proliferation at day 14 resulting in a very small increase in cell numbers.

Each experiment was enumerated with Trypan Blue to calculate the number of dead cells as a percentage of total number of cells as a result of 5-fluorouracil exposure. Comparing these figures from each individual concentration of 5-fluorouracil with the control, it was found that 2.5 mg/mL was the highest concentration of 5-fluorouracil which did not increase cell mortality significantly (p>0.05).

![Figure 3: Growth curves for Dupuytren fibroblasts after exposure to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.](image)

Mean growth curves for 6 Dupuytren fibroblast cell lines (four primary and two recurrent) treated with 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. All experiments were performed in triplicate and mean values and standard error bars were calculated. N = 6 (all male, mean age: 65 +/- 8 years). The total number of cells (in tens of thousands) is represented on the y-axis and the time (days) on the x-axis. The concentration of 5-fluorouracil used is presented in the individual colour coded legend.
This experiment was repeated with 3 non-diseased fascia fibroblast cell lines as above (Figure 4). Proliferation was seen to be inhibited more with rising concentrations of 5-fluorouracil. Cultures rinsed with PBS for 5 minutes acted as control and exhibited an almost linear growth from day 2 to 21, after which the total number of cells declined approximately 10% at day 30. The proliferation in cultures treated with 0.625 and 1.25 mg/mL of 5-fluorouracil was delayed until day 7 and the linear growth from day 7-21 again declined from day 21-30. Cultures exposed to 2.5, 5 and 10 mg/mL of 5-fluorouracil only commenced proliferation after day 14, with very little difference in cell numbers in cultures treated with 2.5 and 5 mg/mL of 5-fluorouracil. Cultures treated with 10 mg/mL 5-fluorouracil resulted in the smallest increase in cell numbers.

Figure 4: Growth curves for non-diseased fascia fibroblasts after exposure to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.

*Growth curves for three non-diseased fascia cell lines treated with 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. All experiments were performed in triplicate and mean values and standard error bars were calculated. N = 6 (all male, mean age: 65 +/- 16 years). The total number of cells (x 10⁴) is represented on the y-axis, the time (days) on the x-axis and the concentration of 5-fluorouracil used is colour coded in the legend.*
The same experiment was repeated with carpal ligament fibroblast cell lines (Figure 5). The proliferation was again inhibited more with rising concentration of 5-fluorouracil, though growth curves in these cultures were similar in doubling time after an initial lag phase dependent on the concentration of 5-fluorouracil used. Control cultures exhibit linear growth after day 2, whilst cultures treated with 0.625 mg/mL of 5-fluorouracil were inhibited till day 7. The proliferation in the remaining cultures were all inhibited to day 14, after which the growth curves differed very little.

Figure 5: Growth curves for carpal ligament fibroblasts after exposure to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes. 
Growth curves for three carpal ligament cell lines treated with 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. All experiments were performed in triplicate and mean values and standard error bars were calculated. N = 3 (two female, one male, mean age: 52 +/- 9 years). The total number of cells ($x\ 10^4$) is represented on the y-axis, the time (days) on the x-axis and the concentration of 5-fluorouracil used is colour coded in the legend.
When only non-treated Dupuytren, non-diseased fascia and carpal ligament fibroblast cell lines were compared (Figure 6), using a paired t-test, there was no significant difference between proliferation in vitro in the cultures under standard culture conditions.

In subsequent experiments using 2.5 mg/mL 5-fluorouracil did not increase the cell mortality, as previously mentioned, whilst still inhibiting proliferation. This concentration was therefore investigated further.

![Graph showing growth curves for Dupuytren's tissue, non-diseased fascia and carpal ligament under standard culture conditions.](image)

**Figure 6:** Dupuytren, non-diseased fascia and carpal ligament fibroblast growth curves under standard culture conditions.

*Growth curves for Dupuytren's tissue, non-diseased fascia and carpal ligament under standard culture conditions.*

*The x-axis represents the number of days the cells were maintained in culture, the y-axis the total number of cells (x 10⁴). Dupuytren's disease (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) cultures are colour coded in the legend which also denotes that the cell lines were not exposed to 5-fluorouracil.*
First carpal ligament fibroblast cell lines were analysed (Figure 7). Using the Mann Whitney-U test, there was no significant difference between treated and un-treated cultures at day 2. From day 7-21 this difference was highly significant, but by day 30 this became insignificant again.

![Graph of growth curves for control carpal ligament cell lines and carpal ligament cell lines exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes compared.]

**Figure 7:** Growth curves for control carpal ligament cell lines and carpal ligament cell lines exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes compared.

*Carpal ligament fibroblast growth curve (n = 3) after 0 or 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The x-axis represents the number of days the cells were maintained in culture, the y-axis the total number of cells (x 10⁴).*
Then non-diseased fascia fibroblast cell lines were investigated in the same manner (Figure 8). Using the Mann Whitney-U test, there was no significant difference between treated and un-treated cultures at day 2. From day 7-30 this difference was highly significant.

![Graph showing growth curves for non-diseased fascia cell lines with and without 5-fluorouracil exposure.](image)

**Figure 8:** Growth curves for control non-diseased fascia cell lines and non-diseased fascia cell lines exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes compared.

*Non-diseased fascia fibroblast growth curve (n = 3) after 0 or 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The x-axis represents the number of days the cells were maintained in culture, the y-axis the total number of cells (x 10⁴).*
When Dupuytren fibroblast cell lines were investigated in the same manner (Figure 9) using the Mann Whitney-U test, there was no significant difference between treated and un-treated cultures at day 2. From day 7-30 this difference became highly significant.

Figure 9: Growth curves for control Dupuytren fibroblast cell lines and Dupuytren fibroblast cell lines exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes compared.

_Dupuytren fibroblast growth curve (n = 6) after 0 or 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The x-axis represents the number of days the cells were maintained in culture, the y-axis the total number of cells (x 10⁴)._
If cell numbers from 5-fluorouracil exposed were compared (Figure 10), bearing in mind that there was no significant difference in the growth curves of the un-treated cell lines it became obvious that all cells were inhibited during the first two weeks after exposure to 2.5 mg/mL 5-fluorouracil. It was furthermore seen that the total number of cells at day 30 in the carpal ligament fibroblasts cultures, representing normal cells, was higher than in the remaining cultures.

![Graph](image)

Figure 10: Growth curves for Dupuytren, non-diseased fascia and carpal ligament fibroblast cell lines exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes compared.

The x-axis represents the number of days the cells were maintained in culture, the y-axis the total number of cells (x 10^4). Dupuytren's disease (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) cultures are colour coded in the legend which also denotes that the cell lines were exposed to 5-fluorouracil (2.5 mg mL).
Furthermore comparing both 5-fluorouracil exposed cell line and controls (Figure 11) it becomes obvious that if the total number of cells in the 5-fluorouracil exposed cultures were calculated as percentage of the corresponding untreated controls, carpal ligament fibroblasts were inhibited by 20% (p>0.05), non-diseased fascia by 50% (p<0.02) and Dupuytren fibroblasts by 55% (p<0.01) by day 30.

![Graph](image)

**Figure 11: Growth curves for control Dupuytren, non-diseased fascia and carpal ligament fibroblast cell lines compared and Dupuytren, non-diseased fascia and carpal ligament fibroblast cell lines exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes compared.**

The x-axis represents the number of days the cells were maintained in culture, the y-axis the total number of cells (x 10⁴). Dupuytren's disease (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) cultures are colour coded in the legend which also denotes whether the cell lines were exposed to 5-fluorouracil (2.5 mg/mL).
5. Discussion

5.1. The use of cell cultures in pre-clinical studies

There are limitations in applying cell culture findings to the situation in the hand after Dupuytren surgery. In vitro the cells are growing in mono-layers on plastic as opposed to cells in a tissue. Furthermore the majority of cells are actively proliferating under the influence of serum, which may make them more susceptible to the effect of an anti-proliferative agent, such as 5-fluorouracil. The initial concentrations and timing of the 5-fluorouracil exposure were based on previous experiments by Khaw et al. (1992 and 1993), whose aim was to inhibit fibroblast proliferation by 50% over the two week period cellular proliferation was considered maximal after surgery. Clinically the aim of these experiments was to achieve the maximal concentration, which did not increase cell death, but decreased Dupuytren fibroblast proliferation for approximately two weeks at the site of operation. As such these findings may have implications for the clinical situation.

5.2. The proliferation of control Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures in vitro

Despite the suggestion that Dupuytren's disease is a proliferative disorder the growth curves for control Dupuytren's disease, non-diseased fascia and carpal ligament cultures were not significantly different in the time period of the experiment (30 days). Had the experiment been conducted over a longer time period potential differences in proliferation may have become obvious. No distinction was made between nodules and cords in Dupuytren's disease tissue specimens and as the curves were the result of the calculated mean cell number at each time point, this may represent a sample error. In general it was not observed that fibroblast cultures were established faster from nodules than cords, though this was not quantified.

5.3. The effect of a single exposure to 5-fluorouracil on fibroblast proliferation in vitro

Despite the control growth curves for Dupuytren's disease, non-diseased fascia and carpal ligament not being significantly different, the effect on fibroblast proliferation of a
single exposure to 5-fluorouracil varied. The cell number at day 30, was significant
decreased for all 5-fluorouracil concentrations tested in Dupuytren fibroblast cultures
(Figure 3), whilst non-diseased fascia fibroblast exhibited a dose-dependent inhibition in
cell numbers (Figure 4). In contrast carpal ligament fibroblast cultures reached cell
numbers of a similar magnitude to controls, independent of all 5-fluorouracil
concentrations tested, at day 30 (Figure 5). This indicates that even a short exposure to
5-fluorouracil has a prolonged effect on fibroblast proliferation in vitro. It furthermore
suggests that Dupuytren fibroblasts are more susceptible to the growth arresting
properties of 5-fluorouracil than carpal ligament. Considering that 5-fluorouracil is
thought to have its greatest effect on rapidly dividing cells (Khaw et al 1992 and 1993),
these results indicate that Dupuytren fibroblasts divide at a faster rate, than carpal
ligament and possibly non-diseased fascia. Khaw et al (1992) furthermore suggested that
a short exposure to 5-fluorouracil selected fibroblast sub-populations, less capable of
proliferating, due to this mechanism.
The duration of 5-fluorouracil exposure was not varied in these experiments, because it
has been suggested that short exposures to high concentrations of 5-fluorouracil may
have the same effect as lower concentrations for longer periods of time (Khaw et al
1992). The optimal concentration for the 5 minute exposure was found to be 2.5 mg/mL
of 5-fluorouracil, because it did not increase cell death significantly as compared to
control cultures. Cell death was not sought increased, to avoid possible necrosis and
therefore increased risk of infection into the proposed delivery site of 5-fluorouracil in
the clinical situation. This concentration furthermore inhibited fibroblast proliferation for
two weeks completely in all cultures and partially for 30 days in Dupuytren and non-
diseased fascia fibroblasts, in contrast to 21 days for carpal ligament cultures. The latter
again suggesting that carpal ligament was inhibited to a lesser degree.
It can however also be argued that this proliferative inhibition would influence the
normal and desired healing of the overlying skin. Timed application of 5-fluorouracil to
the wound only, has been proven not to affect woundhealing in ophthalmic surgery

Though 5-fluorouracil inhibits proliferation in all cells exposed to the drug, by actively
limiting the exposure of 5-fluorouracil a titrated effect could be achieved.
Chapter V

The effects of 5-fluorouracil on α smooth muscle actin production in proliferating Dupuytren's tissue, non-diseased fascia and carpal ligament in vitro
1. **Introduction**

The myofibroblast has been implicated as responsible for the contraction seen in Dupuytren's disease (Schürch et al. 1992) and is easily identifiable by immunohistochemical methods for α-smooth muscle actin (Gabbiani 1995).

