DUPUYTREN'S DISEASE

THE EFFECT OF STEROIDS ON PRO-INFLAMMATORY CYTOKINE PRODUCTION AND CELLULAR APOPTOSIS

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ABSTRACT

Dupuytren's disease is the result of a chronic inflammatory process, producing progressive contracture of patients' fingers. The histology of Dupuytren's tissue has been well studied; demonstrating inflammatory cells in addition to the myofibroblasts within the Dupuytren's nodule itself. These inflammatory cells produce growth factors and cytokines that regulate the proliferation and progressive contracture of the Dupuytren's myofibroblasts.

The mechanism of the migration of these inflammatory cells through the endothelial walls of the blood vessels within the Dupuytren's tissue is not well established. This work examined one possible pathway of inflammatory cell adherence to the endothelial cells and their migration.

The cells in Dupuytren's tissue expressing the integrin $\alpha 4\beta$ 1 and the corresponding ligands, Vascular Cell Adhesion Molecule – 1 (VCAM-1) and HepII/IIICS region of fibronectin known as CS1 were studied.

Transforming Growth Factor–beta (TGF-beta) is an important group of factors linked with fibrosis. The distribution of TGF-beta production in relationship to the expression of CS1 region of fibronectin was also studied in Dupuytren's tissue.

Cells expressing $\alpha 4\beta$ 1 were noted principally in and around the blood vessels expressing VCAM-1 and CS1 fibronectin. VCAM-1 was present within the

endothelium of blood vessels surrounding and penetrating areas of active nodule growth. TGF-beta was noted to be expressed in very similar areas to the CS1 sequence of fibronectin.

Having established this, the effect of methylprednisilone steroid injected locally around the Dupuytren's nodule was studied. The effect of steroids on the expression of growth factors and pro-inflammatory cytokines (Interleukin-1 (IL-1), Interleukin-4 (IL-4), Interleukin –6 (IL-6), Interleukin-8 (IL-8), Tumour Necrosis Factor-alpha (TNF-alpha) and TGF-beta was investigated. In addition, the effect of steroids on the presence of the adhesion molecule VCAM-1, CS1 sequence of fibronectin and $\alpha 4\beta 1$ expressing cells was studied.

In Dupuytren's tissue treated with steroid, the studied growth factor and cytokine expression was reduced, VCAM-1 expression was down regulated in the blood vessels, as was CS1 fibronectin and the number of $\alpha 4\beta 1$ expressing cells was reduced.

As chronic inflammation may represent a failure of inflammatory cells to undergo apoptosis, the rates of apoptosis and proliferation of cells within the Dupytren's nodule was assessed by immunological staining for the Lewis Y marker and the Ki67 antigen respectively.

In addition, cells from Dupuytren's nodules were cultured and studied using flow cytometry measurements of cellular markers of apoptosis (Annexin V binding). This was compared to fibroblast cultures from specimens of palmar fascia taken

at carpal tunnel decompression, where Dupuytren's disease was not present. Fibroblasts were also cultured from specimens of fascia lata taken at hip surgery. These fibroblasts acted as controls. The effect of addition of steroid was again studied.

Steroid produced a marked increase in cellular apoptosis, as represented by Lewis Y marker binding and a reduction in proliferation, as measured by Ki67 antigen binding. In the cell culture experiments, Dupuytren's cells were much more sensitive to steroid induced apoptosis than fibroblasts from normal carpal tunnels.

By understanding the factors involved in controlling inflammatory cell adherence and transendothelial migration and the subsequent failure to undergo normal apoptosis, therapeutic intervention to prevent progression may be possible.

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ABBREVIATIONS

ANNEXIN V	Protein with High Affinity for Phosphatidylserine which Translates to Outer Face of Plasma Membrane at start of Apoptosis
APES	Aminopropyl Triethoxy-Silane
BCIP / NBT	5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium
bFGF	Basic Fibroblastic Growth Factor
CD3	Polypeptide Cell Marker for T Cells
CD68	Glycoprotein Cellular Marker for Macrophages
DNAse	Deoxyribonuclease
EDTA	Ethylenediaminetetracetic Acid
FACS	Fluorescence-Activated Cell Sorter with Flow Cytometric Analysis
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
CS1 Fibronectin	HepII/IIICS Region of Fibronectin
GREs	Glucocorticoid Response Elements
IL-1-alpha / IL-1-α	Interleukin-1 alpha
IL-1-Beta / IL-1-β	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
Ki67	Antigen Present in Nuclei of Proliferating Cell

LDL	Low Density Lipoproteins
Lewis Y	Carbohydrate Antigen Associated with Cell Apoptosis
M	Molar
MAC387	Antigen Marker for Macrophages
ml	Millilitre
mRNA	Messenger Ribonucleic Acid
NF_kB	Transcription Nuclear Factor
eNOS	Endothelial Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
PAS	Periodic Acid-Schiff
PDGF	Platelet Derived Growth Factor
PGF2	Prostaglandin F2
PGE2	Prostaglandin E2
pH	Measurement of Hydrogen Ion Concentration
PI	Propidium Iodide
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
SD	Standard Deviation
TBS	Tris Buffer System
TNF-alpha/ TNF-α	Tumour Necrosis Factor-Alpha

TGF- beta / TGF-β	Transforming Growth Factor Beta
µl	Microlitre
VCAM-1	Vascular Cell Adhesion Molecule – 1
VLA4 / α 4 β 1	Very Long Acting Antigen 4 / Alpha 4 Beta 1 Integrin

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DECLARATION

I submit this thesis as work that I have done and written up by myself

R.M.Dominic Meek

Contractio digitorum ſiniſtræ manus, in volam illius.

INſignis artifex lapicida quidam, ſaxum immenſum voluens, adeò tendines in ſiniſtræ manus vola ad digitos, annularem & minimum definentes, ei attracti ſunt, vt illi à vinculis quib. retinẽtur laxati, eleuatiq́ue, duas chordas ſub cute tenſas in altum referrent, contractiq́ue duo hi digiti & attracti, poſtea ſemper manſerint.

Digiti

Plater's description of Dupuytren's contracture, 1614. 'Contraction of the fingers of the left hand into the palm. A certain well-known master mason, on 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 in forever.'

'Translation by J. B. St. Clair, 1987.'

(Reproduced by kind permission of the Wellcome Institute for the History of Medicine.)

Figure 1

CHAPTER 1

INTRODUCTION

1.1

HISTORY OF DUPUYTREN'S DISEASE

In 1641 the features of Dupuytren's disease were first observed and recorded by Felix Plater of Basel, Switzerland, in his book "*Observationum*", in which he discussed the diseases of the body and soul. He described a stone mason who developed contractures of the ring and little fingers of his left hand. Plater at the time attributed this to tightness of the underlying flexor tendons (Figure 1).

Henry Cline, a pupil of John Hunter, in 1777 was perhaps the first to accurately describe the features of idiopathic contracture of the palmar fascia. Sir Astley Cooper also published descriptions of the condition in 1822, noting that it arose within the aponeurotic layer of the palm. Boyer, 1831, called the contracture 'crispartura tendinum'. It was not until 1832, that Baron Guilaume Dupuytren (1777-1835) delivered his own description of the contracture at his lecture at the Hotel-Dieu, Paris. He later wrote, ' La plupart des individus que cette maladie affecte, ont été obliges de faire des efforts avec la paume de main et de manier des corps durs'. Perhaps due to his flamboyant nature it is his name that is associated with the condition. He described and presented several cases of the disease, including his own coachman, giving his own opinion of the anatomy. He also reviewed unsuccessful therapeutic regimens, and finally considered the results of his own treatment by fasciotomy and splintage. Even immediately after this publication by Dupuytren, the condition provoked debate. Jean Goyrand contested Dupuytren's assertion of the link to manual labour and cited bilateral

disease in his own hospital manager, who had never done a day of hard labour in his life (Goyrand, 1833).

Even today the debate over the aetiology, histology, pathogenesis and treatment is as hotly contested despite over 160 years of research.

1.2

ANATOMY

An understanding of Dupuytren's disease is not possible without knowledge of its relation to the anatomy of the fascia of the palm and digits.

Fascial anatomy of the hand

One of the first descriptions of palmar fascia was by Vesalius (1514-1564). Subsequent work has further delineated the normal anatomy of the fascial structures of the hand.

The palmar fascia is a triangular, dense fibrous structure with an apex towards the interval between the thenar and hypothenar eminence. Its base is parallel to a line joining the metacarpal necks. Proximally it is continuous with the palmaris longus tendon (Absent in 11-23%). Distally it sends slips running centrally and laterally to both sides of each digit. The palmar-digital and digital fascia may be divided into pretendinous bands, spiral bands, natatory ligaments, Grayson's ligaments, Cleland's ligaments and the lateral digital sheath. (Figure 2) Together this palmar and digital aponeurosis stabilises the palmar skin during gripping actions of the hand, preventing displacement that would occur with shear loading of the palmar skin.

In the diseased states of Dupuytren's disease these ligaments, sheaths and bands act as guidance paths for the pathological cords of Dupuytren's tissue. Dupuytren

himself overlooked this as he believed the disease involved only the aponeurosis and overlooked the fact that the normal aponeurosis did not extend into the fingers which frequently contained pathological cords.

The four main named cords are the central cord, the spiral cord, the lateral cord and the retrovascular cord (Figure 3). The retrovascular cord is not illustrated. An abductor digiti minimi cord may extend from the musculotendinous junction or tendon to the ulnar side of the base of the middle phalnyx. Commonly this cord will adhere to the lateral skin. The central cord is a direct extension of the pretendinous cord. The spiral cord develops from the pretendinous aponeurosis, spiral bands, the lateral digital sheaths, the vertical band and Grayson's ligament and usually connects to the middle phalnyx. The lateral cord results from contracture of the lateral digital sheath.The retrovascular cord is described as a condensation of longitudinally orientated fibres deep or dorsal to the neurovascular bundle

The Australian surgeon Hueston (1963) believed that the nodules and bands excised were quite different from the palmar aponeurosis. He felt they arose *de novo*. Now it is accepted that nodules, fascial bands and fibrofatty subcutaneous tissue are in physical and biological continuity making this theory less relevant.

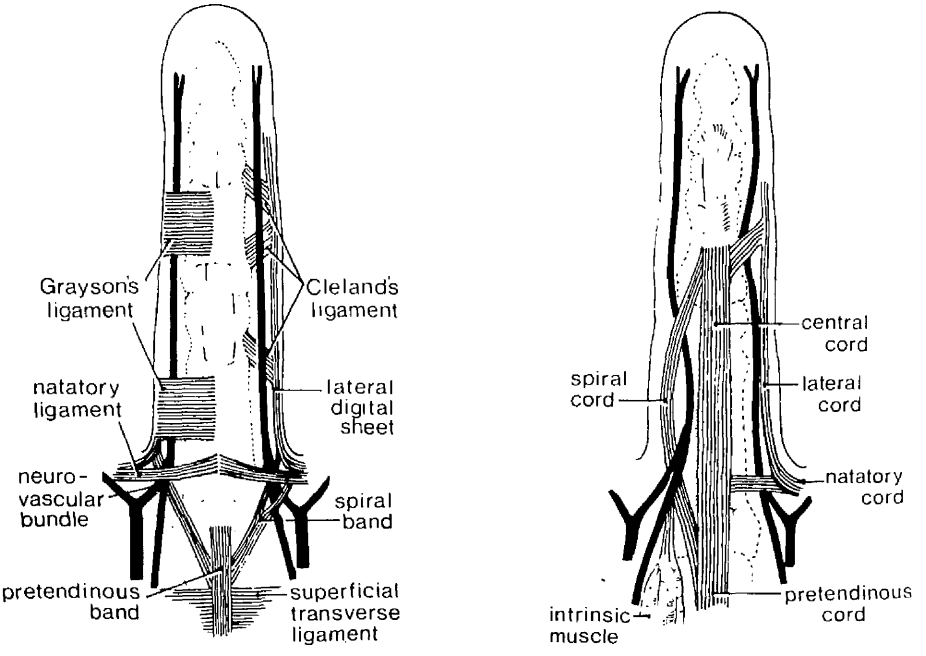


Figure 2 & 3

The normal structures of the finger that may be involved in Dupuytren's Disease are the longitudinal pretendinous bands, natatory ligaments, lateral digital sheaths and Grayson's and Clelands ligaments, as illustrated in figure 2.