2. **Aim**

To investigate the effect of pretreatment with 5-fluorouracil on the production of α-smooth muscle actin in Dupuytren's tissue, non-diseased fascia and carpal ligament.

3. **Methods and Materials**

3.1. **Patients**

The samples examined in this chapter stems from the same patients as in chapter 4 and consist of Dupuytren's disease, non-diseased fascia and carpal ligament.

3.2. **Establishment of monolayer cell cultures**

Glass coverslips were sterilised by immersion in 70% alcohol, then placed in wells and allowed to dry under sterile conditions in a class II hood, designed for human tissue culture. Cells from Dupuytren's tissue, non-diseased fascia and carpal ligament were seeded at a density of 40,000 cells per well in six-well plates, in triplicate for each experiment and allowed to settle for one day before treatment. The cultures were fed twice a week for the duration of the experiment with DMEM (10% foetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin) and kept at in a humid incubator at 37°C, 5% CO₂.

3.3. **Treatment of cell cultures**

The cultures were treated with 5-fluorouracil in serial dilution (0-10 mg/mL) in triplicate for 5 minutes and washed in PBS, before resuspended in media.

3.4. **Fixation of monolayer cultures**

The media is sucked off the cultures, they are rinsed in PBS before suspended in ice cold Methanol for 10 minutes in the freezer (20°C). The coverslips are gently removed from
the wells and fixed to histology slides with nail varnish before stored in the freezer (20°C).

3.5. Staining for alpha smooth muscle actin

The primary antibody (anti alpha actin, Boehringer Mannheim Biochemica) was diluted as follows: 1.3mlPBS:150μl BSA solution (Bovine Serum Albumin, Life Technologies, Paisley): 50μl anti alpha actin and was thereafter kept on ice. One hundred μl of dilute antibody was added to each coverslip and spread over the surface. The slides were incubated for 1 hour in moist conditions before being first washed twice and then immersed in PBS for 10 minutes. The secondary antibody was diluted as follows: 3.59 mL PBS: 400μl BSA: 400μl propidium iodide (Sigma, Poole, UK): 10 μl FITC-conjugated secondary antibody (DAKO, Denmark) and kept on ice in the dark. One hundred μl of dilute secondary antibody was added to each slide and incubated in moist conditions at room temperature for 1 hour in the dark. The slides were then washed and immersed in PBS for 10 minutes before being mounted with DABCO. Specimens were hereafter stored in the freezer until enumeration.

3.6. Enumeration

The cells were counted under UV light, rendering the propidium iodide nuclear stain bright orange and the alpha smooth muscle actin bright green. A minimum of three times 300 cells were counted in each specimen at random according to standard methods and alpha smooth muscle actin positive cells were calculated as a percentage of the total number of cells.

3.7. Statistical analysis

The Wilcoxon paired rank test was used to analyse the effect of 5-fluorouracil exposure on α-smooth muscle actin production.

4. Results

In the same manner as in the previous chapter Dupuytren, non-diseased fascia and carpal ligament fibroblast cell lines were exposed to 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. After immunohistochemical staining the mean percentage of α-smooth muscle actin positive cells was then calculated at each time point. This was
first performed in 3 carpal ligament fibroblast cell lines (Figure 1). α-Smooth muscle actin expression was seen to be inhibited more with rising concentrations of 5-fluorouracil. Cultures rinsed with PBS for 5 minutes acted as control and exhibited a constant low expression of α-smooth muscle actin throughout the experiment after day 2 (0.75 %). Due this low level of α-smooth muscle actin expression no cultures treated with 5-fluorouracil were significantly different from the control (p > 0.15).

Figure 1: The percentage of α-smooth muscle actin positive cells in carpal ligament cultures exposed to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.

Curves for α-smooth muscle actin positive cells as a percentage of total number of cells in carpal ligament fibroblast cell lines, exposed to 5-fluorouracil for 5 minutes in serial dilution ranging from 0-10 mg/mL. All experiments were performed in triplicate. N = 3 (two female and one male, mean age : 52 +/- SD 9 years). The number of α-smooth muscle actin positive cells as percentage of total is represented on the y-axis and the time (days) on the x-axis. The concentration of 5-fluorouracil used is presented in the individual colour coded legend.
The experiment was repeated with non-diseased fascia fibroblast cultures (Figure 2). \( \alpha \)-smooth muscle actin positivity was seen to be inhibited more with rising concentrations of 5-fluorouracil. Cultures rinsed with PBS for 5 minutes acted as control and exhibited a rising expression of \( \alpha \)-smooth muscle actin throughout the experiment after day 2. The myofibroblast differentiation in cultures treated with 0.625 mg/mL of 5-fluorouracil was delayed until day 7 and the subsequent rise in levels attained non-treated levels at day 30. Cultures treated with 1.25, 2.5 and 5 mg/mL 5-fluorouracil exhibited an inhibition of \( \alpha \)-smooth muscle actin positivity until day 14 after which the fibroblasts recommenced differentiation. The subsequent rise in levels was smaller with increasing 5-fluorouracil concentration. Cultures treated with 10 mg/mL 5-fluorouracil were inhibited until day 21 after which the \( \alpha \)-smooth muscle actin positivity again began to rise.

![Graph showing the percentage of \( \alpha \)-smooth muscle actin positive cells in non-diseased fascia cultures exposed to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.](image)

**Figure 2:** The percentage of \( \alpha \)-smooth muscle actin positive cells in non-diseased fascia cultures exposed to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.

Curves for \( \alpha \)-smooth muscle actin positivity in 3 non-diseased fascia fibroblast cell lines treated with 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. All experiments were performed in triplicate. \( N = 3 \) (all male, mean age: 65 +/- SD 16 years). The number of \( \alpha \)-smooth muscle actin positive cells as percentage of total is represented on the y-axis and the time (days) on the x-axis. The concentration of 5-fluorouracil used is presented in the individual colour coded legend.
This experiment was again repeated with Dupuytren fibroblast cell lines (Figure 3). Proliferation was seen to be inhibited more with rising concentrations of 5-fluorouracil. Cultures rinsed with PBS for 5 minutes acted as control and exhibited an almost constant expression of α-smooth muscle actin throughout the experiment after day 2. The α-smooth muscle actin positivity in cultures treated with 0.625 and 1.25 mg/mL of 5-fluorouracil was delayed until day 7 though the subsequent rise in α-smooth muscle actin was smaller with increasing 5-fluorouracil concentration. The α-smooth muscle actin expression was inhibited until day 14 in cultures treated with 2.5 mg/mL 5-fluorouracil after which the levels began to rise. This concentration proved to cause significant (p < 0.03) inhibition of the myofibroblast differentiation in Dupuytren fibroblasts. Concentrations of 5 and 10 mg/mL exhibited a paradoxical decline in α-smooth muscle actin positivity from day 2 to day 14, for thereafter to rise. Both cultured were significantly inhibited compared to control (p < 0.007).

![Graph showing the percentage of α-smooth muscle actin positive cells in Dupuytren fibroblast cultures exposed to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.](image)

**Figure 3:** The percentage of α-smooth muscle actin positive cells in Dupuytren fibroblast cultures exposed to 5-fluorouracil in serial dilution (0-10 mg/mL) for 5 minutes.

*Curves for α-smooth muscle actin positivity in 6 Dupuytren fibroblast cell lines (four primary and two recurrent) treated with 5-fluorouracil for 5 minutes in serial dilution from 0-10 mg/mL. All experiments were performed in triplicate. N = 6 (all male, mean age: 65 +/- 8 years). The number of α-smooth muscle actin positive cells as percentage of total is represented on the y-axis and the time (days) on the x-axis. The concentration of 5-fluorouracil used is presented in the individual colour coded legend.*
From the above results the mean α-smooth muscle actin positivity was calculated as a percentage of total number of cells at each time point in Dupuytren’s tissue, non-diseased fascia and carpal ligament under standard culture conditions, representing control values (Figure 4).

The percentage of myofibroblasts were seen to be relatively high in Dupuytren's disease (mean = 3.5 %) throughout the experiment, in contrast to carpal ligament which were consistently low (mean = 0.75 %). The percentage of myofibroblasts in non-diseased fascia was 0 at day 2, for thereafter to rise in a linear fashion until reaching Dupuytren's disease levels (3.5 %) at day 30 (mean = 2 %).

Figure 4: Myofibroblasts in un-treated Dupuytren’s disease, non-diseased fascia and carpal ligament

The x-axis represents the number of days the cells were maintained in culture, the y-axis the number of alpha smooth muscle actin positive cells as a percentage of total. Dupuytren’s disease (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) cultures are colour coded in the legend which also denotes that the cell lines were not exposed to 5-fluorouracil.
From previous experiments (Chapter IV) it was shown that 2.5 mg/mL 5-fluorouracil did not increase the cell mortality and the effect on α-smooth muscle actin production arising from the use of this concentration was therefore investigated further. The number of alpha smooth muscle actin positive cells as percentage of total number of cells in carpal ligament fibroblasts treated with 2.5 mg/mL of 5-fluorouracil were first compared to untreated controls (Figure 5). The un-treated fibroblasts exhibited a very low rate of differentiation ( <1%) and hence the treatment with 5-fluorouracil became insignificant (Wilcoxon signed rank test p>0.8).

![Graph](image)

**Figure 5: Number of alpha smooth muscle actin positive cells as percentage of total number of cells in carpal ligament fibroblasts exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes and un-treated controls.**

*α-smooth muscle actin positive cells in carpal ligament cultures (n = 3) after 2.5 mg/mL 5-fluorouracil exposure for 5 minutes and control. The x-axis represents the days the experiment lasted and the y-axis the number of α-smooth muscle actin positive cells as a percentage of total number of cells.*
The effect of the same concentration of 5-fluorouracil was investigated in non-diseased fascia cultures (Figure 6). The control cell-lines exhibited an ever increasing amount of alpha smooth muscle actin related to cell density and time. The 5-fluorouracil treated cells were significantly suppressed at day 30 only at this concentration of 5-fluorouracil (Wilcoxon signed rank test day 2-21, p>0.05, day 30, p<0.0039).

![Graph showing number of alpha smooth muscle actin positive cells as percentage of total number of cells in non-diseased fascia fibroblasts exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes and untreated controls.](image)

Figure 6: Number of alpha smooth muscle actin positive cells as percentage of total number of cells in non-diseased fascia fibroblasts exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes and un-treated controls.