The three pathologic cords that may develop during the progressive palmar fibromatosis of Dupuytren's disease are the central, the spiral and the lateral cords, as illustrated in figure 3.

(Reproduced with permission from McFarlane RM: Patterns of the diseased fascia in the fingers of Dupuytren's contracture. *Plast Reconstr Surg* 54:31, 1974 and from Chiu HP, McFarlane RM: Pathogenesis of Dupuytren's contracture: A correlative clinical-pathological study. *J Hand Surg* 3:1-10, 1978)

1.3

HISTOLOGY

Much of the previous histological work has been disappointing in that it has contributed little to the understanding of Dupuytren's disease (Nezelof, 1974). It has confirmed fibrosis, as expected from clinical observation and from operation, but lent nothing to suggest its aetiology or histogenesis. With modern tools available such as immunological and molecular biological analyses, more meaningful interpretation of the gross changes will be obtained.

The work of Hueston, 1963, suggested the nodules were histologically separate from fascial tissue and that Dupuytren's disease resulted from an extrinsic pathogenic process to the palmar fascia. However now it is generally accepted that the nodules, fascial bands and fibrofatty subcutaneous tissue are in physiological and biological continuity (Baird, 1994)

It appears that the disease evolves through several phases. In the palmar aponeurosis area the disease may be in different phases at any one time, separated by only millimetres.

Nodule Phase

Nodular areas of thickening are the actively developing and progressing areas, characterised by individual nodules of about equal size, round or oval and of 0.5 to 1 mm in diameter. The nodules are highly cellular, consisting predominantly of fibroblasts arranged in an irregular but more or less concentric pattern. They

are highly vascular and closely arranged together in the central part of the lesion, containing a network of fibres. On exiting the nodule, the fibres unfurl and elongate and increase in size to lie in a criss-cross fashion to form a dense complex mesh that with the myofibroblasts nodules constitutes the main component of the lesion.

Intermediate Phase

The intermediate lesions are characterised by morphological differentiation of these fibroblasts, which proliferate and migrate away from the vicinity of blood vessels. The fibroblasts develop elongated nuclei and acquire increased amounts of cytoplasm assuming morphological appearance of myofibroblasts. A more detailed discussion on the nature of these myofibroblasts is deferred to the section on chronic inflammation. Gradually the tissue becomes less cellular and the collagen content increases as does the ratio of type III to I collagen, and may spread to involve the dermis and epidermis. Foci of haemosiderin are sometimes noted resulting from micro-haemorrhages. A variable inflammatory cell infiltrate is seen, consisting of lymphocytes, monocytes, macrophages and masts cells.

Cord Phase

The cords are surmised to be areas where involution and maturation have occurred. The cords have heterogeneous texture with regular fasciculation. Cellular density is low, the few recognisable cells being fibrocytes lying longitudinally between collagen fibres. The collagen fibres are wide, elongated, and birefringent when seen through polarised light and slightly arched. The more peripheral fibres seem looser and less ordered and are Periodic Acid-Schiff

(PAS) test negative. The central fibres are thinner and more tortuous and are PAS positive and contain no elastin fibres. Strands occasionally run into the fat and adjacent tissue.

A summary classification of this was presented by Luck (1959) who divided Dupuytren's tissue into three stages: (1) proliferative (2) involutional (3) residual. In this the nodule represented the earliest stage of the disease in the process of proliferation. The involutional stage consisted of the fibroblasts orientating themselves along the major stress axes in the palmar fascia. Finally the residual stage ensued with the disappearance of the fibrous nodule, leaving only focal dense adhesions and relative acellular fibrous cords, almost similar to tendons. (Figure 4 a, b)

Figure 4 a

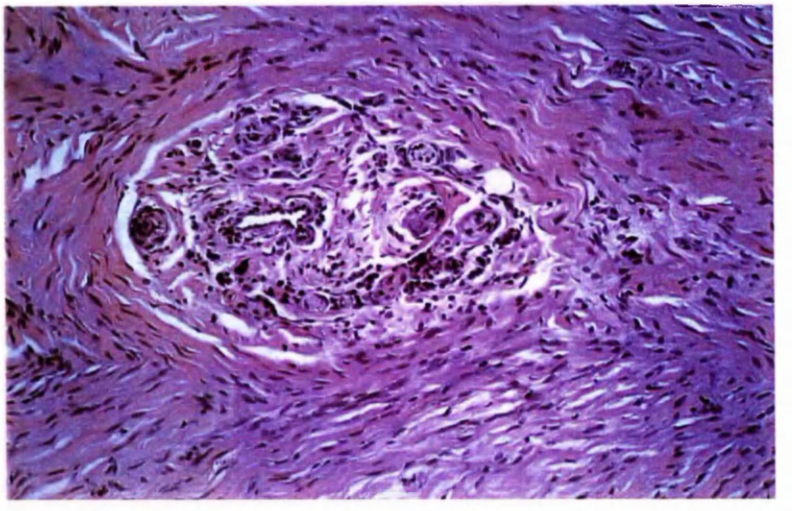


Figure 4 b

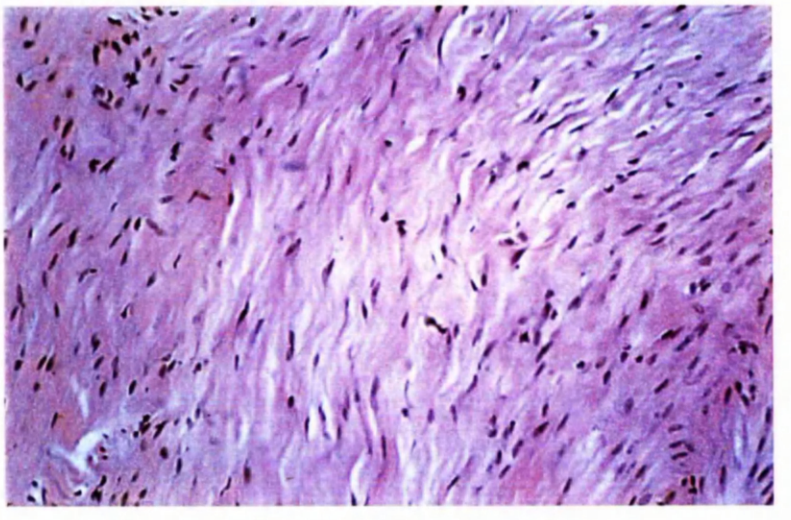


Figure 4 a

A proliferative nodule: note the thickened vessel walls, some occluded, and the proliferating cells surrounding these vessels (Baird, 1994, reproduced with permission).

Figure 4 b

Residual phase of Dupuytren's contracture: note the hypocellular tissue with parallel bundles of collagen fibres resembling tendons (Baird, 1994, reproduced with permission).

1.4

AETIOLOGY

Associated conditions

1 Alcoholism and Hepatic Cirrhosis

2 Epilepsy

3 Pulmonary fibrosis

4 Tuberculosis

5 Smoking

6 Diabetes Mellitis

7 Hypercholestaemia

8 HIV infection

9 Trauma

1 Alcohol and Cirrhosis

Dupuytren (1834) implied the link between Dupuytren's contracture and high alcohol intake. Certainly the incidence of alcoholism in populations of Dupuytren's contracture patients and the frequency of Dupuytren's contracture in populations of alcoholics has been studied. No universal agreement has yet been reached but correlation between alcoholic patients and higher rates of Dupuytren's contracture seems reproducible (Noble *et al.*, 1992; Attali *et al.*; 1987; Houghton *et al.*, 1983). It should be noted that correlation between alcohol induced hepatic cirrhosis and Dupuytren's contracture is positive (Rafter *et al.*, 1980; Noble *et al.*, 1992). This suggests alcoholics probably do have a higher rate of Dupuytren's contracture and that this effect may be largely due to the liver disease caused by alcohol abuse in genetically predisposed individuals.

2 Epilepsy

There is a statistical correlation between Dupuytren's contracture and epilepsy (Lund, 1941; Skoog, 1948) and idiopathic epilepsy seems to have a stronger correlation than post-traumatic epilepsy. The correlation does not appear to be due to anti-epileptic drugs (Noble *et al.*, 1991), although there is some in vitro evidence that phenobarbitone causes dose related changes in factors that influence fibroblast growth and differentiation (Baird, 1994).

3 Pulmonary Fibrosis and Chronic Obstructive Airways Disease

An association between pulmonary fibrosis and chronic obstructive airways disease and Dupuytren's disease has been observed (Parrella *et al.*, 1987; Despierres *et al.*, 1966).

4 Tuberculosis

Dupuytren's contracture has been reported to commence in many patients within one month of immobilisation in bed for pulmonary tuberculosis (Hueston, 1991), and this association was noted as early as 1966 (Despierres *et al.*, 1966)

5 Smoking

There is an increased incidence of Dupuytren's contracture in cigarette smokers (An *et al.*, 1988).

6 Diabetes Mellitis

Diabetes mellitus has been noted to be associated with Dupuytren's contracture for over a 100 years (Viger, 1883). The incidence of Dupuytren's contracture in patients with diabetes mellitus has been estimated to approach 40% (Noble *et al.*, 1984). There appears to be no significant difference in Dupuytren's contracture incidence between non-insulin dependent and insulin dependent diabetics. Duration of diabetes appears to affect the incidence, as the incidence naturally

increases with age; however it also increases with age in idiopathic cases. Suggestions have included an inherited predisposition to both diabetes mellitus and Dupuytren's contracture or that microangiopathy secondary to the diabetes mellitus may provoke fibroblast differentiation and proliferation.

7 High LDL

Diabetes mellitus and hypercholestaemia, alcohol disease and smoking all lead to elevated lipid levels. Serum lipid levels were studied prospectively in relation to Dupuytren's disease. Dupuytren's patients had significantly higher fasting serum cholesterol and triglyceride levels than controls (Sanderson *et al.*, 1992).

8 HIV Infection

A study of 50 male patients admitted with complications of their HIV infection found an incidence of Dupuytren's contracture of 36% compared to 0% for an age matched control group (Bower *et al.*, 1990). The authors related this increased incidence to the elevated free radical activity noted in HIV infection, but the modulation of the pro-inflammatory cytokine profiles may also be involved, as will be discussed later.

9 Trauma

Dupuytren's contracture has for years raised a controversy between whether acute traumatic injury or cumulative biomechanical work exposure could contribute to the development of this disorder.

Arguments against Dupuytren's being directly related to trauma include the observation that the condition is not any more common in manual workers than clerical workers. It appears bilaterally in some 40% of patients and the dominant hand is not more frequently affected than the non-dominant hand. It also has a familial incidence reportedly the same in identical twins and it is associated with fibrous growth elsewhere not associated with trauma e.g. Peyronie's disease. In addition, injuries to the palmar fascia are certainly not always followed by Dupuytren's contracture. There are no reported accounts of increased incidence in sports people who wield clubs or rackets in a repetitive and traumatic manner (Fisk, 1985). Some histological studies have demonstrated circumstantial evidence of repetitive microtrauma, such as partial fascial ruptures, scarring in the subcutaneous tissue and haemosiderin deposits. However these could equally result as a consequence of Dupuytren's contracture rather than represent its cause.