α-smooth muscle actin positive cells in non-diseased fascia cultures \( (n = 3) \) after 2.5 mg/mL 5-fluorouracil exposure for 5 minutes and control. The x-axis represents the days the experiment lasted and the y-axis the number of α-smooth muscle actin positive cells as a percentage of total number of cells.
Analysing the effect of the same concentration in Dupuytren fibroblasts (Figure 7) it was shown that the \( \alpha \)-smooth muscle actin production in control cultures maintained a relatively high proportion of myofibroblasts throughout the experiment (mean = 3.5\%). Exposed to 2.5 mg/mL 5-fluorouracil the expression of alpha smooth muscle actin was suppressed from day 0-30 to a significant degree (Wilcoxon signed rank test, \( p<0.003 \)).

**Figure 7:** Number of alpha smooth muscle actin positive cells as percentage of total number of cells in Dupuytren fibroblasts exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes and un-treated controls.

\( \alpha \)-smooth muscle actin positive cells in Dupuytren's disease cultures (\( n = 6 \)) after 0 or 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The x-axis represents the days the experiment lasted and the y-axis the number of \( \alpha \)-smooth muscle actin positive cells as a percentage of total number of cells. Number of alpha smooth muscle actin positive cells as percentage of total in non-diseased fascia fibroblasts treated with 2.5 mg/mL 5-fluorouracil and un-treated controls.
The effect of 2.5 mg/mL 5-fluorouracil on myofibroblast differentiation in Dupuytren, non-diseased fascia and carpal ligament was then compared (Figure 8).

It was shown that 5-fluorouracil almost suppressed myofibroblast differentiation in carpal ligament cultures for 30 days. In contrast the effect lasted for approximately two weeks in Dupuytren and non-diseased fascia fibroblasts. The latter two cultures exhibited a similar rise in α-smooth muscle actin positive cells after this time point, which was significantly different (p < 0.05) from the level found in carpal ligament fibroblasts.

Figure 8: Number of α-smooth muscle actin positive cells as percentage of total number of cells in Dupuytren, non-diseased fascia and carpal ligament fibroblasts treated with 2.5 mg/mL 5-fluorouracil for 5 minutes.

α-smooth muscle actin positive cells in Dupuytren's disease cultures (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) after 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The x-axis represents the time in days of the duration of the experiment and the y-axis the number of α-smooth muscle actin positive cells as a percentage of total number of cells.

Finally the percentage of α-smooth muscle actin positive cells in cultures treated with 2.5 mg/mL 5-fluorouracil from Dupuytren, non-diseased fascia and carpal ligament fibroblast
cultures were compared with controls (Figure 9). It became evident that carpal ligament fibroblasts exhibited the lowest proportion of myofibroblast differentiation of all cultures, irrespective of 5-fluorouracil exposure. Control Dupuytren fibroblast differentiation was therefore consistently and significantly (p< 0.0005) elevated compared to both 5-fluorouracil treated and un-treated carpal ligament differentiation throughout the experiment. In contrast control non-diseased fascia fibroblast myofibroblast differentiation only became significantly different from control and treated carpal ligament cultures after day 14. Control Dupuytren fibroblasts was significantly higher than control non-diseased fascia until day 14 only, but remained significantly higher than 5-fluorouracil exposed non-diseased fascia for 30 days.

![Graph showing myofibroblast differentiation in different tissues with and without 5-fluorouracil exposure.](image)

**Figure 9:** Myofibroblast in all tissues with and without 5-fluorouracil exposure

α-smooth muscle actin positive cells in Dupuytren's disease cultures (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) after 0 or 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The x-axis represents the time in days of the duration of the experiment and the y-axis the number of α-smooth muscle actin positive cells as a percentage of total number of cells.
Representative micrographs of 5-fluorouracil (2.5 mg/mL) exposed and control cultures were also obtained after immunohistochemical staining for α-smooth muscle actin positive cells (Figures 10-11). The apparent morphology of the treated cells did not seem to change significantly, though the occasional formation of ‘blebs’ in the cytoplasmic extensions was observed after 5-fluorouracil exposure. This change diminished with time and was not quantified.

Figure 10: Micrograph α-smooth muscle actin positive cells in control Dupuytren fibroblast cultures under standard cultures conditions.

Monolayer culture of control Dupuytren fibroblasts at day 14 after staining for α-smooth muscle actin positive cells at x400 magnification. The nuclei were counterstained with propidium iodide and appear orange, whilst the α-smooth muscle actin became bright green under ultraviolet light.
Figure 11: Micrograph α-smooth muscle actin positive cells in Dupuytren fibroblast cultures after 5-fluorouracil exposure.

Monolayer culture of 5-fluorouracil exposed Dupuytren fibroblasts at day 14 after staining for α-smooth muscle actin positive cells at x400 magnification. The nuclei were counterstained with propidium iodide and appear orange, whilst the α-smooth muscle actin became bright green under ultraviolet light.
5. Discussion

5.1. Myofibroblast differentiation in vitro

As discussed in the previous chapter there are limitations in applying cell culture findings to the situation in the hand after Dupuytren surgery. The differentiation of myofibroblasts may not only be influenced by specific cytokines in the serum (the role of TGFβ1 will be investigated in chapter VI), but also by other factors in the local cellular environment. Furthermore the myofibroblast, has been found to be more or less prominent at different stages of Dupuytren's disease as divided by Luck's classification (Luck 1959). The myofibroblast was suggested to be very prominent in the proliferative (stage I), less so in the involutional (stage II) and almost, if not completely absent in the residual (stage III) (Schürch et al 1992). Tissue utilised in these experiments was classified as Lucks stage III. It may however be argued that the stage of the tissue, from which the cell-lines were established becomes immaterial when optimal cellular conditions are provided in vitro. However, in standardising these experiments, the comparison of the results was facilitated.

5.2. The myofibroblast differentiation in control Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures in vitro

α-smooth muscle actin production representing the metamorphosis of fibroblasts to myofibroblasts was shown to be significantly different in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures. Carpal ligament expressed below 1 % α-smooth muscle actin throughout the experiment. This is in accordance with the suggestion that carpal tunnel syndrome does not arise as a complication following contraction of the ligament (Paulson et al 1986). Non-diseased fascia fibroblast cultures were shown to increase the percentage of myofibroblasts with time and increasing cell density. Myofibroblast levels in non-diseased fascia was therefore found to be similar to carpal ligament fibroblast cultures from day 2-14 and similar to those found in Dupuytren fibroblasts from day 21-30. This could potentially be seen as an indication of non-diseased fascia fibroblasts' ability to attain α-smooth muscle actin expression akin to that seen in Dupuytren fibroblasts under specific circumstances, related to time and cell density. Dupuytren's disease fibroblasts expressed significantly (p< 0.0005) more α-
smooth muscle actin than any other cell lines throughout the experiment, independent of cell density. The absolute mean of myofibroblasts present in Dupuytren fibroblast cultures was 4.5%. This was significantly less than the 1-26% (mean 14 +/- 8%) of α-smooth muscle actin which Tomasek and Rayan (1995) found in fibroblast cultures obtained by explant from Dupuytren nodules. Cultures in these experiments were not classified according to whether the original tissue was nodule or cord, which may represent a sample error and explain large standard error values.

Tomasek and Rayan (1995) furthermore utilised cultures below passage 10, whilst cultures in these experiments had not been passaged more than four times. Furthermore they assayed cultures after only 2 days of culture on glass coverslips, whilst the fibroblasts in these experiments were assayed at day 2, 7, 14, 21 and 30 days. These factors may explain part of the difference in the results obtained.

5.3. The effect of a single exposure to 5-fluorouracil on fibroblast to myofibroblast differentiation in vitro

Hitherto the suggested effects of 5-fluorouracil has been limited to cytostatic and cytotoxic mechanisms, depending on the dose utilised. In these experiments it was shown that 5-fluorouracil exposure decreased the percentage of fibroblasts expressing α-smooth muscle actin in vitro and it is therefore proposed that 5-fluorouracil may influences the production of proteins. This is in accordance with previous suggestions (Blumenkranz et al. 1984) that 5-fluorouracil may be converted into its corresponding ribophosphate, which is then incorporated into RNA. This would result in the production of abnormal ribosomes, altered translation from messenger RNA and abnormal protein synthesis.

The effect on α-smooth muscle actin production was shown to be significantly decreased in a dose-dependant manner in Dupuytren fibroblast cultures throughout the time period of the experiment. Non-diseased fascia fibroblast cultures also exhibited a dose-dependent inhibition of myofibroblast differentiation after day 14, which reached significant levels as control cultures obtained a measurable level of α-smooth muscle actin. Insufficient numbers of carpal ligament fibroblasts expressed α-smooth muscle actin for 5-fluorouracil to effect a significant decrease.

With a 5-fluorouracil induced decrease in the percentage of α-smooth muscle actin positive cells, it may furthermore be hypothesised that the myofibroblast production of collagen type III (Gabbiani et al. 1976) may be reduced. This would ultimately alter the
collagen ratio in Dupuytren's disease. The same could be hypothesised for the non-
diseased fascia, which has been shown to contain an increased ratio of type III to type I
collagen, even before clinical symptoms and signs of Dupuytren's disease appear
(Fitzgerald et al 1995). This aspect of 5-fluorouracil exposure remains to be investigated,
but may merit further study.

*5-fluorouracil have been shown to inhibit myofibroblast differentiation in Dupuytren
and non-diseased fascia fibroblasts in a dose-dependent manner. By actively limiting
the exposure of 5-fluorouracil a titrated effect could be achieved.*
Chapter VI

The effect of Transforming Growth Factor $\beta_1$ on proliferation and production of alpha smooth muscle actin in Dupuytren's tissue, non-diseased fascia and carpal ligament and the effect of 5-fluorouracil treatment
1. Introduction

Hypertrophic scar fibroblasts exhibit greater sensitivity to the fibroblast-myofibroblast differentiation effects of Transforming Growth Factor β1 (TGFβ1) than fibroblasts derived from normal skin or normal scars. Furthermore TGFβ1 appears to inhibit normal fibroblast growth, whereas it appears to induce growth of hypertrophic scar fibroblasts (unpublished data from Dr C Linge, RAFT).

2. Aim

The aim of these experiments was to examine the effect of TGFβ1 on proliferation and alpha smooth muscle actin production in fibroblasts grown from Dupuytren's tissue, non-diseased fascia and carpal ligament and whether any stimulatory effect could be inhibited by 5-fluorouracil.

3. Patients, Material and Methods

3.1. Patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of Patients</th>
<th>Gender Distribution</th>
<th>Mean Age (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dupuytren's disease</td>
<td>6</td>
<td>one female and five male</td>
<td>57 ± 4 years</td>
</tr>
<tr>
<td>Non-diseased fascia</td>
<td>3</td>
<td>one female and two male</td>
<td>63 ± 6 years</td>
</tr>
<tr>
<td>Carpal ligament</td>
<td>3</td>
<td>one female and two male</td>
<td>64 ± 17 years</td>
</tr>
</tbody>
</table>

Table 1: Patient data for in vitro experiments with TGFβ1

3.2. Establishment of monolayer cultures with TGFβ1

Glass coverslips were sterilised by immersion in 70% alcohol, then placed in wells and allowed to dry under sterile conditions in a class II hood, designed for human tissue culture. Cells from Dupuytren's tissue, non-diseased fascia and carpal ligament were seeded at a density of 20,000 cells per well in six-well plates, in triplicate for each experiment and allowed to settle for one day before treatment. The cultures were suspended in DMEM (10% foetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin) with 2 ng/mL TGFβ1 (human, recombinant, Sigma), left undisturbed for five days in a humid incubator at 37°C, 5% CO₂. After treatment the
media, now without TGFβ₁, was changed twice a week. DMEM was found to contain 500 pg TGFβ₁ by ELISA techniques by Dr C Linge and Mr J Shelton and was therefore not thought to influence the outcome of the results.