An argument in favour of trauma being associated is Dupuytren's increasing incidence with age, as this perhaps indicates that chronic minor trauma to the hand has a cumulative effect. Of more relevance is its association with some injuries to the hand and arm and its complications, for example, after Sudeck's dystrophy (Plewes, 1956) and frozen shoulder (Early, 1962). Indeed, Dupuytren's

contracture has been reported to occur within weeks or months of a specific incidence such as a Colles fracture (Kelly *et al.*, 1992) or metacarpal fracture (McFarlane and Shum, 1990a). One mechanism proposed is the tethering effect of the scar tissue within the palmar tissue. An alternative theory is the recruitment of a pool of inflammatory cells within the injured limb of a genetically susceptible individual, which then promotes a localised fibrotic response. This will be elaborated on later.

1.5

GENETICS

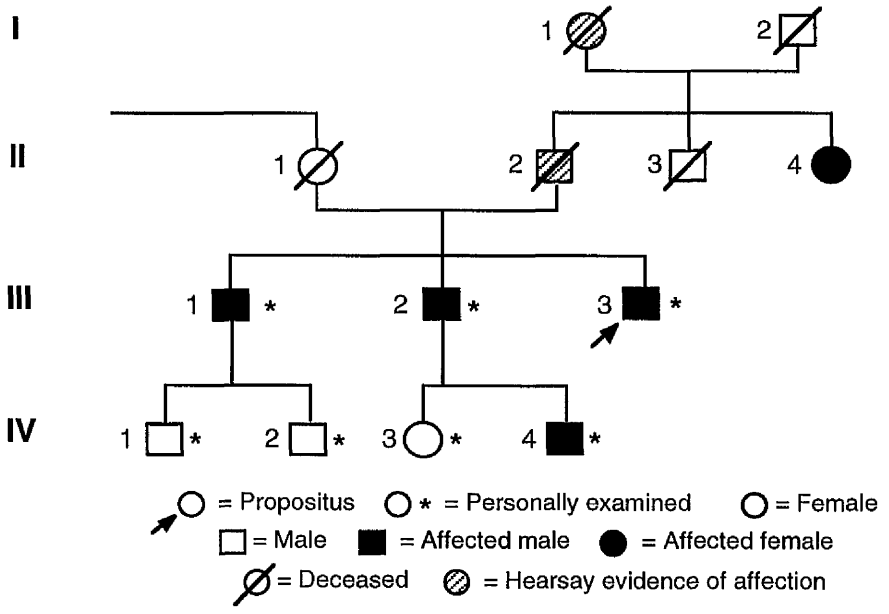
Dupuytren's contracture affects both men and females but in 10:1 ratio in incidence. Ling (1963) studied 50 patients from the Edinburgh hand clinic and obtained knowledge of the whereabouts of 1st/2nd/3rd-degree relatives, amounting to 832 relatives for further surveying. Dupuytren's contracture lent itself to such a survey, as examination of the hands and feet of people at home without X-rays is satisfactory and therefore large numbers may be screened. However such surveys are problematic, in that many individual relatives will be too young to manifest Dupuytren's contracture, although they may well develop it. Despite these drawbacks, when weight adjusted for age, the results of the survey correlated very well with those that would be expected for an autosomal dominant inheritance (Figure 5). This has since been supported by other work (James, 1985). Other patterns of inheritance are unlikely as the ratio of affected males to females is unchanged in first degree relatives making sex-linked transmission unlikely and as the rates amongst parents, siblings and children are comparable it lessens the likelihood of polygenic inheritance.

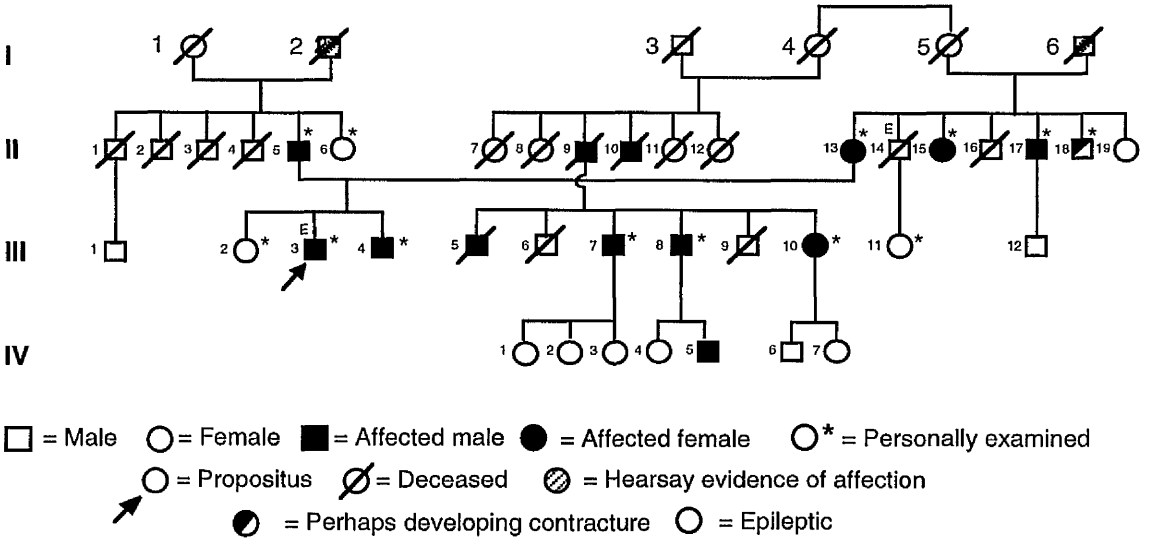
Several cytogenetic studies have been completed. Chromosomal abnormalities are commonly detected in cell cultures from Dupuytren's tissue but the only consistent finding so far have been for trisomy of chromosome 8 (Bonnici *et al.*, 1992). Interestingly, chromosomal aberrations including trisomy 8 have also been noticed in palmar fascia from carpal tunnel decompressions, suggesting a potential for chromosomal instability in this fibrous structure. Trisomy 8 has also

been detected in some large bowel tumours and mixed salivary gland neoplasms, the latter resembling Dupuytren's contracture in their high frequency of local recurrence following excision. Trisomy 8 has also been noted in the penile fibrosis of Peyronie's disease. However as yet there is no definitive evidence to suggest chromosomal abnormalities alone cause transformation of the palmar fascia in Dupuytren's contracture.

Figure 5

Two illustrative family trees from Ling's series are portrayed here. They illustrate the autosomal dominant pattern of inheritance. The second family tree it is probable that the trait was brought together by both the father and the mother in generation II and the siblings are homozygous for this abnormality; severe Dupuytren's occurred early in several of the family. It would seem possible that expression in homozygotes for this disease is earlier than average. Such individuals cannot be rare in so common a dominant inherited disease.


I
II
III
IV
= Propositus
* = Personally examined
= Female
= Male
= Affected male
= Affected female
= Deceased
= Hearsay evidence of affection


I
II
III
IV
= Male
= Female
= Affected male
= Affected female
* = Personally examined
= Propositus
= Deceased
= Hearsay evidence of affection
= Perhaps developing contracture
= Epileptic

1.6

EPIDEMIOLOGY

Dupuytren's disease has a suggested prevalence of 1-3% (Viljanto, 1973), but the prevalence varies widely in different parts of the world (Strickland *et al.*, 1990). It is virtually confined to Caucasians with only very occasional reports in black individuals with a prevalence of only 0.0007% in South Africa, (Mennen, 1986). The true incidence of Dupuytren's contracture is also difficult to establish. In the United Kingdom, an incidence was determined in those over 75 years of 18.1% for men and 9% for females (Early 1962).

Certainly there is a comparatively steady increase of Dupuytren's contracture with advancing age (Skoog, 1948). Why women are significantly less often affected by palmar fibrosis than men is unknown. Male to female ratios is reported as approximately 9 to 1 or 7 to 1, changing with progressive age to 3.7 to 1 (Skoog, 1948; Berge and Pohl, 1988). The reason for such varied sex ratios may be partly explained due to earlier affliction of males with Dupuytren's disease. If they are operated on more often at an early stage, then surgical statistics will overestimate the true male: female ratio. Estimates of the sex ratio should only be accepted with some reserve.

Dupuytren's contracture is not coincidental to the dominant versus non-dominant handiness (Hueston, 1987). Two thirds of patients show bilateral affliction (McFarlane, 1990b). More operations are undertaken on the right hand as the

majority of patients are right handed and contracture of the dominant hand will have a greater effect on function, leading them to present to the surgeon.

1.7

SURGICAL INTERVENTION

The mere presence of Dupuytren's nodules and cords is not an indication to operate. The aim of surgery is to restore function. Increasing palmar changes lead to flexion contracture which results in the functional impairment that the patient complains of is an indication for surgery, and intervention is definitely indicated where increasing induration and cord formation produces distinct extension deficit.

No one method to treat Dupuytren's contracture is perfect and all have disadvantages and associated morbidity.

Astley Cooper, ten years prior to Dupuytren's naming the disease, performed a percutaneous fasciotomy, which is simple division of the cord. Later Luck (1959) advocated removing the nodule in addition to a fasciotomy. Indications for fasciotomy were reported by Colville (1983), the chief criterion being a well defined pretendinous cord with bow stringing across the palm, said to indicate the absence of deep fascial connections. If the skin and cord were extensively attached, preventing the cord sliding distally after the division, then a fasciotomy was contraindicated. Although there was short-term improvement in metacarpal-phalangeal joint contracture, the long-term results were not as good for metacarpal-phalangeal joint contracture as those expected from a limited fasciectomy (McFarlane and Botz, 1990c), which is correction by removal of the fascia. However, fasciotomy may be acceptable in elderly and infirm patients

where the risk of recurrence may be accepted because of the simple uncomplicated nature of fasciotomy.

McCash (1964) described an open palm technique with a transverse palmar wound left open. In a primary case there is no loss of skin, healing occurring by contracture of the transverse palmar wound drawing the skin out to its original length and not by growth of new epithelium over the wound (Lubahn *et al.*, 1984). This technique gives good access for removal of diseased palmar fascia but it is controversial in its use in recurrent disease, as although allowing good drainage, it will not overcome a true shortage of skin. In addition, it does require convalescence period of 4 to 6 weeks as opposed to 2 weeks if a closed technique is used.

Some complications and unsatisfactory results

The only sure way to prevent a surgical complication is to not operate. Bearing this in mind, complications, which are due to the surgeon's intervention, include the following: incisional skin contracture, skin necrosis neurovascular damage and joint stiffness.

Incisional scar contracture is dependent on the siting of the incision. Longitudinal incisions over the finger allow easy identification and protection of the neurovascular bundles but necessitates interruption by Z plasties or full thickness skin grafts at closure to prevent contraction. Zigzag and V-Y exposures combine longitudinal access with a low risk of scar contracture, but with flap tip necrosis as a risk. Long transverse incisions of the palm leave no problem of scar contracture but need meticulous haemostasis for closure or the resulting haematoma may cause flap necrosis.

Skin necrosis is a consequence of raising flaps over extensive and invasive Dupuytren's tissue. A balance has to be maintained between excision of all diseased tissue and preservation of the dermis of the flap. If the patient's diathesis is very strong then dermofasciotomy and replacement with full thickness skin graft may be the preferred management. Z plasties in longitudinal excisions should be placed at the end of the operation once flap viability is ascertained.

Neurovascular damage should be avoided and the bundle is rarely affected in primary disease. In recurrent disease, the digital nerve and artery are often involved and it is better to leave a little tissue surrounding the bundle than damage it. Such damage can result in persistent cold sensitivity and even distal gangrene.