The concentration of TGFβ₁ used was derived from experiments conducted by Dr C Linge and Mr J Shelton at the RAFT Institute of Plastic Surgery in the same manner as described above (Figure 1). TGFβ₁ concentrations ranging from 0.001-1 ng/mL produced a marked increase in myofibroblast differentiation. This maximal increase was not significantly different for TGFβ₁ concentration between 1-5 ng/mL.

![Graph](image)

**Figure 5:** Titration of TGFβ₁ effects on normal cutaneous fibroblasts

One experiment conducted in triplicate with normal skin fibroblasts derived by explant from a 34 year old female. TGFβ₁ was added to the culture medium as described above. The x-axis represents the different concentrations of TGFβ₁ studied, whilst the y-axis represents the number of α-smooth muscle actin positive cells as a percentage of total number of cells.

### 3.3. 5-fluorouracil treatment of cell cultures

On day five the cell cultures were treated with 2.5 mg/mL of 5-fluorouracil (David Bull Laboratories, Warwick) for five minutes, then washed in PBS, whilst the control cultures were washed in PBS only.
3.4. Staining for alpha smooth muscle actin

The staining procedure for α-smooth muscle actin has been described in Chapter V.

3.5. Enumeration

The method of enumeration has been described in Chapter V.

3.6. Statistical analysis

The Mann Whitney U test was used to analyse the differences in TGFβ1 treated cultures and controls, whilst the Wilcoxon paired rank test was used to analyse the effect of 5-fluorouracil exposure.

4. Results

4.1. The influence of TGFβ1 on myofibroblast differentiation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

The myofibroblast differentiation after TGFβ1 stimulation in Dupuytren, non-diseased fascia and carpal ligament fibroblasts was first compared at day 2 (Figure 2). Dupuytren fibroblasts exhibited the highest number of α-smooth muscle actin positive cells as a percentage of total number of cells in culture (13 % +/- SE 2 %) after TGFβ1 stimulation. This was found to be highly significant (p < 0.0001).

In contrast carpal ligament fibroblasts exhibited the lowest percentage of myofibroblast differentiation (3.6 % +/- SE 0.6 %) after TGFβ1 exposure, but this was also found to be highly significant (p < 0.001). Due to the minimal myofibroblast proportion present in control non-diseased fascia cultures at day 2, the increase in percentage of α-smooth muscle actin positive cells following TGFβ1 treatment (7.2 % +/- SE 2.2) in this cell line is the greatest of the tissues examined (p < 0.0001).

If the α-smooth muscle actin positive cells as a percentage of total number of cells in TGFβ1 treated cell lines are compared with non TGFβ1 treated, the increase in both Dupuytren and carpal ligament fibroblasts is approximately four-fold, whilst there is an approximately 70 x increase in non-diseased fascia.
Figure 2: Number of α-smooth muscle actin positive cells as a percentage of total number of cells in Dupuytren, non-diseased fascia and carpal ligament fibroblasts at day 2 after TGFβ1 treatment.

The number of α-smooth muscle actin positive cells as a percentage of total number of cells in cell cultures derived from Dupuytren's disease (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) at day 2 with and without TGFβ1 (2 ng/mL TGFβ1 for 5 days) stimulation. The x-axis represents data from the tissues examined, a solid column denoting control and the striped column representing TGFβ1 treated cultures as represented in the legend.
In order to further investigate the longterm effect of a single exposure to TGFβ₁ on Dupuytren fibroblast cultures the results for the whole duration of the experiment were compared with controls (Figure 3).

Initially the percentage of myofibroblasts as a percentage of total number of cells declined from day 2 to 7, to stabilise at approximately 8%. The increase in myofibroblast differentiation shown at day 2 (figure 2) was however sustained at a significant level (p< 0.05) in Dupuytren cultures throughout the time period examined.

Figure 3: Number of α-smooth muscle actin positive cells as a percentage of total number of cells in Dupuytren fibroblasts from day 2-30 after TGFβ₁ exposure.

Dupuytren fibroblast cultures exposed to TGFβ₁ for 5 days and controls (n = 6).
The x-axis represents time in days and the y-axis represents the number of number of α-smooth muscle actin positive cells as a percentage of total. The legend is colour coded for TGFβ₁ exposure.
The same experiment was performed with non-diseased fascia fibroblast cultures (Figure 4). In contrast to Dupuytren fibroblast cultures, non-diseased fascia cultures the initial significant increase at day 2 (Figure 1) in myofibroblasts after TGFβ1 exposure became in-significant after day 2 (p>0.1). This was due to the control cultures increasing the alpha smooth muscle actin production with increasing cell density and time. The effect of TGFβ1 was seen to diminish after day 2, unlike Dupuytren's disease (Figure 2), but at day 30 the non-stimulated culture has increased the myofibroblast differentiation to a significant degree compared to the TGFβ1 stimulated cell line (p<0.0002).

Figure 4: Number of α-smooth muscle actin positive cells as a percentage of total number of cells in non-diseased fascia fibroblasts from day 2-30 after TGFβ1 exposure.

Non-diseased fascia cultures (n = 3) exposed to TGFβ1 for 5 days and controls. The x-axis represents time in days and the y-axis represents the number of number of α-smooth muscle actin positive cells as a percentage of total.
In accordance with the previous chapter these experiments were finally conducted with carpal ligament fibroblast cultures (Figure 5).

As with non-diseased fascia and in contrast to Dupuytren fibroblasts the TGFβ1 induced increase in carpal ligament fibroblast differentiation is only evident at day 2, but not at later time points (p > 0.2)

![Figure 5: Number of α-smooth muscle actin positive cells as a percentage of total number of cells in carpal ligament fibroblasts from day 2-30 after TGFβ1 exposure.](image)

Non-diseased fascia cultures (n = 3) exposed to TGFβ1 for 5 days and controls. The x-axis represents time in days and the y-axis represents the number of number of α-smooth muscle actin positive cells as a percentage of total.

4.2. The influence of 5-fluorouracil on TGFβ1 treated myofibroblast differentiation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

Having investigated the effect of 2 ng/mL TGFβ1 for 5 days on cell cultures derived from Dupuytren's disease, non-diseased fascia and carpal ligament, these cultures were subjected to 5-fluorouracil exposure. The effect of 5-fluorouracil was primarily studied on day two for Dupuytren fibroblast cultures (Figure 6). At this time point there was no significant difference in total number of cells.
When comparing TGFβ1-stimulated and non-stimulated Dupuytren cultures with their 5-fluorouracil inhibited parallel cultures, it became evident that despite the increase in myofibroblast differentiation after stimulation, 5-fluorouracil still inhibited this differentiation to a significant degree at this time point (p<0.009). Further comparing other cultures it became evident that control Dupuytren fibroblast cultures exposed to 5-fluorouracil were significantly different from cultures treated with TGFβ1 and exposed to 5-fluorouracil (p < 0.0001).

Figure 6: Number of α-smooth muscle actin positive cells as a percentage of total number of cells for TGFβ1 treated Dupuytren fibroblast cultures and controls compared with cultures exposed to 5-fluorouracil at day 2.

Cell cultures of Dupuytren’s disease (n = 6) day 2 with and without TGFβ1 (2 ng/mL TGFβ1 for 5 days) stimulation and 2.5 mg/mL 5-fluorouracil. The x-axis represents Dupuytren fibroblast cultures with and without TGFβ1 treatment, the legend indicates whether these cultures have been treated with 5-fluorouracil and the y-axis represents the number of α-smooth muscle actin positive cells as a percentage of total number of cells. Cultures were exposed to TGFβ1 for 5 days prior to 5-fluorouracil treatment, control cultures were not exposed to TGFβ1 prior to 5-fluorouracil treatment.
In the same manner as before the whole time period of the experiment was furthermore studied for Dupuytren (Figure 7), non-diseased fascia and carpal ligament fibroblasts. TGFβ1-stimulated Dupuytren fibroblasts exhibited a decrease in myofibroblast differentiation from day 2 to 7 after removal of TGFβ1. After this time the level stabilised at approximately 8%. Fibroblasts exposed to TGFβ1 and then 5-fluorouracil exhibited a parallel drop to a lower level from day 2 to 7 and appeared to recommence differentiation to reach non-5-fluorouracil treated cultures at day 21.

When TGFβ1 stimulated cultures were treated with 2.5 mg/mL 5-fluorouracil for 5 minutes, the ensuing decrease in myofibroblast proportion was significant (p<0.009), until day 14, after which it ceased to be significant (p>0.1).

**Figure 7: Number of α-smooth muscle actin positive cells as a percentage of total number of cells for TGFβ1 treated Dupuytren fibroblast cultures from day 2-30, compared with cultures exposed to 5-fluorouracil.**

_Dupuytren's disease fibroblast cultures (n = 6) treated with 2 ng/mL TGFβ1 for 5 days prior to 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The graph is colour coded for TGFβ1 treatment and the legend is colour coded for 5-fluorouracil treatment._
In the same manner results from non-diseased fascia fibroblast cultures treated with TGFβ1 and 5-fluorouracil were analysed further (Figure 8). TGFβ1 treated non-diseased fascia fibroblast cultures without 5-fluorouracil exposure exhibited a decrease in myofibroblast numbers from day 2 to 7 and thereafter stabilised at approximately 2%. The TGFβ1 and 5-fluorouracil treated cultures exhibited a parallel curve, but the decrease in myofibroblast differentiation after 5-fluorouracil treatment in TGFβ1 stimulated cultures of non-diseased fascia was insignificant (p>0.1).

![Graph](image)

**Figure 8**: Number of α-smooth muscle actin positive cells as a percentage of total number of cells for TGFβ1 treated non-diseased fascia fibroblast cultures from day 2-30, compared with cultures exposed to 5-fluorouracil.

*Non-diseased fascia fibroblast cultures (n = 3) treated with 2 ng/mL TGFβ1 for 5 days prior to 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The graph is colour coded for TGFβ1 treatment and the legend is colour coded for 5-fluorouracil treatment.*
Finally the same analysis was performed for carpal ligament fibroblast cultures (Figure 9). The apparent TGFβ1 induced increase in myofibroblasts decreases from day 2 to 7 for both 5-fluorouracil treated cultures and controls and thereafter stabilised at a low level. The myofibroblast differentiation in carpal ligament fibroblasts treated with TGFβ1 and 5-fluorouracil was significantly decreased at day 2 (p<0.02), but ceased to be significant thereafter (p>0.05).

Figure 9: Number of α-smooth muscle actin positive cells as a percentage of total number of cells for TGFβ1 treated carpal ligament fibroblast cultures from day 2-30, compared with cultures exposed to 5-fluorouracil.

Carpal ligament fascia fibroblast cultures (n = 3) treated with 2 ng/mL TGFβ1 for 5 days prior to 2.5 mg/mL 5-fluorouracil exposure for 5 minutes. The graph is colour coded for TGFβ1 treatment and the legend is colour coded for 5-fluorouracil treatment.

4.3. The influence of TGFβ1 on proliferation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

The effect of TGFβ1 on fibroblast cultures derived from Dupuytren, non-diseased fascia and carpal ligament was also investigated. The effect of TGFβ1 was first studied in Dupuytren fibroblast cultures (Figure 10).

Growth curves for both TGFβ1 treated and control cultures exhibited an almost linear growth throughout the experiment. Non TGFβ1 treated cells however reached a static
number of cells at day 21, which did not increase to day 30. In contrast TGFβ1 treated cell numbers increased in the same time period.

Treatment with TGFβ1 was seen to inhibit the proliferation significantly from day 7-21 (p<0.04) only. At day 30 these cell cultures had proliferated to a degree that cell numbers were not significantly different from control cultures (p > 0.08)

**Figure 10: Growth curves for TGFβ1 treated and control Dupuytren fibroblasts.**

*Dupuytren's disease fibroblast growth curve (n = 6) for TGFβ1 treated fibroblasts and un-treated controls. The x-axis represents time in days, whilst the y-axis represents total number of cells in 10⁶. The legend is colour coded for TGFβ1 exposure.*
The same study was performed in non-diseased fascia fibroblast cultures (Figure 11). Non TGFβ1 exposed fibroblasts exhibited a linear increase in cell number from day 2 to 14, this increase became steeper from day 14 to 21, for thereafter to decrease at day 30. TGFβ1 treated fibroblast exhibited a linear growth curve throughout the 30 days. This was parallel with non TGFβ1 treated cultures from day 2 to 14 only. Therefore the treatment with TGFβ1 inhibited proliferation significantly from day 21 to 30 only (p<0.02).

**Figure 11: Growth curves for TGFβ1 treated and control non-diseased fascia fibroblasts.**

*Non-diseased fascia fibroblast growth curve (n = 3) for TGFβ1 treated fibroblasts and un-treated controls. The x-axis represents time in days, whilst the y-axis represents total number of cells in 10⁴. The legend is colour coded for TGFβ1 exposure.*
This experiment was repeated with carpal ligament fibroblast cultures (Figure 12). Both control and TGFβ₁ treated carpal ligament fibroblast cultures exhibited linear growth curves, but treatment with TGFβ₁ was seen to inhibit proliferation from day 7-30 (p<0.03).

![Growth curves for TGFβ₁ treated and control carpal ligament fibroblasts.](image)

**Figure 12: Growth curves for TGFβ₁ treated and control carpal ligament fibroblasts.**

*Carpal ligament fascia fibroblast growth curve (n = 3) for TGFβ₁ treated fibroblasts and un-treated controls. The x-axis represents time in days, whilst the y-axis represents total number of cells in 10⁴. The legend is colour coded for TGFβ₁ exposure.*
Finally the longterm effect of TGFβ₁ on proliferation was analysed at day 30 (Figure 13), in accordance with the analysis of the effect of 5-fluorouracil on proliferation in chapter IV.

It was seen that the inhibitory effect of TGFβ₁ on proliferation had ceased to obtain significant results in Dupuytren fibroblast cultures ($p > 0.08$). This was in contrast to both non-diseased fascia and carpal ligament fibroblast cultures which were still inhibited significantly at this time point ($p < 0.02$, $p < 0.03$).
4.4. The influence of 5-fluorouracil on proliferation in TGFβ1 treated fibroblasts and controls from Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

In accordance with previous experiments the effect of 2.5 mg/mL on proliferation was assessed in TGFβ1 treated cultures and controls in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures. Dupuytren fibroblast cell lines were assessed primarily (Figure 14).

The TGFβ1 only treated culture exhibit linear growth whilst cultures subsequently treated with 5-fluorouracil barely double in cell number for 30 days. The reduction in proliferation effected by TGFβ1 was therefore further suppressed after 5-fluorouracil treatment from day 7-30 to a significant level (p<0.0005).

![Figure 14: Growth curve for TGFβ1 treated Dupuytren fibroblast cultures exposed to 5-fluorouracil and controls.](image)

*Growth curve for TGFβ1 treated Dupuytren's disease fibroblast (n = 6) exposed to 5-fluorouracil and controls. The x-axis represent the duration in days of the experiment and the y-axis represents the total number of cells in 10⁴. The legend is colour coded for 5-fluorouracil exposure.*
The same experiment was performed with non-diseased fascia fibroblast cultures (Figure 15). TGFβ1 only treated control cultures exhibit a linear growth for 30 days, whilst the additional exposure of 5-fluorouracil inhibited proliferation to a degree which only allowed a sufficient number of cells to divide to maintain the initial cell number. Therefore non-diseased fascia fibroblast cultures the inhibition of proliferation effected by TGFβ1 was further suppressed by the addition of 5-fluorouracil to a significant level (p<0.03) from day 7 onward.

![Graph](image)

**Figure 15: Growth curve for TGFβ1 treated non-diseased fascia fibroblast cultures exposed to 5-fluorouracil and controls.**

*Growth curve for TGFβ1 treated non-diseased fascia fibroblast (n = 3) exposed to 5-fluorouracil and controls. The x-axis represent the duration in days of the experiment and the y-axis represents the total number of cells in 10⁴. The legend is colour coded for 5-fluorouracil exposure.*
Finally the same experiment was performed with carpal ligament fibroblast cultures (Figure 16). Cultures treated only with TGFβ1 exhibited a linear growth throughout the experiment, whilst the addition of 5-fluorouracil inhibited proliferation for 21 days, after which the cells recommenced division. The inhibition of proliferation effected by TGFβ1 as seen previously, was further suppressed by the 5-fluorouracil exposure to a significant level from day 7-30 (p<0.004).

Figure 16: Growth curve for TGFβ1 treated carpal ligament fibroblast cultures exposed to 5-fluorouracil and controls.

*Growth curve for TGFβ1 treated carpal ligament fibroblast (n = 3) exposed to 5-fluorouracil and controls. The x-axis represent the duration in days of the experiment and the y-axis represents the total number of cells in 10⁴. The legend is colour coded for 5-fluorouracil exposure.*
As with previous proliferation experiments, the effect of 5-fluorouracil on TGFβ₁ treated fibroblast at day 30 were analysed for Dupuytren, non-diseased fascia and carpal ligament cultures (Figure 17).

In all cultures the exposure to 5-fluorouracil effected a significant decrease in cell numbers at this time point, despite the inhibition by TGFβ₁. Dupuytren fibroblasts: p < 0.0005, non-diseased fascia fibroblasts: p < 0.03, carpal ligament fibroblasts: p < 0.004.

![Graph showing fibroblast numbers](image)

Figure 17: Number of fibroblasts at day 30 after TGFβ₁ treatment in Dupuytren, non-diseased fascia and carpal ligament cultures.

The number of fibroblasts in cell cultures derived from Dupuytren’s disease (n = 6), non-diseased fascia (n = 3) and carpal ligament (n = 3) at day 30 after TGFβ₁ (2 ng/mL TGFβ₁ for 5 days) stimulation and 5-fluorouracil exposure at day 30. The x-axis represents data from the tissues examined, a solid column denoting TGFβ₁ treated and the striped column representing cultures exposed to 5-fluorouracil as represented in the legend.

Finally the effect on proliferation of TGFβ₁ and 5-fluorouracil was compared in Dupuytren, non-diseased fascia and carpal ligament cultures at day 30 (Figure 18).
To summarise previous results the proliferation of Dupuytren fibroblasts was no longer significantly inhibited by TGFβ1 at day 30. Proliferation in both TGFβ1 treated and control cultures were however significantly inhibited by 5-fluorouracil at this time point. Furthermore the decreased number of fibroblasts effected by 5-fluorouracil was significantly lower than after TGFβ1 treatment ($p < 0.006$). In non-diseased fascia cultures TGFβ1 caused a significant decrease in proliferation. 5-fluorouracil exposure decreased proliferation to a significant degree in both TGFβ1 treated and control cultures, but the final cell number after either TGFβ1 or 5-fluorouracil was not significantly different ($p > 0.26$). In carpal ligament cultures TGFβ1 caused a significant decrease in cell numbers at day 30, whilst 5-fluorouracil did not. This was in contrast to TGFβ1 treated cultures, where 5-fluorouracil did decrease proliferation to a significant degree. The cell numbers after TGFβ1 or 5-fluorouracil exposure did however not differ significantly ($p > 0.5$).

![Graph](image)

**Figure 18: The effect on proliferation at day 30 of TGFβ1 and 5-fluorouracil compared in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures.**

*The number of fibroblasts in cell cultures derived from Dupuytren’s disease ($n = 6$), non-diseased fascia ($n = 3$) and carpal ligament ($n = 3$) at day 30 after TGFβ1 (2 ng/mL TGFβ1 for 5 days) stimulation, 5-fluorouracil exposure at day 30 and controls. The x-axis represents data from the tissues examined and the colour coded legend denotes whether the cultures have been exposed to TGFβ1, 5-fluorouracil or both.*
5. Discussion

5.1. TGFβ₁ induced myofibroblast differentiation in vitro.

As discussed in the previous chapters there are limitations in applying cell culture findings to the situation in the hand after Dupuytren surgery. The differentiation of myofibroblasts may not only be influenced by TGFβ₁, but also by other factors in the local cellular environment. As the timing and exact physiological concentrations of TGFβ₁ during the development of Dupuytren's disease it is not known, it can be hypothesised that these levels fluctuate in the disease process. A review of the results obtained in the literature may elucidate the action of TGFβ₁ on Dupuytren's disease, but the complex nature of the disease process merits further investigation. In order to fully investigate the role of TGFβ₁ further studies are suggested including pulsed and continuous exposure at different concentrations of various combinations of growth factors.

5.2. The effect of TGFβ₁ on myofibroblast differentiation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures.

A concentration of 2 ng/mL TGFβ₁ for 5 days was seen to significantly increase the myofibroblast differentiation in non-diseased fascia and carpal ligament cultures for two days only, but caused a sustained increased expression of α-smooth muscle actin in Dupuytren fibroblast cultures. This was in accordance with the results obtained by Linge et al (unpublished data), which suggested that fibroblasts obtained from hypertrophic scar tissue exhibited a greater sensitivity to the myofibroblast inducing effect of TGFβ₁. It may be therefore be suggested that these results support the hypothesis of Dupuytren's disease being a reparative process. The experiments with TGFβ₁ in this chapter were based upon concentrations found to be optimal in normal skin fibroblasts for α-smooth muscle actin production. The total percentage of myofibroblast differentiation obtained with normal skin fibroblasts proved to be far greater than that seen with Dupuytren, non-diseased fascia and carpal ligament fibroblasts. Badalamente et al (1996) investigating both TGFβ₁, TGFβ₂ and a combination of both, found that the initial plating density and the stage (proliferative vs involutional) was very important for the resulting
myofibroblast differentiation, adding one picomolar \(10^{-12}\) TGF\(\beta_1\) every 48 hours for 6 days. It was found that TGF\(\beta_1\) increased the myofibroblast differentiation less than TGF\(\beta_2\) irrespective of plating densities (5000 cells/cm\(^2\) vs 20,000 cells/cm\(^2\)), but that a combination of both growth factors at increased myofibroblast differentiation significantly only at plating densities of 20,000 cells/cm\(^2\). Badalamente et al (1996) found that TGF\(\beta_1\) increased the myofibroblast proliferation by 18% at the low and 19% at the high plating density, which was much lower than the increase in \(\alpha\)-smooth muscle actin positive cells in these experiments (400%). The material used for obtaining the Dupuytren myofibroblasts was nodules, found to be in either the proliferative or involutional stage, in contrast to material used in these experiments which consisted of fibrotic stage samples only. In comparing these results it must however be noted that the plating density, the histological stage of the tissue utilised for establishment of cell cultures, the concentration of and the timing of TGF\(\beta_1\) addition was different to the presented experiments. Badalamente et al (1996) resuspended the Dupuytren fibroblasts in DMEM with Foetal Bovine Serum, which according to Linge et al (unpublished data) contain 500 picomolar of TGF\(\beta_1\) as assessed by the ELISA technique. This may explain the difference in results obtained.