It is important to avoid joint stiffness, as it determines the functional level of the hand. Early postoperative proximal inter-phalangeal joint stiffness is associated with a swollen hand or local painful focus such as stitch or scar adhesion or nerve irritation and can usually be easily corrected. Some patients, more often women, may develop fusiform swelling and pain of the proximal phalangeal joints 2 or 3 weeks after uncomplicated surgery. Despite protracted physiotherapy many have residual loss of grip strength and function.

Any open operation may result in infection and for the avoidance of necrosis, haematoma formation and good surgical technique is required.

RECURRENCE

Recurrence is defined as the development of new Dupuytren's tissue appearing in the previously operated area, and should be distinguished from extension of the disease to an unoperated area. Rates of recurrence have greatly varied in different series from 20 to 68% (Leclercq and Tubiana, 1993). However, there now seems to be a general consensus that the longer the follow up the higher the rate of recurrence (Leclercq and Tubiana, 1993). Many factors appear to influence the

chance of recurrence. The two most important are age of presentation, a young age of first operation being associated with a high risk of recurrence, and the existence of previous recurrence. Other factors that increase the chance of recurrence include: a family history, ectopic lesions, epilepsy, incomplete correction of the contracture, female sex and surgical complications (Table 1).

Table 1

Influence of various factors on recurrences (Leclercq and Tubiana, 1993)

Factors	Patients (no.)	Recurrence (no.)	Percent (%)
Age			
<45	16	16	100
45-65	18	12	66
>65	9	4	44
Family History			
Positive	14	11	78
Negative	24	14	58
Previous Recurrence	6	6	100

An almost total absence of recurrence has been noted under full thickness skin grafts and these have therefore been advocated for young or strong diathesis patients or in recurrent cases (Leclercq and Tubiana, 1993). The underlying mechanism for this is not entirely clear.

1.8

ALTERNATIVE MANAGEMENTS

Since the time of Dupuytren himself, attempts have been made to either alleviate the symptoms or stop progression of Dupuytren's contracture with drugs and alternative remedies, ranging from calomel lotion to simple sulphur and leeches, all without significant beneficial effect. Such treatments represent a desire to avoid the risk of operating. Gilbert (1913) and Wainwright (1926) gave thyroid extract and Noix (1946) used fibrinolysin thiocimin electroiontophoretically, but not effectively, to soften the collagen contracture.

In contrast to mere softening, collagen degradation was tried with pepsin (Hesse 1931) and later with trypsin, α chymotrypsin and hyaluronidase (Bassot 1969; Hueston 1971). Despite pronounced effects of this chemical fasciotomy the treatment was unpredictable and had associated morbidity including neurovascular damage and occasional skin necrosis and was not widely accepted.

Vitamin E has also been used in Dupuytren's contracture, having been successfully used in the treatment of Peyronie's disease (Burford and Burford, 1957). Treatment of Dupuytren's however, produced variable results from good (Steinberg, 1946; Steinberg, 1951) to ineffective (King 1949; Richards 1952).

Recently, radiotherapy has been tried to prevent disease progression from early nodule formation (Keilholz *et al.*, 1996). Long-term follow-up of treated patients

suggested dramatically reduced progression rates and reduced symptoms, with no recorded acute or late treatment radiation toxicity.

Steroids have been used successfully on other fibromatosis such as Peyronie's disease, hypertrophic and keloid scarring (Rothfield and Murray, 1967; Bodner *et al.*, 1954; Ketchum *et al.*, 1974). Steroids certainly appeared to halt progression of Dupuytren's disease, but did not affect established contractures. In addition, it facilitated movement and reduced oedema post-operatively and increased the rate of rehabilitation generally (Bernstein, 1954). This certainly suggested that ways to prevent disease progression from early nodule formation exist and could prevent otherwise necessary surgical procedures being performed in advanced stages of Dupuytren's contracture.

1.9

CURRENT THEORIES

Current theories individually fail to explain all the features observed in Dupuytren's contracture.

Hueston, as mentioned previously, proposed an intrinsic and extrinsic theory of pathogenesis (Hueston, 1963). The intrinsic component was thought to lie within the aponeurosis itself, resulting from presumed microruptures in the fascial bands and the resulting altered physical compliance of the palmar fascia as mechanical initiating factors. The extrinsic contribution comprised the development of primary nodules in the subcutaneous fat, superficial to the aponeurosis. It now appears these two structures are in physical and biological continuity, so this theory adds little to our understanding of Dupuytren's contracture.

Another theory is that Dupuytren's contracture is a sequence of responses of the palmar fascia to injurious stimuli, rather than a disease in its own right (Flint and McGrouther, 1990). Factors such as diabetes and alcoholism by altering the properties of the palmar fascia facilitated pathological change. The Dupuytren's contracture nodule was viewed as a localised repair reaction in the fascia. The early 'trigger' event is proposed as a loss of bulk in the subcutaneous fat pad resulting in increased stress on the intra-fascial 'core' collagen fibres. This is an elaborate adaptation of the 'cumulative trauma theory', which failed to adequately explain similar rates of occurrence in manual labourers and office workers or similar rates for bilateral contracture, or the racial and geographical differences.

Some degree of fascial rupture will occur for most people every day, yet only a minority develops Dupuytren's contracture.

Tissue hypoxia has also been proposed, but it is not clear if the micro-circulatory changes are a primary or secondary effect of Dupuytren's disease. Tissue oxygen perfusion changes were investigated (Kischer and Speer, 1984) and Murrell *et al.*, went even further, suggesting localised ischaemia results in elevated levels of the purine bases xanthine and hypoxanthine (Murrell *et al,* 1987). This promotes conversion of xanthine hydrogenase to xanthine oxidase, which catalyses the oxidation of these bases and releases superoxide O^{2-} radicals and hydrogen peroxide, both of which can be toxic to cells. It was theorised that O^{2-} free radicals may trigger fibroblast proliferation and centrifugal chemotaxis from the angiocentric nodules. A positive feedback could be created where free radicals cause further damage to the pericytes, thickening of capillary basal laminae and sustained fibroblast proliferation. Against this theory is the fact there is no increased incidence of Dupuytren's contracture in gout where levels of xanthine and hypoxanthine are certainly elevated (Adam and Loynes, 1992). The true role of free radicals needs further evaluation.

1.10
CHRONIC INFLAMMATION

Tissue responds to insult with an attempt to repair itself. This response can be divided into an inflammatory response, a fibroproliferative response and a remodelling response. In the pathological state, acute inflammation becomes chronic with resulting excessive fibroproliferation and subsequent fibrosis. Fibrosis is characterised by a qualitative and a quantitative alteration of extracellular matrix deposition, and with excessive accumulation of mesenchymal cells.

A similar sequence of events occurs in wound healing with the transient formation of limited normal granulation tissue, whereas in fibrosis a maladaptive repair leads to an extensive, exaggerated process with consequent functional impairment. Inflammatory cells (predominantly macrophages, but also T lymphocytes, neutrophils, eosinophils), platelets, and fibroblasts / myofibroblasts play a direct and indirect role in this tissue injury and repair.

1.10.1
Inflammatory Response with Transendothelial migration

The composition and distribution of the inflammatory infiltrate at sites of inflammation seem to be mediated by a variety of factors relating to the causative agent, its nature, location, duration and the histo-specific regulation of the local inflammatory responses. The local production of pro-inflammatory agents, the selective expression of cell type-specific adhesion molecules and the generation

of specific gradients of chemotactic and haptotactic molecules have been recognised playing a key role in leukocyte recruitment, efficient routing and persistence at sites of inflammation

1.10.2

Inflammatory cells in Dupuytren's disease

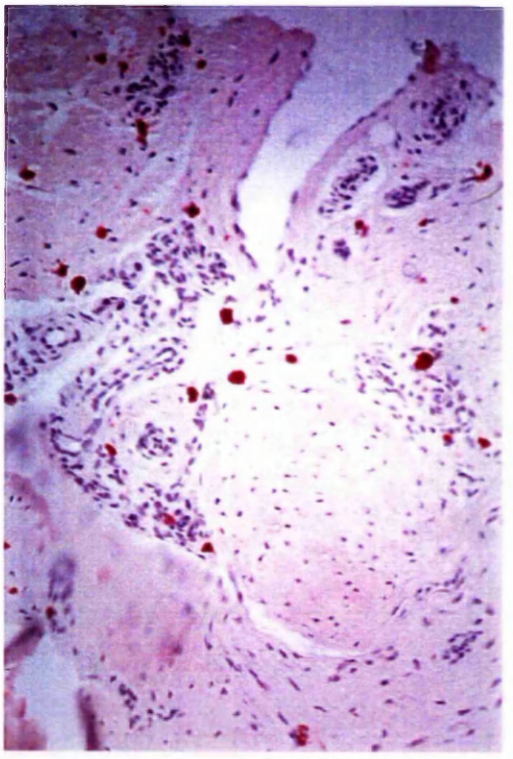
The presence of inflammatory cells has been noted in Dupuytren's tissue by many authors (Andrews *et al.*, 1991). Work in this department demonstrated the heterogeneous nature of the cells present in Dupuytren's contracture (Baird *et al.*, 1993a). Using flow cytometry, the peri-nodular tissue was demonstrated to contain a surprisingly high proportion of T lymphocytes and macrophages (Baird, 1993a). This was later confirmed in tissue sections using histology and immuno-histochemistry, where having looked at seventeen samples of Dupuytren's tissue, all stained positively for CD3, CD68 and MAC387 antibody markers (Figure 6). This compared with only 1 positive histology section (for CD3 only) out of 5 control samples of palmar fascia. T lymphocytes were the most prominent inflammatory cells present, usually occurring in large clusters next to blood vessels and between the fibres in the nodule itself. Macrophages and mast cells were in much higher numbers in the Dupuytren's tissue than in palmar fascia control sections, and lay between the fibroblasts and around the blood vessels in the connective tissue, which penetrated into the nodule.

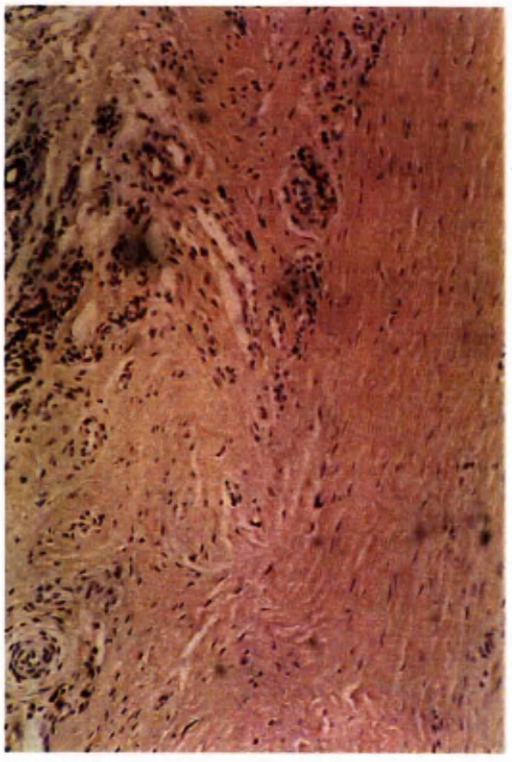
Figure 6

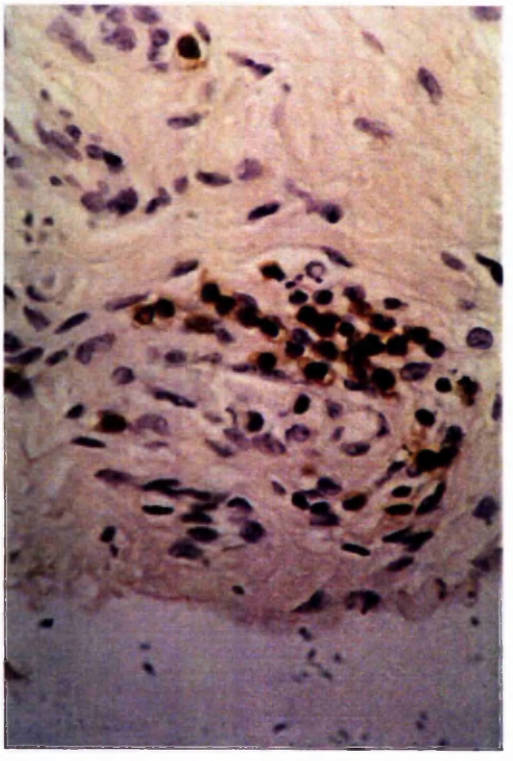
Sections of Dupuytren's tissue stained by immuno-histochemistry: demonstrating CD3 antigen for human T cells positive cells in (a) on fluorescein staining (× 320 magnification); Staining for CD68 antigen, another T cell marker, in (b) demonstrating positive cells in the peri-nodular Dupuytren's tissue (× 128 magnification); (c) MAC387 antigen for human macrophages stained several cells around many blood vessels with peroxidase immuno-histochemical staining (× 128 magnification); (d) Again CD3 antigen positive cells demonstrated lying along blood vessels (× 320 magnification), this time stained by peroxidase immuno-histochemistry. These sections represent unpublished work from Baird *et al.*

a

b







c

d

1.10.3

Fibroproliferative Response

Several pro-inflammatory cytokines participate in the local injury and inflammatory reaction (IL-1, TNF-alpha, PDGF, TGF-beta, bFGF and IL-8) and these have been demonstrated to be present in Dupuytren's tissue (Baird, 1994).

Inflammatory Cell Signalling Molecules In Dupuytren's Disease

Abnormal local regulation of cytokines and growth factors, which stimulate Dupuytren's fibroblasts and matrix synthesis, has been demonstrated by several authors now. Specifically PDGF (Badalamente, 1992, Aliota, 1994, Terek, 1995, Baird, 1993b), IL-1-alpha and beta (Baird, 1993b), TGF-beta (Baird, 1993b) and bFGF (Baird, 1993b). The todate unpublished results of this departments immunohistochemistry studies of IL-1-alpha and beta, IL-4, IL-6 and TNF-alpha is expressed in Table 2.

Table 2

Immunohistochemistry results for Dupuytren's tissue

Patients Sample	IL-1-alpha	IL-1- beta	IL-4	IL-6	TNF-alpha	bFGF
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	-	-	wk	-	wk	wk
6	+	+	+	wk	+	+
7	+	+	+	+	+	wk
8	+	+	+	+	+	+
9	+	+	+	+	wk	+
10	+	+	+	+	+	+

IL-1-alpha stained positively on immunohistochemistry in the endothelial cells of many blood vessels entering the nodule and was particularly strong in small capillaries. It also appeared in the Dupuytren's fibroblasts in mature, large nodules and very positive in macrophages around the blood vessels and nodules. IL-1-beta had a similar staining pattern to IL-1-alpha (Figure 7a).

TNF-alpha was detected again in the endothelial cells of many blood vessels, including some but not all of the small capillaries. Tissue macrophages and migrating monocytes stained positively but the fibroblasts did not express TNF-alpha. Sweat glands stained strongly positive (Figure 7a).

IL-4 stained positively in inflammatory cells generally and in particular in lymphocytes present in clusters (Figures 7a & 7b). IL-6 was present in the endothelial cells of small capillaries, but in general the cells of the main blood vessels were negative.

bFGF was strongly staining in the sweat glands, but also present in the endothelium of capillaries and larger blood vessels. Some Dupuytren's fibroblasts, mainly in the centre of the nodules, also expressed bFGF. (Figure 7a)

Sections from five palmar fascia control patients demonstrated either no positive staining or only very weak and non-specifically localised results

Figure 7a

Sections of Dupuytren's tissue stained for IL-1 alpha / beta, TNF-alpha, IL-4, bFGF and IL-8 by and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive).

(1) IL-1-alpha production is demonstrated in fibroblasts and endothelial cells of blood vessels of the fibrofatty area of Dupuytren's tissue (×25 magnification).

(2) TNF-alpha distribution at the edge of a nodule is mainly in the blood vessels and migrating monocytes (×10 magnification).

(3) IL-4 stained endothelium and inflammatory cells at one of the areas where blood vessels penetrate the nodule (×25 magnification).

(4) bFGF stained strongly in the endotheilum of capillaries and larger blood vessels at a fibrofatty area of Dupuytren's tissue (×25 magnification).

(5) IL-1 -beta is expressed in a similar distribution to IL-1-alpha production in fibroblasts and endothelial cells at the edge of a nodule (×25 magnification).

(6) IL-8 production in the fibrofatty area was seen in both the endothelial cells of blood vessels and by some Dupuytren's fibroblasts (×25 magnification).

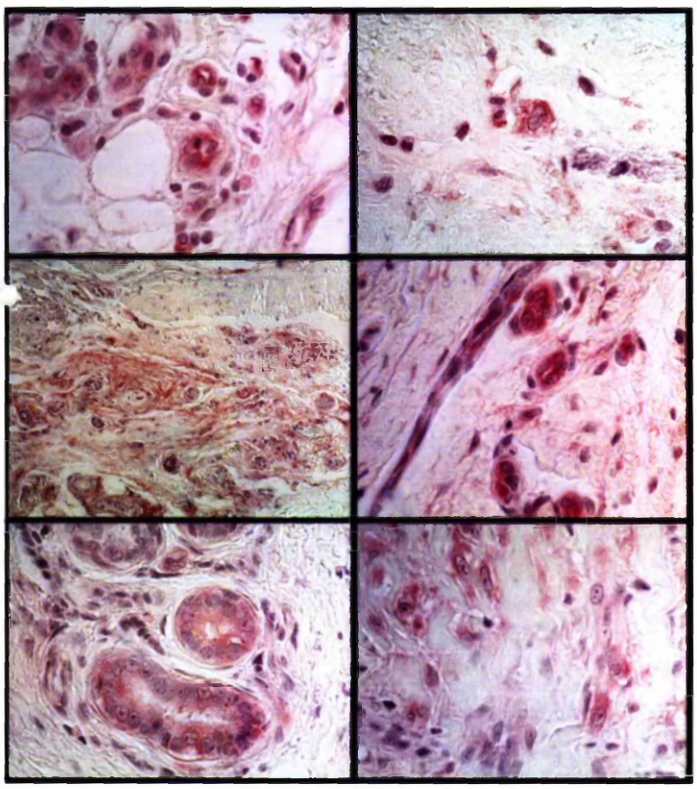
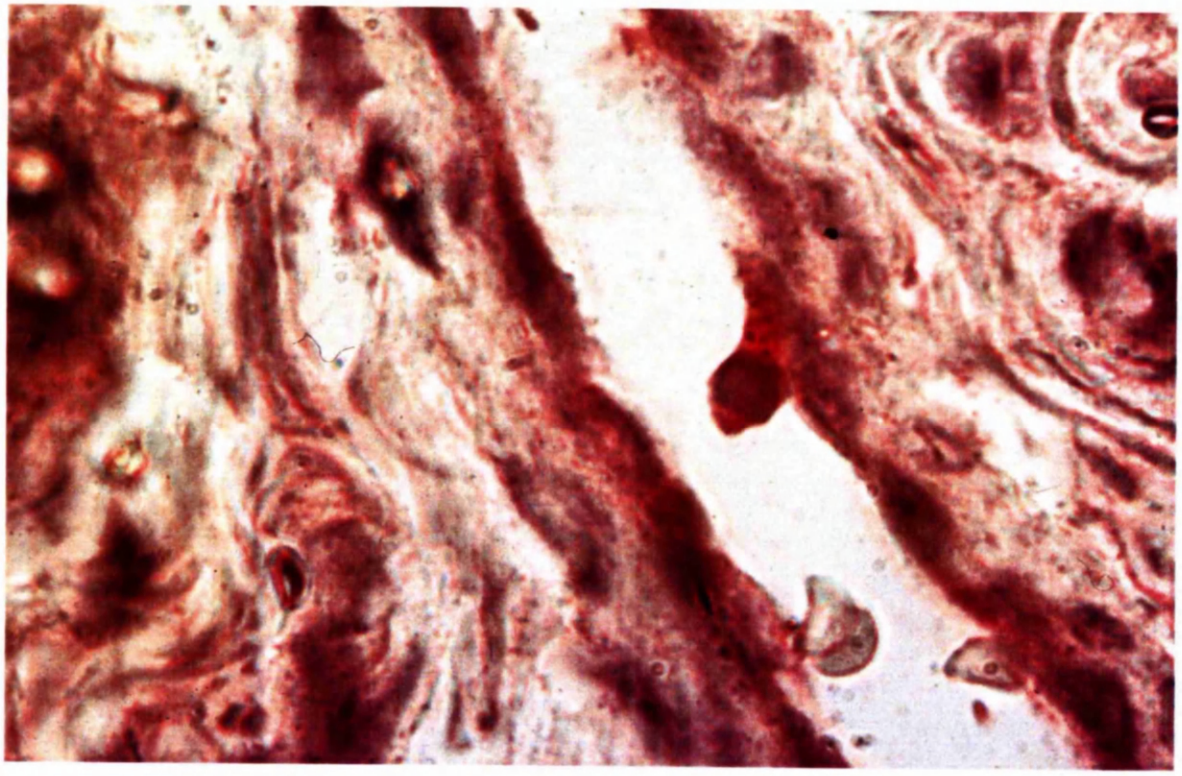

1
2
3
4
5
6

Figure 7b

Section of Dupuytren's tissue stained by immuno-histochemistry for IL–4 antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). Expression of IL-4 by a T cell contained in a blood vessel and starting to penetrate into the nodule (Magnification ×320).



Transforming Growth Factor Beta

TGF-beta is a group of cytokines, of which isoform types 1 and 2 are known to stimulate the synthesis of extracellular matrix and integrin expression in vitro, and has been identified in chronic inflammatory sites (Roberts *et al.*, 1986; Ignovitz and Massague, 1986; Wahl *et al.*, 1993; Massague *et al.*, 1991; Raghow *et al.*, 1987). TGF-beta by augmenting integrin expression may promote leukocyte adhesion to vessel walls and stimulate mononuclear cells to release further cytokines, refuelling the cycle. Functionally TGF-beta promotes monocyte adhesion to type IV collagen, laminin and fibronectin. This represents one mechanism as to how TGF-beta may facilitate monocyte margination and accumulation at a TGF-beta producing site (Wahl *et al.*, 1993). TGF-beta also increases gelatinase type IV collagenase and this could potentiate monocyte migration through the extracellular matrix (Wahl *et al.*, 1993).

TGF-beta also can act as an immunosuppressant modulator. Kulkarni demonstrated that TGF-beta knockout mice have remarkable infiltrations of mononuclear leukocytes into multiple organs (Kulkarni and Karlsson, 1993). This suggests TGF-beta can on occassion promote fibrosis while diminishing inflammation. It may be the balance between the three known TGF-beta isoforms and their receptors that underlies this balance. These paradoxical functions of the TGF-beta molecule do not detract from its pivotal role in inflammation and fibrosis. Understanding these complex relationships and their subsequent modulation will be necessary to control fibrosis.

Interleukin-8

IL-8 is part of the chemokine family. Chemokines are divided into two subfamilies depending on the arrangement of the first two of four conserved cysteines, which are separated by one amino acid into the CXC chemokines and CC chemokines. IL-8 is a member of the CXC chemokines.