Control non-diseased fascia cultures obtained higher levels than TGF\(\beta_1\) treated cultures at day 30, suggesting that TGF\(\beta_1\) exposure may induce a finite production of \(\alpha\)-smooth muscle actin in vitro independent of cell numbers in contrast to control cultures. Carpal ligament fibroblast cultures exhibited an initial rise in \(\alpha\)-smooth muscle actin positive cells only after TGF\(\beta_1\) exposure, for thereafter to diminish to the low levels of control cultures. It is therefore proposed that carpal ligament fibroblasts express \(\alpha\)-smooth muscle actin only under the influence of TGF\(\beta_1\), but that both control and TGF\(\beta_1\) treated cultures were independent of cell density.

It may therefore be hypothesised that TGF\(\beta_1\) induces a variable response in the different strains of fibroblasts in the hand in Dupuytren's disease.

5.3. The influence of 5-fluorouracil on TGF\(\beta_1\) treated myofibroblast differentiation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

The myofibroblast differentiation in Dupuytren fibroblast cultures was inhibited for 14 days after a single exposure to 5-fluorouracil. In contrast myofibroblast differentiation
was not affected in non-diseased fascia fibroblast by 5-fluorouracil after TGFβ1 exposure and carpal ligament cultures were inhibited at day 2 only. In non-TGFβ1 stimulated cultures the concentration of 2.5 mg/mL was seen to inhibit myofibroblast differentiation for 30 days, suggesting the potency of TGFβ1 as a myofibroblast inducing agent. Further experiments with higher concentrations of 5-fluorouracil may overcome this, but defeat the purpose of using this concentration, which was investigated because it did not increase the number of cell deaths in culture.

The different response of the various cell cultures may be hypothesised to suggest varying level of sensitivity to TGFβ1 and 5-fluorouracil, because a significant decrease was not shown to depend on the absolute percentage of myofibroblasts induced at day 2.

The myofibroblast differentiation in Dupuytren and carpal ligament cultures was significantly inhibited, despite respective control levels being significantly different (Dupuytren: 13.8 % +/- SE 2 %, carpal ligament: 3.6 % +/- SE 0.6 %). In non-diseased fascia cultures however, control levels at day 2 (7.2 % +/- SE 2.2 %) were not significantly inhibited by 5-fluorouracil. This may be due to the larger increase in α-smooth muscle actin positive cells seen in non-diseased fascia cultures after TGFβ1 exposure (70 fold), but it was shown that the single exposure of 5-fluorouracil decreased the level of myofibroblast differentiation in cultures not treated with TGFβ1 at day 30 only. The longterm effect of a single exposure to 5-fluorouracil in non-diseased fascia may therefore not be possible due to the depletory effect of TGFβ1 on α-smooth muscle actin production as discussed above.

### 5.4. The influence of TGFβ1 on proliferation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

TGFβ1 significantly decreased proliferation in Dupuytren's disease from day 2-21. As in the experiments preceding these, Kloen et al (1995) also used normal skin fibroblasts to illustrate the effects of TGFβ1 on proliferation. Kloen et al (1995) utilised TGFβ1 concentrations of 5 ng/mL for 24 hours and found that this concentration stimulated mitogenesis up to five-fold in some Dupuytren cultures the following day, whilst the effect was less in normal fibroblasts. Kloen et al (1995) furthermore observed that simultaneous addition of TGFβ1 and EGF (Epidermal Growth Factor) resulted in synergistic stimulation of DNA-synthesis in Dupuytren cultures, whereas this effect was only additive in normal fibroblasts. Alioto et al (1994) investigated the effect of TGFβ
(not specified) on Dupuytren fibroblasts and found that the optimal concentration of TGFβ for inducing proliferation, measured the following day, was 0.01-1 ng/mL for 24 hours, whilst 2 ng/mL, as used here for 5 days, did not change the proliferation rate significantly. This is in accordance with the results obtained by Linge et al (unpublished data), which suggested that TGFβ1 increased the proliferation in fibroblasts obtained from hypertrophic scar tissue, despite the concentration of TGFβ1 being different. It is however in contrast with the results obtained from the presented experiments. The concentrations used and the time period investigated in the literature differ from those used in these experiments, but serve the purpose of illustrating the variable response of Dupuytren fibroblast proliferation to TGFβ1 exposure. Furthermore over the 30 days investigated, TGFβ1 was seen to decrease proliferation in Dupuytren fibroblasts from day 2-21, in contrast to non-diseased fascia fibroblasts, which were inhibited from day 21-30 and carpal ligament cultures, which were inhibited from day 7-30. This supports the observed difference in the fibroblast cultures investigated found in previous chapters and furthermore suggests that the effect of TGFβ1 on fibroblast proliferation differs from the effect on myofibroblast differentiation. Despite the late effect of TGFβ1 on proliferation in non-diseased fascia and carpal ligament cultures, it is proposed that Dupuytren fibroblasts are more sensitive to TGFβ1 than other fibroblasts investigated, because the inhibitory effect was significant over a longer time period.

5.5. The influence of 5-fluorouracil on proliferation in TGFβ1 treated fibroblasts and controls from Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures

The proliferation in Dupuytren, non-diseased fascia and carpal ligament fibroblast cultures treated with TGFβ1 was decreased further to a significant degree from day 7-30, after 5-fluorouracil exposure. This suggests a synergistic effect on proliferation by TGFβ1 and 5-fluorouracil. Of interest was the observation that at day 30 the cell number in Dupuytren fibroblast cultures was significantly lower after 5-fluorouracil than after TGFβ1 exposure, whilst no significant difference was found in non-diseased fascia and carpal ligament cultures. It is therefore suggested that the inhibitory effect by 5-fluorouracil on proliferation in Dupuytren fibroblast cultures lasts longer and is greater than the effect obtained with TGFβ1. This is in contrast with results from carpal ligament
fibroblast cultures, where the duration of the inhibitory effects was reversed. It is therefore suggested that these observations are in accordance with the suggestion that Dupuytren fibroblasts represent a different population of fibroblasts from non-diseased fascia and carpal ligament. It is furthermore proposed that these results may imply that non-diseased fascia and carpal ligament fibroblasts also differ.

*At the investigated concentrations a single exposure to TGFβ₁ reduced proliferation and increased myofibroblast differentiation, whilst 5-fluorouracil decreased both proliferation and myofibroblast differentiation in Dupuytren fibroblast cultures to a varying degree. This suggests that the influence of TGFβ₁ varies in the pathological process of Dupuytren's disease and that the optimal effect of 5-fluorouracil may be achieved by a single exposure.*
Chapter VII

The contractility of Dupuytren’s fibroblasts and the effect of 5-fluorouracil
1. **Introduction**

A means of studying the contractile forces produced by Dupuytren's contracture cells in 3-dimensional collagen gels is provided by the Culture Force Monitor (Eastwood et al 1994), which measures the quantitative forces generated by cells in culture.

2. **Aim**

To quantify the contraction produced by Dupuytren fibroblasts in a 3-dimensional collagen gel and investigate the effect of pre-treatment with 5-fluorouracil on their contractile potential.

3. **Patients, Materials and Methods**

3.1. **Patients and tissue**

<table>
<thead>
<tr>
<th>Dupuytren's disease</th>
<th>6 patients four primary, two recurrent,</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(one female and 5 male, age: 59+/−SD 8 years)</td>
</tr>
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</table>

**Table 1: Patient data for contraction studies**

3.2. **Establishment of cell cultures**

Cell lines from Dupuytren's disease were grown via explant (see chapter V).

All cells used were below passage 4.

3.3. **The Culture Force Monitor**

The Culture Force Monitor (CFM) was developed to measure cellular contractile forces (Eastwood 1994). Transducer class strain gauges were attached to a central measuring arm, constructed from strips of Copper-beryllium sheet (Goodfellow Metals, Cambridge, UK). As very low deflection was expected a high input signal was applied by means of a ripple free 12 V power supply with the strain gauges having a very high resistance (5000Ω). The output signal was increased by a strain gauge amplifier (RS Components, Rugby, UK), then channeled into a digital autoranging voltmeter, to give a constant visual reading and then into an analog to digital converter (Bytronic, Sutton Coldfield, UK) installed into a PC (40 Mb hard disk, 4Mb RAM) with 386 processing chip. Data was collected at one reading per second of force and time. Post processing of the
experimental data was performed with purpose written software, which averages 600 readings to produce an acceptable smoothing and stores this value against time.

Cell chambers were constructed by casting silicone elastomer (Dow Corning, Milton Keynes, UK) into a polymethylpentene petri dish around a rectangular mold. The silicone elastomer was degassed for 30 minutes prior to casting and 20 minutes after casting. The petri dish and silicone elastomer were then placed at 37°C for 24 hours to harden. After setting the central mold was removed to reveal a channel, hydrophobic in nature, to inhibit cell attachment.

Attachment bars to form physical contact to the collagen gel containing the cells were made from hydrophilic microporous Vyon (Porcair, Lings Lynn, UK) cut into strips and bound together with nylon thread. The attachment bars were connected to the CFM via ‘A’ frames, made from stainless steel suture wire (0.35 mm diameter), taking care all angles were kept at 90° (Figure 1).

Figure 1: Schematic representation of culture force monitor set-up.

*For explanation see text.*
Figure 2: Culture force monitor loaded with gel

For explanation see text and Figure 1.
3.4. **Preparation of gel**

The collagen gel was prepared by mixing 4 mL of native acid soluble type I rat tail collagen (Advanced Protein Products, West Midlands) with 0.5 mL 10 X DMEM (Flow Laboratories, UK). A balanced pH, indicated by a dramatic colour change, was achieved by the dropwise addition of NaOH.

Dupuytren cells were harvested (see S.II.iii) and $4 \times 10^6$ cells aliquoted. The cells were resuspended in 0.5 mL DMEM, with 10% foetal calf serum, 1% glutamine and 1% streptomycin/penicillin, before gently mixing with the neutralised gel (final collagen concentration 1 mg/mL). The collagen/gel suspension was poured into the silicone elastomer chamber between the two Vyon attachment bars, and allowed to set in a humidified incubator (5% CO$_2$, 37°C) for 15 minutes. The cell chamber was then topped up with 15 mL of culture medium before connected to the CFM for 24 hours.

3.5. **Addition of 5-fluorouracil**

Dupuytren fibroblasts in tissue culture flasks were treated with 5-fluorouracil (2.5 mg/mL) for 5 minutes, washed with PBS, after which $4 \times 10^6$ cells were harvested and suspended in collagen gel.

A further set of experiment were conducted with 2 mL 2.5 mg/mL 5-fluorouracil being added to the central channel at 16 hours. This was not washed off as with previous experiments and the cells were thus exposed to 5-fluorouracil for 8 hours after application.

3.6. **Fixation and staining of gels**

Gels were fixed in 2.5 % glutaraldehyde whilst maintaining the tension for a minimum of 3 hours before being stored in PBS.

The gels were stained with Toluidine Blue for 30 seconds before decolouring in distilled water for one week before examined under a 3-D microscope at the Department of Anatomy at University College London.

3.7. **Statistical analysis**

The paired student t-test was used to analyse the effect of 5-fluorouracil exposure on contraction.
4. Results

The contractile curve for the control Dupuytren fibroblasts possessed the steepest gradient within the first three hours of the experiment. Thereafter the gradient diminished, though the cells continued to contract for 24 hours in a near-linear manner. The contraction of the 5-fluorouracil treated fibroblasts appeared to be identical to that of the un-treated cells in the first two hours. Thereafter the cells appeared not to increase their contraction but remain static.

![Graph showing contraction curve](image)

**Figure 3:** Contraction curve of control Dupuytren fibroblast in collagen gel and after 5 minutes of 2.5 mg/mL 5-fluorouracil exposure prior to suspension in gel

Typical contractile curve produced by CFM showing the gross contractile forces produced by Dupuytren fibroblasts as gross force with and without short 5-fluorouracil exposure for a single experiment. Each data point is the average of 600 readings. The x-axis represents time in hours for the duration of the experiment, whilst the y-axis represents the force exerted by the cells measured in dynes ($10^{-3}$ N). The Dupuytren fibroblasts were exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes before suspended in the collagen gel. The time point of 5-fluorouracil treatment was therefore set to 0.
These results presented as the contraction of the treated Dupuytren fibroblasts calculated as a percentage of their un-treated controls show that at 0 hours both 5-fluorouracil treated cells and control have not commenced contraction therefore the starting value was equal. At two hours the 5-fluorouracil treated cells were seen to contract 73% of controls, which was not a significant reduction (p > 0.05). At 12 and 24 hours 5-fluorouracil treated cultures contracted respectively 62% and 61% of controls, which was highly significant (paired t-test: p<0.0007 and p< 0.0004).

![Graph showing contraction of treated Dupuytren fibroblasts as a percentage of untreated cells over 24 hours.](image)

**Figure 4:** Contraction as measured by the CFM of treated Dupuytren fibroblasts as percentage of un-treated cells over 24 hours

*Individual results were calculated as the gross force of contraction produced by 5-fluorouracil treated fibroblasts as a percentage of their corresponding control and represented as a mean of the total number of experiments (n = 6). The x-axis represents the duration of contraction (0-24 hours), whilst the y-axis represents the contraction of 5-fluorouracil treated fibroblast as percentage of control.*

If then looking at the micrographs of the gels of untreated Dupuytren fibroblasts (Figure 5), it was observed, but not quantified that the fibroblasts appeared to be mostly bipolar and exhibited long cytoplasmic extensions, but compared with a micrograph of treated Dupuytren fibroblasts suspended in a collagen gel (Figure 6), it was again observed, but
not quantified that fibroblasts appeared rounded with short with numerous cytoplasmic extensions.

Figure 5: Control Dupuytren fibroblasts suspended in collagen gel after contraction for 24 hours

Image of control Dupuytren fibroblasts after contraction suspended in collagen gel. Gels were stained with Toluidine Blue and a green filter was employed for better visualisation for stereomicroscopic examination (x 400) on an Edge High Definition Stereo Light Microscope (Edge Scientific Instrument Corporation, Los Angeles, CA).
Figure 6: Dupuytren fibroblasts after 5 minutes of 5-fluorouracil exposure prior to suspension in collagen gel after contraction for 24 hours

Image of 5-fluorouracil pre-treated Dupuytren fibroblasts after contraction suspended in collagen gel. Gels were stained with Toluidine Blue and a green filter was employed for better visualisation for stereomicroscopic examination (x 400) on an Edge High Definition Stereo Light Microscope (Edge Scientific Instrument Corporation, Los Angeles, CA).
Experiments were furthermore carried out with the addition of 2.5 mg/mL 5-fluorouracil for 8 hours at the end of the experiment. This contractile curve for the control Dupuytren fibroblasts is similar to the curve described in Figure 3. When 2 mL of 2.5 mg/mL 5-fluorouracil was added to the experiment after 16 hours on the CFM, the obtained contraction at this time was seen to immediately diminish and thereafter remain static.

Figure 7: Contraction curve of control Dupuytren fibroblast in collagen gel and after addition of 2.5 mg/mL 5-fluorouracil at 16 hours

Typical contractile curve produced by CFM showing the gross contractile forces produced by Dupuytren fibroblasts with and without the addition of 5-fluorouracil at 16 hours for a single experiment. Each data point is the average of 600 readings. The x-axis represents time in hours for the duration of the experiment, whilst the y-axis represents the force exerted by the cells measured in dynes (10^3 N). Two mL of 2.5 mg/mL 5-fluorouracil was added at 16 hours to the collagen gel in the central channel and the experiment was left undisturbed for a further 8 hours. The time point of 5-fluorouracil treatment was therefore set to 16.
Micrographs from Dupuytren fibroblasts exposed to 5-fluorouracil for 8 hours were observed to show a marked translucency and fragmentation with pyknotic nuclei and poor cytoplasmic extensions.

Figure 8: Dupuytren fibroblasts in collagen gels after 6 hours of 5-fluorouracil

Image of Dupuytren fibroblasts treated with 5-fluorouracil at time = 16 hours. treated Dupuytren fibroblasts after contraction suspended in collagen gel. Gels were stained with Toluidine Blue and a green filter was employed for better visualisation for stereomicroscopic examination (x 400) on an Edge High Definition Stereo Light Microscope (Edge Scientific Instrument Corporation, Los Angeles, CA).
5. Discussion

Eastwood et al (1994) showed that the increase in force due to collagen gel maturation reached a steady state of \(20 \times 10^5\) N after three hours. In experiments with fibroblasts suspended in the gel, cells were therefore found to contribute little to the net force produced within the first three hours. After this time period a near-linear increase in force was observed until the end of the experiment at 24 hours.

Even after subtraction of the force exerted by the collagen gel maturation in the first three hours the contraction curve produced by the control Dupuytren fibroblasts could be divided into two stages. A rapid contraction lasting approximately 0-3 hours, followed by a slower near-linear contraction lasting to the end of the experiment (24 hours). Eastwood et al (1994) suggested that the first rapid stage of contraction consisted of two phases on the basis of reviewing experiments utilising cytoskeletal poisons and inhibitors of cell function. The first phase of the initial force generation was suggested to stem from the basic cell matrix binding and possibly cytoskeletal assembly to integrins and the second part from normal microtubule formation.

When Dupuytren fibroblasts were exposed to 2.5 mg/mL 5-fluorouracil for 5 minutes only, prior to suspension in collagen gel, the mean contraction was observed to obtain only 75% of the value of the control cultures at two hours. Utilising the paired Student t-test this was statistically insignificant (\(p > 0.05\)). After approximately two hours the contraction curve of the 5-fluorouracil exposed fibroblasts was seen to plateau and remain constant until the end of the experiment at 24 hours. Subsequent calculation of 5-fluorouracil exposed fibroblast contraction as a percentage of control indicated a mean inhibition of contraction of 38% at 12 hours and 39% at 24 hours (paired Student t-test: \(p < 0.0007\) at 12 hours and \(p < 0.0004\) at 24 hours).

Even if the force contributed by collagen gel maturation (\(20 \times 10^5\) N) is taken into account, the contraction values obtained would still imply that 5-fluorouracil is able to inhibit cell matrix binding and perhaps microtubular formation. Micrographs of the Dupuytren fibroblasts suspended in collagen gel after contraction indicated that the cytoskeleton had been disrupted, changing the cellular morphology from elongated cells with ample cytoplasmic extensions to rounded multinucleate cells.
When two mL of 2.5 mg/mL 5-fluorouracil was added at 16 hours after which the gel was not rinsed with PBS, the contraction obtained by the fibroblasts at this time point was seen immediately to diminish and remain static to the end of the experiment at 24 hours. Considering that the gel have matured and the fibroblasts were presumed to have obtained both cell matrix binding and formed microtubules at this time, any of these factors could be influenced by 5-fluorouracil. The micrographs of these cells suspended in collagen gel after contraction, exposed to 2.5 mg/mL 5-fluorouracil for 8 hours was observed to contain pyknotic nuclei, disrupted cytoplasmic extensions and vacuoles in the cytoplasm, indicating cell death.

Eastwood et al (1994) reviewed experiments showing a rise in contraction after Colchicine application and suggested that force was released as a peak of contraction as the microtubules were disrupted. This component appeared to be stored within the cell as part of the output of the actin-myosin motor elements of the microfilaments. It was proposed that a component of the total force generated is carried as a compressive load in the microtubules, probably as a function of cell shape. However when Colchicine was added prior to cell attachment to the collagen lattice restricted the normal development of cell processes and cell shape, reducing contraction by up to 50 %.

It could therefore be hypothesised that if 5-fluorouracil application caused disruption of the microtubules, this would be apparent as a rise, rather than a fall in contraction, as showed here. However the micrographs of Dupuytren fibroblasts exposed to 5 minutes of 5-fluorouracil prior to suspension in collagen gel demonstrated the same morphology as described in Colchicine treated cells.


Fishkind (1995) showed that actin filaments pull the cell membrane inwards to form a cleavage furrow, necessary to complete cytokinesis and Goldman (1976) proposed that actin filaments played an important role in cell contractility.
In chapter IV 5-fluorouracil was shown to influence the production of alpha smooth muscle actin which has been shown to be relevant for the contractile potential of Dupuytren fibroblasts in cultures (Tomasek and Rayan 1995). The experiments conducted in this chapter only ran over 24 hours, perhaps inadequate time for the 5-fluorouracil to have exerted the desired effect on alpha smooth muscle actin production, necessitating an additional influence on the cytoskeleton itself to explain the reduced contractility.

*It is therefore suggested that 5-fluorouracil, in its cytostatic capability, inhibits the production of certain proteins, like Rho and actin filaments and therefore reduces the contractile potential of treated cells, especially in view of the morphological characteristics seen after a short 5-fluorouracil exposure.*
Chapter VIII

General discussion and conclusion
1. General discussion

1.1. Is Dupuytren's disease a proliferative disorder?

Luck (1959) suggested that the initial stage in Dupuytren's disease was characterised by proliferating fibroblasts and Murrell (1992) suggested that the disease on a cellular level was caused by proliferation of fibroblasts, rather than an alteration in phenotype. No immunohistochemical evidence of proliferation was however found and it may therefore be hypothesised that the disease sampled at time of surgery may differ from the initial pathogenic event. The specimens were furthermore not differentiated into nodules and cords, which may represent an error in analysis of the results.