Increased expression of IL-8 has been reported in a number of inflammatory disorders characterised by leukocyte infiltration, including rheumatoid arthritis (Seitz *et al.*, 1991), psoriasis (Sticherling *et al.*, 1991), gout (Terkeltaub *et al.*, 1991) and idiopathic pulmonary fibrosis (Car *et al.*, 1994). Both the interleukin protein itself and the two identified IL-8 receptors appear to have critical roles. Anti-IL-8 antibodies reduce tissue damage from inflammation in animal models (Harda *et al.*, 1994).

IL-8 can degranulate neutrophils releasing substances, which attract T lymphocytes and monocytes (Taub *et al.*, 1996). IL-8 is produced by several cell types, including endothelial cells (Sica *et al.*, 1990), epithelial cells (Elner *et al.*, 1990, Standiford, *et al.*, 1990), fibroblasts (Larsen *et al.*, 1989), neutrophils (Schroeder *et al.*, 1988), peripheral blood monocytes (Yoshimura *et al.*, 1987), tissue macrophages (Koch *et al.*, 1991), T lymphocytes (Schroeder *et al.*, 1988), keratinocytes (Larsen *et al.*, 1989), and chondrocytes (Recklies and Golds, 1992).

Prostaglandin E_2 (PGE_2) enhances IL-8 production by synovial fibroblasts after stimulation with IL-1 (Agro *et al.*, 1996) and PGE_2 production is elevated in Dupuytren's tissue (Badalamente *et al.*, 1988).

Human macrophages loaded with cholesterol produce more IL-8 mRNA and IL-8 itself into the medium (Wang *et al.*, 1996). High levels of LDL are seen in hypercholestraemia, diabetes mellitis and smoking all associated with increased incidence of Dupuytren's disease (Cathcart *et al.*, 1985).

In addition to IL-1 alpha and beta and TNF-alpha, Dupuytren's fibroblasts have now been shown by culture experiments and confocal microscope results to produce significantly more IL-8 than skin fibroblasts on IL-1 stimulation (Meek *et al.*, 1996), (Figure 8). This might suggest that Dupuytren's patients have a genetic defect resulting in the presence of a clone of palmar fibroblasts that when stimulated with increased levels of IL-1 or TNF-alpha produces sustained levels of IL-8 (Meek *et al.*, 1996). Such prolonged production of IL-8 would lead to chronic inflammatory cell infiltration by virtue of its leukocyte chemoattraction and promotion of angiogenesis. The fibrogenic cytokines subsequently produced by both the inflammatory cells and fibroblasts would lead to fibroblast proliferation, matrix synthesis and ultimately fibrosis.

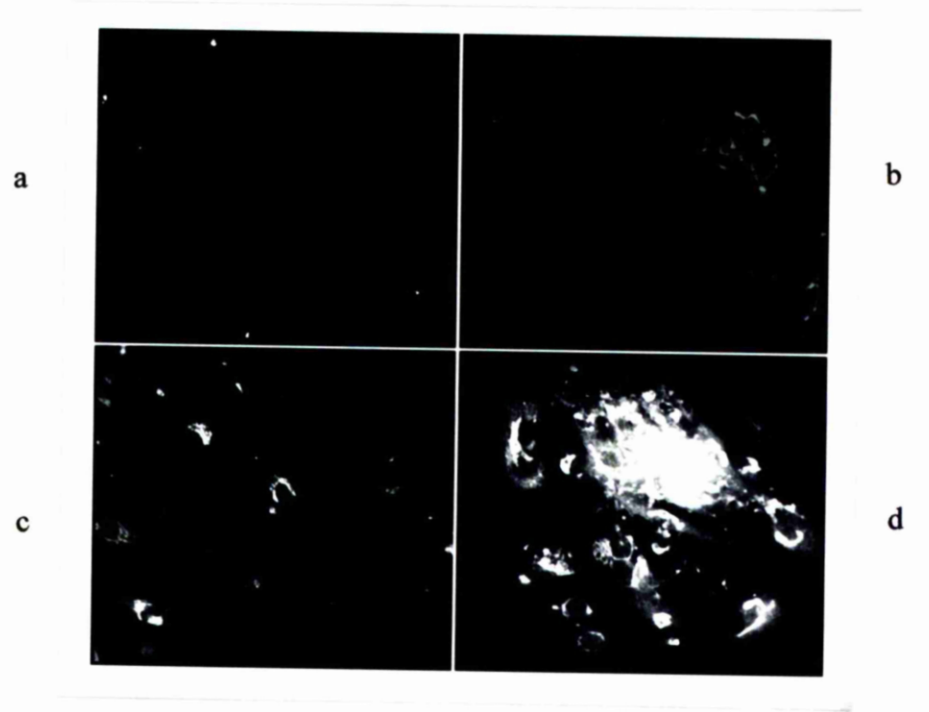


Figure 8

IL-8 fluorescent-labelled antibody binding analysed by a confocal microscope for normal skin cultures and Dupuytren's cell cultures. The top two squares show control cell cultures of normal skin cells obtained from penile foreskin. (a) is un-stimulated and (b) has been stimulated with lipopolysaccharide. There is a little increase in IL-8 expression. This compares with the Dupuytren's cultures below. Even in the un-stimulated Dupuytren's cultures (c) there is a higher baseline production of Interlukin-8 than the stimulated normal skin cells. Once stimulated (d) there is a dramatic increase in Interleukin-8 production. The confocal microscope allowed quantification of this production by calibrated light units.

Parallel to Dupuytren's disease, idiopathic pulmonary fibrosis can be divided into a subacutely progressive phase and a chronically progressive phase. This is similar to the nodule (proliferating) and cord (endstage) of Dupuytren's disease. IL-8 protein levels from bronchoalveolar lavage fluid were increased in subacutely progressive state but not in patients with the chronic stage. However if macrophages were isolated from the lavage of chronic phase patients and then stimulated with lipopolysaccaride they produced significantly more IL-8 than macrophages from control subjects. These patients had macrophages primed for IL-8 production even if they were not producing it in the chronic state. (Nakamura *et al.*, 1995). This is also true for peripheral blood monocytes and endothelial cells in chronic pulmonary disease (Streiter *et al.*, 1990).

This may explain why there is an increased incidence of Dupuytren's disease in people suffering from chronic pulmonary airway disease, idiopathic pulmonary fibrosis and hypercholestaemia, as these conditions are associated with cells with increased sensitivity in producing IL-8. This genetic predisposition may also predispose the Dupuytren's fibroblasts for IL-8 production as well.

1.10.4

Extracellular matrix

The extracellular matrix is composed of various cell-adhesive glycoproteins, such as fibronectin, laminin and different types of glycosaminoglycans and proteoglycans. Collagen is the major protein of the extracellular matrix (Burgeson, 1988). These building blocks are linked together to form a dense and complex tissue that fills the interstitial spaces. This is the medium that immune cells find themselves situated in during inflammation. Recognition of extracellular matrix molecules by the immune cells is mediated by very late activation (VLA) receptors (Hynes, 1992). Like cytokines, the cell-adhesive glycoproteins of the extracellular matrix affect cell activation, adhesion, proliferation and differentiation (Cahalon *et al.*, 1994).

Of all the matrix proteins, fibronectin is of particular relevance as it anchors the myofibroblast of inflammatory tissue to the extracellular matrix and thus is integral to the contraction process. In addition fibronectin is now known to bind to many of the inflammatory cells found in chronic inflammatory conditions and is present on the endothelium as well as in the extracellular matrix. Fibronectin shows a widespread pattern of expression in development as well as inflammation and repair. Cell surface receptors exist in fibronectin for many integrins and cell-surface proteoglycans and the integrin receptors have been mapped to specific sites in the fibronectin molecule. Work on the three dimensional structure of fibronectin defined repetitive sequences that were responsible for integrin recognition (Ffrench-Constant, 1995). For example, CS1

and CS5 sequences of the IIICS region of fibronectin is the receptor for $\alpha 4\beta 1$ and the RGD sequence lying within the type III repeat is recognised by $\alpha 5\beta 1$, (Figure 9).

The extracellular matrix in Dupuytren's

In the normal palmar fascia, the fibroblasts are embedded in an extracellular matrix containing parallel bundles of collagen fibrils. In the Dupuytren's diseased fascia, the nodules are characterised by abundant myofibroblasts and these are surrounded by a tight network of thicker and longer branched collagen filaments. At distances greater from the cells, the collagen fibrils become irregularly orientated. In the cords, there are only a few myofibroblasts, which are closely packed between large bundles of collagen fibrils. In the nodules, the matrix around the myofibroblasts usually contains predominantly proteoglycans. The proteoglycans connect the cells with the collagen fibrils and to each other (Badalamente *et al,* 1983; BrickleyParson *et al*, 1981; Chiu and MacFarlane, 1978).

Fibronectin is of particular interest, as studies have demonstrated that it is the anchoring protein for the myofibroblasts in Dupuytren's tissue (Tomasek *et al.*, 1986). Fibronectin has one particular site that binds to a subgroup of inflammatory cells carrying the $\alpha 4\beta 1$ integrin and is called the CS1 domain. The $\alpha 4\beta 1$ integrin is common to the predominant inflammatory cells found in Dupuytren's tissue and is also the ligand for VCAM-1 adhesion molecules expressible on endothelial cells.

Other work has looked at the extracellular matrix around the myofibroblast in Dupuytren's tissue specifically (Berndt *et al.*, 1994) demonstrating fibronectin within the whole palmar aponeurosis and in particular within the proliferative areas, whereas laminin, collagen type IV and tenascin labelling was restricted to the alpha-smooth muscle actin positive proliferative nodules.

The integrin $\alpha 4\beta 1$is a cell surface heterodimer expressed on most mononuclear leukocytes, eosinophils and basophils (Helmer *et al.*, 1990; Bochner *et al.*, 1991). It has been shown to mediate cell adhesion to VCAM-1 (Elices *et al.*, 1990; Carlos *et al.*, 1991; Hakkert *et al.*, 1991; Tiisla *et al.*, 1993; Osborn *et al.*, 1992a; 1992b; Vonderheide and Springer, 1992) as well as the HepII/IIICS region of fibronectin at which the CS1 site has the greatest $\alpha 4\beta 1$ binding activity (Guan and Hynes, 1990; Wayner *et al.*, 1989; Humphries *et al.*, 1986; Humphries *et al.*, 1987; Humphries, 1990; Mould and Humphries, 1991).

Fibronectin expression has been studied in Dupuytren's disease (Tomasek and Haahsama, 1991; Berndt *et al.*, 1994; Halliday *et al.*, 1994) and its co-expression with $\alpha 4\beta 5$ integrin expression on stromal cells (Magro *et al.*, 1995). This study demonstrated the distribution of the CS1 site of fibronectin and VCAM-1 along with $\alpha 4\beta 4$ integrin expression to which they both bind and relate this with the production of the TGF-beta. It has already been demonstrated that in vitro cultured Dupuytren's cells produce TGF-beta, and that TGF-beta stimulates Dupuytren's fibroblasts to grow (Kloen *et al.*, 1995).

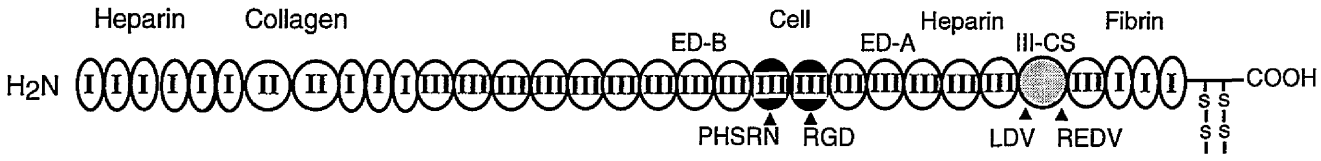


Figure 9

Model of the structure of fibronectin. Fibronectin is composed of three types of internal repeating modules designated type I, type II, and type III. The ED-A, ED-B, and IIICS modules can be present or absent in some forms of fibronectin as a result of alternative splicing. There are interchain disulfide bonds at the carboxy-terminal end of fibrinonectin. The binding domains of fibrinonectin are indicated at the top. The central cell binding domain consists of the ninth and tenth type III modules containing the minimal PHSRN and RGD cell recognition sequences, respectively. The IIICS module contains a cell adhesive site that functions independently of the central cell binding region and contains the LDV and REDV minimal cell recognition sequences.

1.10.5

Myofibroblasts

Myofibroblasts occur in granulation tissue and healing wounds. Their properties are associated with tissue contraction and hypertrophic healing. A reduction in their numbers is associated with the start of the maturation phase of repair and abatement of contraction.

Myofibroblasts have several ultrastructural and biochemical features of smooth muscle with microfilament bundles and alpha smooth muscle actin. The micro-filaments are associated with pH dependent triphosphate necessary for cellular contraction in muscle. These anchoring strands of actin bundles bind to collagen fascicles in the extracellular matrix by strands of fibronectin (Bauer and Parks, 1983). The cells contain vimentin and desmin. Four different types of heterogeneous phenotypes of myofibroblasts exist in different tissues based on the contained combination of vimentin, desmin and α smooth muscle actin (Skalli *et al.*, 1989).

When chronically inflamed tissue resolves into a scar then alpha smooth muscle actin containing myofibroblasts disappear, possibly by apoptosis (Schmitt-Graff *et al.*, 1994). If the tissues remain inflamed the myofibroblasts remain and together with increased extracellular matrix production result in contraction. Myofibroblasts accumulation in fibrosis may be at least in part due to local proliferation (Hewitson *et al.*, 1995).

Previously fibroblasts have been thought of as a homogenous non-differentiating cell population. When analysed by molecular biological methods, fibroblasts appear to exists as 11 cell types with biological and biochemical individuality developed along 11 different stages of differentiation sequences in 5 compartments under the control of genetic programs (Bayreuther *et al.*, 1995). This differentiation is maintained in fibroblast populations in pathological manifestations, from fibrosis to Duchenne muscular dystrophy or neoplastically transformed cell lineages. All these cell systems appear to be stem cell systems with a design resembling the design of the fibroblast stem cell system. Are certain cells preprogrammed to become myofibroblasts?

Alternatively, if myofibroblasts are transformed cells then what controls the alpha smooth actin expression in fibroblasts? Factors appear to include increased TGF-beta, heparin binding sites, or a reduction in α interferon or cell density (Tomasek *et al.,* 1999; Gressner and Bachem, 1994).

The activation of lipocytes and fibroblasts to a myofibroblast is triggered in the liver by the release of platelet-derived growth factor (PDGF) and TGF-beta from activated Kupffer cells (Schuppan *et al.*, 1995). TGF-beta-1 also differentiated ocular trabecular meshwork cells to myofibroblasts phenotype (Tamm *et al.*, 1996). Bachem showed that TGF-beta-1 and TNF-alpha could stimulate the trans-differentiation of fat storing cells in the rat liver into myofibroblasts in association with increased fibronectin synthesis (Bachem *et al.*, 1993). Cells with

the myofibroblast phenotype are more abundant in fibrotic lung using a bleomycin-treated model and these cells possessed greater contractile capacity in vitro at least in part due to increased endogenous TGF-beta-1 gene expression (Zhang *et al.*, 1996).

Myofibroblast differentiation can be induced in fibroblasts by plating them at low density and these myofibroblast enriched cultures in turn produce more latent and active TGF-beta than high density fibroblast enriched cultures (Masur *et al.*, 1996). This suggests a cell density model of myofibroblast differentiation, where loss of cell contact could stimulate TGF-beta production.

Myofibroblasts In Dupuytren's Tissue

The myofibroblast characteristic of Dupuytren's tissue has been an area of much research. The origin of this cell in Dupuytren's tissue as with other myofibroblasts remains uncertain (Gown, 1990; Kisher and Speer, 1984), with some suggesting they arise from the 'pericyte' or perivascular cells. Others suggests it is simply a transient form of fibroblast (Andrews *et al.*, 1991) or it is derived from smooth muscle cells (Darby *et al.*, 1990) or both (Schürch *et al.*, 1990). Ehrlich, (1990) believed the myofibroblasts orientated and fixed the collagen (Ehrlich and Rajaratnam, 1990). Whatever its origin, the expression of smooth muscle actin phenotype correlates with increased contractility in Dupuytren's myofibroblasts (Tomasek and Rayan, 1995). Dupuytren's fibroblasts are thought to have increased sensitivity to oxygen free radicals leading to myofibroblast differentiation (Murrell, 1992)

Dupuytren's myofibroblast aggregates contract in response to PGF2 alpha and relax in response to PGE2. PDGF was associated with myofibroblast as shown by immuno-histochemical staining (Badalamente *et al.*, 1992). Both bFGF and PDGF were mitogenic for Dupuytren's cell cultures, and TGF-beta potently stimulated collagen production by Dupuytren's fibroblasts (Alioto *et al.*, 1994). These 3 factors are elevated in Dupuytren's tissue and they and other macrophage mediated growth factors with their interrelated effects on Dupuytren's cells needs further delineation. The questions is, are there different myofibroblast phenotypes in Dupuytren's nodules, or are they the same phenotype at different temporal stages?

1.10.6

Nitric Oxide

Nitric oxide (NO) is a free radical gas, which is implicated as a secondary messenger molecule in many biological pathways (Clancy and Abramson, 1995). NO is generated by the conversion of the amino acid L-arginine to L-citrulline in a reaction catalysed by a family of isoenzymes, arising from three distinct genes. These types are (1) neuronal (nNOS), (2) inducible (iNOS) and (3) endothelial (eNOS). The inducible form iNOS is induced by many pro-inflammatory cytokines and is thought to be calcium dependent. iNOS is generally seen in pathological conditions where its induction results in the production of large quantities of NO. NO in low levels of production can act as a scavenger of superoxide anion (Midorikawa and Ogata, 1996) and in this role could serve as a defensive function by protecting tissues against super-oxide-induced inflammatory cell release of fibrogenic mediators. The NO molecule in high levels contributes to tissue damage by producing peroxynitrite anion through its interaction with superoxide. Peroxynitrite is a powerful long-lived oxidant, which is known to cause acute inflammation and oedema (Salvemini *et al.*, 1996).

In addition to NO, other free radicals produced by inflammatory cells include superoxide anion radical, hydrogen peroxide and the extremely reactive hydroxyl radicals. These free radicals are produced in anaerobic conditions and are known to play a key role in the aetiology of fibrosis (Cerutti *et al.*, 1992). Superoxide anion induces inflammatory cells to degranulate and release mediators important

in the development of fibrosis including TGF-beta and TNF-alpha (Gordon and Galli, 1994). Reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and cytokines are frequent companions at sites of acute inflammation. A clear link exists between certain cytokines production and subsequent generation of ROI and RNI (Remick and Villarete, 1996). Recent data suggests ROI, RNI and NO not only act as an end-stage effector molecule, but also as an initiator of acute inflammation. For example ROI and RNI will upregulate IL-8 production specifically via other cytokines. In addition, nitric oxide synthesis inhibitors decrease the production of IL-8 in stimulated whole blood and fibroblasts and exogenously added ROI increasing IL-8 production by the same cells (Remick and Villarete, 1996).

TGF-beta is involved in stimulating extracellular matrix production and healing by fibrosis. Many cytokines (particularly IL-1 and TNF-alpha) stimulate production of nitric oxide from various cells. TGF-beta can also suppress the NO production by reducing iNOS expression (Vodovotz and Bogdan, 1994). Some cells demonstrating this suppression include macrophages, smooth muscle cells, endothelial cells, fibroblasts and mesangial cells (Vodovotz and Bogdan, 1994, Junquero *et al.*, 1992; Finder *et al.*, 1995; Lavnikova and Laskin, 1995; Ikeda *et al.*, 1994). The relationship of these interactions in Dupuytren's tissue has not been established.

Recent evidence is accumulating that implicate nitric oxide as a secondary messenger in intracellular signal transduction pathways involved in triggering many cells, including inflammatory cells, to undergo apoptosis (Nishio *et al.*,

1996; Um *et al.*, 1996; Shimaoka *et al.*, 1995; Palluy and Rigaud, 1996; Messmer *et al.*, 1996). This may offer another avenue of intervention once the exact mechanism is understood. Importantly, current pharmacological agents can control nitric oxide production.

1.10.7

Steroids

Steroids are the most potent and effective agents in controlling chronic inflammatory diseases (Barnes and Adcock, 1993). Steroids interact with a cytoplasmic receptor, part of a supergene family, present in almost all cells but in differing densities. The regulation of these receptors expression can be at the transcriptional, translational or post-translational stages, as seen in the down-regulation of steroid receptors in circulating monocytes and lymphocytes after steroid therapy (Wilckens, 1995).

Gene transcription

In responsive cells, steroids regulate transcription of certain target genes usually of 10-100 genes per cells (Owens *et al.*, 1991). Within the nucleus the receptor forms a dimer and binds to the DNA at the glucocorticoid response elements (GREs). The receptor can promote or suppress transcription by steric hindrance or it can reduce mRNA stability (Barnes and Adcock, 1993).

Anti-inflammatory effects

Cytokines

Steroids decrease the transcription of cytokines e.g. IL-1, TNF-alpha, Granulocyte macrophage colony stimulating factor (GM-CSF), IL-3, IL-4, IL-5, IL-6, IL-8. The mechanism involves GREs and reduced mRNA stability (Guyre

et al., 1988). Steroids also block cytokines' effects in other ways, including by decreasing IL-2 receptors and inactivating transcription factors e.g. NF_kB (Barnes and Adcock, 1993). Dexamethasone downregulates expression of IL-8 mainly by decreasing mRNA stability. That is, they repress genes in conditions of stress (Standiford *et al.*, 1992).

Nitric oxide

iNOS in macrophages is potently inhibited by steroids. The inhibitory effect is on the iNOS gene itself (Gilbert and Herschman, 1993).

Adhesion molecules

Steroid induced reduction of adhesion molecule expression is mainly secondary to reduced cytokine production e.g. IL-1 beta or TNF-alpha. However, they also have a direct inhibitory effect on expression of adhesion molecules at the level of gene transcription (Cronstein *et al.*, 1992). Aziz demonstrated this in endothelial cells, where dexamethasone reduced VCAM-1 expression (Aziz and Wakefield, 1996).

Therapeutic implications for chronic inflammation

As all glucocorticoids bind to the same cytoplasmic receptor it is unlikely that more selective steroids will be developed. Their most important effect is the repression of genes encoding for cytokines and lipid mediator enzymes.

Individual control of subsequent steps in the sequence of events may give more selective results without the potential side effects of steroids.

1.10.8

Steroid Effect On Inflammatory Components In Relation To Dupuytren's Contracture

The histopathology of Dupuytren's disease has been documented as already stated previously (Meyerding *et al.*, 1941, Luck, 1959). In addition to the myofibroblasts from the nodule itself (Gabbiano and Majno, 1972; Iwasaki *et al.*, 1984; Scürch *et al.*, 1992; Azzarone *et al.*, 1983), clusters of macrophages and T lymphocytes have been observed in Dupuytren's tissue (Baird *et al.*, 1993a). These inflammatory cells have been demonstrated to produce a spectrum of cytokines (Baird, 1993b), which regulate migration, proliferation, and contracture of Dupuytren's myofibroblasts (Baird, 1994). These cytokines include bFGF, IL-1alpha / beta, TGF-beta 1 & 2, TNF-alpha, IL-8.