Comparing proliferation in the various cell lines investigated, under standard tissue culture conditions, no difference was found. This indicates that excessive proliferation in Dupuytren fibroblast is not inherent, even under optimal conditions in vitro. Highly proliferative tissues however, are more sensitive to anti-proliferative agents and 5-fluorouracil inhibited proliferation over a longer time period in Dupuytren's and non-diseased fascia fibroblasts, than in carpal ligament fibroblasts.

If Dupuytren fibroblasts were conceived as transformed cells, it could be hypothesised that the high level of c-myc expression shown in tissue samples of primary Dupuytren's disease would only result in increased proliferation if the availability of mitogens was increased as shown in vitro with fibroblast cell lines stimulated by serum (Pledger et al 1977, Campisi et al 1984, Waters et al 1991, Harrington et al 1994) or in the post-operative environment, stimulated by wound healing factors.

*It is therefore proposed that Dupuytren's disease is characterised by proliferation, depending on the local extracellular environment.*

1.2. Is Dupuytren's disease a tumour or is it a reparative process?

1.2.1. Tumour?

Luck (1959) recognised the difficulty in distinguishing between neoplasia and dysplasia. Dupuytren's disease has histologically been mistaken for fibrosarcoma (Erdmann et al 1995) and c-myc levels were not found to be significantly different from those in malignant tumours, such as fibrosarcoma. As in malignant tumours there is no demarcated tissue plane in Dupuytren's disease and it is not encapsulated as a benign
tumour, which may be perceived as local invasion, yet some fibres are not involved (McGrouther 1990).

Dupuytren's disease recurs after surgery, perhaps due to incomplete excision. Skin overlying infiltrating breast carcinoma is excised because malignant cells may have invaded and the skin is therefore a potential source of recurrence (Dixon and Mansel 1992). Dermofasciectomies have lower recurrence rates than fasciectomies, and McCann et al (1993) suggested recurrence stemmed from myofibroblasts in the overlying skin. Skin replacement however also alters the dermal structure in the palm, by supplying a different collagen network (Flint and McGrouther 1990).

1.2.2. Scar?

Dupuytren fibroblasts exhibited a high sensitivity to the fibroblast-myofibroblast differentiation effects of TGFβ₁ in the same manner as hypertrophic scar fibroblasts (Linge et al unpublished data) and furthermore did not exhibit the imbalance between proliferation and apoptosis postulated as the basis for tumour growth. What is perceived as local invasion, may be explained by the finer anatomical extensions of the gross fascial structures involved in Dupuytren's disease, complete the palmar continuum and it is therefore not unreasonable to expect the disease to begin in any compartment (McFarlane 1990).

If Dupuytren's disease is perceived as a connective tissue response, then recurrence may be explained by surgery perpetuating this process. In a small personal series it was observed that my grandfather had and my father has Dupuytren's disease, but my brother has keloid scars. They share the same occupation and gene-pool. My grandfather and father passively hyper-extended the involved digits from the first sign of Dupuytren's disease and did/does not have flexion contractures. This is in accordance with the clinical practice to avoid scar contractures.

The different levels of c-myc expression in Dupuytren's disease may be an indication of the different stages of disease evolution, treatment response or suggest that recurrent and primary disease are different diseases. It is therefore proposed that primary Dupuytren's disease is a tumour and recurrent disease a reparative process and it may therefore be hypothesised that the pathway for primary and recurrent disease only is common in parts.
1.3. Is the Dupuytren fibroblast different from other fibroblast in the hand?

Dupuytren fibroblasts reacted in a different manner to 5-fluorouracil and TGFβ1 exposure, than carpal ligament, but non-diseased fascia fibroblasts exhibited a similar degree of myofibroblast differentiation as Dupuytren fibroblasts at high densities. C-myc levels were elevated in primary Dupuytren's disease and this is in accordance with chromosomal studies (Würster-Hill et al 1988), which suggested that the most common abnormality in Dupuytren's disease was trisomy 8, which coincide with the location of myc oncogenes (McFarlane 1990).

*It is suggested that Dupuytren fibroblasts are different, but that non-diseased fascia fibroblasts may behave like Dupuytren fibroblasts under specific circumstances.*

1.4. What influences the fibroblast to become Dupuytren's disease?

TGFβ1 has been suggested to upregulate ECM deposition (Sporn and Roberts 1992) and increase proliferation in fibroblasts (Kloen et al 1995), whilst down-regulating c-myc and proliferation in epithelial cells (Pitenpol et al 1990, Waters et al 1991). The timing and concentration of TGFβ1 was concluded to be important in the cellular response obtained and varied with cell type investigated. The results obtained with the specific TGFβ1 concentration studied in Dupuytren fibroblasts, may be proposed to be in accordance with the involutional stage (Luck 1959). The cellular response may therefore depend on the timing and concentration of TGFβ1, apart from fibroblast phenotype. This would be theoretically expected by analogies to other complex biological processes and underlines the importance of timing in any intervention study aimed at biological response modification.

High density cultures of non-diseased fascia fibroblasts exhibited the same myofibroblast differentiation as Dupuytren fibroblasts, as opposed to carpal ligament. The disease may therefore rely on cell density to progress. Proliferation was however not significantly different for 30 days in all three tissues.

Mechanical loading could be suggested to influence the formation of fibrotic cords (McGrouther 1990). The contractile properties of myofibroblasts in the nodule are therefore suggested to retain the spherical form of the nodule, whilst the movement of the hand is suggested to deform the nodule along the longitudinal axis of the hand.
(longitudinal fibres are affected, not transverse). This ‘tug-of-war’ is therefore proposed to result in joint contracture.

The clinically potentially significant results of these experiments are therefore supported by this in vitro model of the pathogenic events in Dupuytren's disease.

1.5. Is surgery the right treatment?

Ever since the earliest descriptions of Dupuytren's contracture, the optimal surgical treatment for the disease has been discussed. Whether to incise or excise. Knowledge concerning the basic cellular pathology and disease process involved in Dupuytren's disease has since opened possibilities of adjuvant therapy and non-surgical treatments. Surgery produces a scar and we cannot as yet control scar formation. Recurrent Dupuytren's disease may be hypothesised to represent a scar and the operation to provide a different environment for the cells, with a subsequent different disease process. Primary and recurrent disease may not be the same process and may therefore require different treatment.

The main aim of surgical treatment is to correct the contracture characteristic for Dupuytren's disease, but adjuvant therapy may influence the diathesis of the disease.

1.6. What is required of an adjuvant treatment?

The ideal treatment has no side-effects and high specificity. The main problems of Dupuytren's disease are contracture and recurrence.

1.6.1. Contraction

If it is accepted that myofibroblasts are intricate to the contractile process in Dupuytren's disease, any reduction in the number of myofibroblasts would be beneficial. 5-fluorouracil reduces the myofibroblast differentiation in vitro. Furthermore micrographs of the 5-fluorouracil treated Dupuytren fibroblasts suspended in collagen gel after contraction indicated that the cytoskeleton had been disrupted, changing the cellular morphology from elongated cells with ample cytoplasmic extensions to rounded multinucleate cells.

5-fluorouracil may therefore not only change the initial, but also later cellular processes leading to contraction.
1.6.2. Recurrence

If fibroblast proliferation is pathognomonic in Dupuytren's disease, then a reduction of proliferation could be hypothesised to change the pathological process. This was achieved by a single exposure to 5-fluorouracil in vitro.

It was proposed that the Dupuytren fibroblast proliferation, induced by the normal wound-healing process post surgery for Dupuytren's disease was the initial step in the pathogenesis of recurrent disease. Such a mechanism would be in good accordance with the histological suggestion that early Dupuytren's disease is characterised by fibroblast proliferation (Luck 1959). The effects of 5-fluorouracil applied directly post-operatively may be due to a selection of fibroblast subpopulations, less capable of proliferating, because 5-fluorouracil is thought to mostly influence rapidly dividing cells (Khaw et al 1992, 1993). It is suggested that if hypo-proliferative sub-populations of Dupuytren fibroblasts were selected by 5-fluorouracil exposure and proliferative wound-healing response could be inhibited selectively in Dupuytren fibroblasts, this would reduce recurrent disease.

*Primary disease is suggested to begin as fibroblast proliferation and it is proposed that operating perpetuates this process by inducing a proliferative wound-healing. This proliferation was successfully inhibited by 5-fluorouracil in vitro.*
1.7. **Proposed mechanism of Dupuytren's disease and adjuvant treatment targets**

![Diagram](image)

*Figure 1: Proposed mechanism of Dupuytren's disease and adjuvant treatment.*

The combined results of these investigations suggested that c-myc may be deregulated in Dupuytren's disease. Furthermore, in vitro studies using both morphological markers and the culture force monitor model suggested that a short exposure to 5-fluorouracil decreased proliferation, differentiation, and contraction of Dupuytren fibroblasts in vitro.

2. **Conclusion**

These studies were entirely pre-clinical and will require appropriate clinical testing before treatment protocols could be proposed. One of the problems envisaged for pharmacological treatment in the future is that it may require precise delivery at defined sites within the palmar fascial fibres to obtain the desired effect. On the basis of a detailed anatomical and cellular knowledge of the pathological process of Dupuytren's disease, pharmacological treatment may be possible in the future to reduce both disease progression and recurrence.
Appendix
1. Suggestions for further study
In writing this thesis many other problems have been brought to light, which could possibly be of interest in further research. The main triade of problems with Dupuytren's disease is hyperproliferation of fibroblasts, contraction and inappropriate production of collagen. With the effect of 5-fluorouracil investigated with the respect to proliferation, contraction and myofibroblast differentiation it remains to examine the effect of 5-fluorouracil treatment on collagen production. Further studies on the long-term effects of 5-fluorouracil on contraction would be advisable, since these experiments were conducted with cells immediately after 5-fluorouracil treatment to see whether this effect was sustained.

The contractile process in Dupuytren's disease may be connected to the integrins, a hetero dynamic group of cell adhesion molecules. α5β1 integrin is a specific cellular receptor for fibronectin and in linking fibronectin to stromal cells of both proliferative and involutinal phases and may therefore be involved in the contractile processes occurring in Dupuytren's disease (Magro 1995). Cord retraction seem to depend on the interactions among fibroblast-like cells and matrix components and among matrix macromolecules themselves (Pasquali-Ronchetti et al 1993).

Integrins have been implicated as part of the contractile process and further work using the CFM producing gels would be ideal for examining the levels of integrins, the effect of integrin-antibodies and 5-fluorouracil on contraction.

Multiple growth factors have been implicated in Dupuytren's disease, but few have been investigated in combination. Only TGFβ1 was investigated in this thesis, but further experiments with a range and combination of growth factors would emulate the pathological wound healing process better in vitro.

The possibility of a block in the pathway regulating proliferation was illustrated with findings of high levels of c-myc oncogene, without evidence of mitotic activity, in Dupuytren tissue samples. Though this pathway is complex and the factors influencing it are numerous, further studies including p16 and p53 may be of benefit.

The main aim of this thesis was to prepare the ground for clinical trials and it is hoped that this can be commenced in the near future.
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