Some clinicians had noted the effect of locally injected steroid could sometimes halt the progressive proliferation of Dupuytren's nodules macroscopically and indeed cause them to regress in size (Ketchum *et al.*, 1972). Therefore, the effect of preoperatively locally injected steroid on the above interrelated extracellular matrix protein, adhesion molecule, integrin and cytokines required clarification.

1.10.9

Steroid Induced Apoptosis In Dupuytren's Disease

In normal granulation tissue myofibroblasts disappear by apoptosis. In excessive scarring tissue myofibroblasts persist (Desmouliere, 1995). This differing behaviour of myofibroblasts is dependent on environmental factors controlled by inflammatory cells.

Thus chronic inflammatory conditions depend on the capacity to inhibit an intrinsic suicide pathway in these inflammatory cells or in the myofibroblasts. In inflammatory tissue undergoing normal resolution a decrease would be expected in monocyte population by apoptosis and in subsequent cytokine production. For example, the normal resolution of muscle inflammation macrophages undergo such selective apoptosis (Tidball and St Pierre, 1996)

Regulation of monocyte and lymphocyte apoptosis

Cellular Adherence

Collagen, fibronectin and laminin increase monocyte survival by blocking apoptosis (Mangan *et al.*, 1993). The extracellular matrix glycoprotein CS1 fibronectin is present in large amounts in Dupuytren's tissue and is a natural ligand for monocytes.

Chemotactic factors

Chemotactic factors including TGF-beta 1 are extremely potent at recruiting monocytes to sites of inflammation, although it appears that additional activation is required to block apoptosis. TGF-beta stimulates IL-1 production but also IL-1 receptor antagonist protein and thus has little net effect. The pro-inflammatory cytokines, IL-1 and tumour necrosis factor alpha are independently able to inhibit monocyte apoptosis (Mangan *et al.*, 1993; Maier *et al.*, 1996). Both IL-1 and TNF-alpha are expressed by Dupuytren's cells (Baird *et al.*, 1993b).

Steroids

Steroids have been shown to induce inflammatory cell depletion by apoptosis in the intestine of rats (Soda *et al.*, 1993) and augment T cell apoptosis in inflammatory lesions of the peripheral nervous system in the rat (Zettl *et al.*, 1995). This may be an augmentation to their anti-inflammatory effects mediated by direct inflammatory cell apoptosis. The subsequent down-regulation of growth factors and cytokines itself may induce apoptosis indirectly as another mechanism of effect.

There may well be a direct effect of steroids on fibroblasts and other non-inflammatory cells as well. For example, Lutton demonstrated rat osteoclasts underwent morphological changes resembling apoptosis after 2-4 hours of exposure to dexamethasone and corticosteroid (Lutton *et al.*, 1996).

1.11

Hypothesis

Dupuytren's disease is a chronic inflammatory disease characterised by the presence of increased levels of pro-inflammatory cells and growth factors. This is theorised as due to the persistence of inflammatory cells, which normally would undergo apoptosis. The aim of this work was to record the effect of steroids on Dupuytren's tissue pro-inflammatory cytokines, growth factors, extracellular matrix and the rate of apoptosis of inflammatory cells and fibroblasts. Understanding the individual pathways involved may allow more accurate targeting of therapeutic intervention to prevent the subsequent fibrosis. This may have relevance to many inflammatory conditions that lead to fibrosis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Patients

2.2 Immunohistochemistry

2.3 Cell culture & Flow cytometry

2.4 Steroids

2.5 Materials

2.1

PATIENTS

The patients were all taken from referrals to the hand clinic of the Western Infirmary, Glasgow, Scotland. They represented a typical cross section of the West of Scotland population. All patients entered were primary presentations and had not undergone previous surgery. Ethical permission had been previously granted for the study on pro-inflammatory cytokines by Mr Baird in the early 1990's. As all sample material would normally be disposed of, no results were made aware to the patient and the treatment of nodules with steroids is a recognised management (Ketchum *et al.*, 1974), there was no objection from the local ethical committee at that time. All patients gave consent for the use of their tissue for this study. The consultant, the specialist hand registrar, or myself performed all the surgery.

The patients were studied as follows. The immunohistochemistry study of inflammatory cytokines in Dupuytren's tissue and the effect of steroids were studied in 20 patients with Dupuytren's disease undergoing routine fasciectomy and 4 patients undergoing routine carpal tunnel decompression.

The immunohistochemistry study of the extracellular matrix and adhesion factors in Dupuytren's tissue and the effects of steroids were studied in 30 patients and the same control tissue was used from the carpal tunnel decompressions.

Thirdly, the effect of steroids on proliferation and apoptosis in Dupuytren's tissue was studied by immunohistochemistry in 32 patients.

Finally, flow cytometry was used to study the effect of steroids on apoptosis of cultured Dupuytren's cells, cultured palmar fascia cells and of cultured fascia lata cells. 6 patients undergoing fasciectomy had cultures grown from the excised tissue, 6 patients undergoing routine carpal tunnel decompression had cultures grown from excised palmar fascia and finally 2 patients undergoing fractured neck of femur surgery had cultures grown from samples of fascia lata.

In the study of steroid changes on the inflammatory cytokines 20 patients were used. Ten Dupuytren's patients (7 men and 3 women) were entered into the non-steroid treated group and 10 Dupuytren's patients (8 men and 2 women) into the group preoperatively injected with steroid. The average age of the untreated group was 47 years +/-11 years SD (29 to 63 years range) and was comparable with the steroid treated group age average of 52 years +/- 13 years SD (36 years to 67 years range). The patient group treated with steroid had 20mg depomedrone, in a volume of 0.5ml, injected into the perinodular area of the palm at five days prior to operation at the pre-operative assessment clinic. All patients were at a comparable stage of Dupuytren's contracture and activity. Surgery was performed routinely under regional anaesthesia and tourniquet control. 4 patients undergoing routine carpal tunnel decompression had a representative sample of palmar fascia excised. Surgery was performed under identical conditions, again with regional anaesthesia and tourniquet control.

Samples of excised Dupuytren's tissue and palmar fascia control tissue were placed in 10% neutral buffered formalin solution.

In the study of steroid changes on the extracellular matrix and adhesion factors 30 patients were used. 20 Dupuytren's patients (17 men and 3 women) were entered into the non-steroid treated group and 10 Dupuytren's patients (8 men and 2 women) into the group preoperatively injected with steroid. The average age of the non-steroid treated group was 58 years +/- 14 years SD (40 to 77 years' range) and was comparable with the steroid treated group age average of 54 years +/- 16 years SD (31 years to 75 years range).

For the study of steroid effect on apoptosis in tissue, 32 patients undergoing fasciectomy were studied in two groups of 16. One group of 16 Dupuytren's patients (14 men and 2 women) were entered into the non-steroid treated group and the other group of 16 Dupuytren's patients (14 men and 2 women) into the group preoperatively injected with steroid. The average age of the untreated group was 54 years +/- 15 years SD (42 to 70 years range) and was comparable with the steroid treated group age average of 57 years +/- 18 years SD (35 years to 75 years range). The patient group treated with steroid had 20mg depomedrone, in a volume 0.5ml, injected into the perinodular area of the palm five days prior to operation at the pre-operative assessment clinic. All patients were at a comparable stage of Dupuytren's contracture and activity clinically, and on subsequent histological assessment. Surgery was performed routinely under regional anaesthesia and tourniquet control. Samples of excised Dupuytren's tissue were placed in 10% neutral buffered formalin solution.

Tissues for culture experiments were collected as follows.Tissue from 6 patients undergoing palmar fasciectomy for Dupuytren's disease was used for culture experiments. The tissue was placed in a transport medium immediately after excision and processed within the hour. Again for culture experiments, 6 patients undergoing routine carpal tunnel decompression had a representative sample of palmar fascia excised. Surgery was performed under identical conditions, again with regional anaesthesia and tourniquet control. None of these patients had received preoperative steroid injections. Fascia lata samples were obtained at wound closure from two patients undergoing routine dynamic-hip screw fixation of fractured necks of femur. Again, no preoperative injections at the site of operation had been performed. The fascia lata samples were used for culture experiments only and were again stored in transport medium (Hanks medium supplemented with 2% foetal calf serum (FCS) and antibiotics) and identically processed prior to culture. No patients in any group had been receiving oral or parental steroids.

2.2

IMMUNOHISTOCHEMISTRY

I performed all histological preparations. The analysis of sections was independently performed by one of the laboratory technicians and verified by myself.

Samples of Dupuytren's and control tissue had been collected at the time of surgery in 10% formalin. All subsequent procedures were carried out at room temperature unless otherwise stated. The samples were dehydrated sequentially through a gradation of alcohol and histoclear solutions prior to embedding in paraffin wax. The tissue blocks were then cut and 7μm sections mounted onto 3-Aminopropyl triethoxy-silane (APES) coated slides. Immunohistochemistry was performed on the sections by the sandwich technique employing the avidin-biotin alkaline phosphatase complex. Sections were dewaxed with histoclear, rehydrated in graded alcohol solutions to 0.05 M Tris Buffer (pH7.2), and then pretreated with trypsin (Sigma, 1.0mg dissolved in 1ml of deionised water) for 15 minutes at 37°C. After 3 washes with TBS pH 7.2 the sections were incubated with primary antibody for 1 hour. The sections were then incubated with biotinylated secondary antibody (Sigma Immuno Chemicals; 1:60 dilution) for 45 minutes followed by 3 washes with TBS pH 7.2 and then finally incubation with extravidin alkaline phosphatase (Sigma Immuno Chemicals; 1:400 dilution) took place for 45 minutes with final 3 washes with TBS pH 7.2. Bound alkaline phosphatase was visualised using BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) at room temperature until visualisation was

complete or background changes were evident. This gave a bluish / black / brown colour depending on the intensity of reaction. Alternatively, bound alkaline phosphatase was visualised using Fast Red TR/Napthol AS-MX Phosphate (4-Chloro-2-methylbenzenediazonium/3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate), producing an intense red stain. The section was placed in distilled water to stop this reaction and then counter-stained with (0.5%) neutral red. Control negative sections were run without primary antibody. The sections were then mounted in 50% glycerol solution with a coverslip.

The antibodies used were rabbit anti human TNF-alpha (R&D systems; 1:60 dilution), sheep anti human IL-1 alpha (R&D systems; 1:60 dilution), sheep anti human IL-1 beta (R&D systems; 1:60 dilution), rabbit anti human IL-4 (R&D systems; 1:60 dilution), goat anti human IL-6 (R&D systems; 1:60 dilution), goat anti human IL-8 (R&D systems; 1:50 dilution), goat anti human bFGF (R&D systems; 1:60 dilution), mouse anti human CS1 fibronectin (Chemicon; 1:100 dilution), goat anti human VCAM-1(R&D systems; 1:200 dilution), mouse anti human VLA-4 (alpha 4 beta 1 integrin) (Serotec; 1:100 dilution), and chicken anti human TGF-beta 1 and 2 (R&D systems,1:60 dilution), Lewis Y (Sigma 1:60; dilution) and Ki 67 (DAKO; 1:60 dilution) antigen markers for apoptosis and proliferation respectively.

Ki67 is a nuclear protein, which is expressed in the proliferating cells during late G1-, S-, M-, and G2-phases of the cell cycle, while cells in the G0 (quiescent) phase are negative for this protein (Rose *et al.*, 1994). Lewis Y antibody recognises a carbohydrate identified as a marker of specific types of cell and

specific stages of differentiation. Expression of Lewis Y has been shown to correlate closely with the process of apoptosis, but not with cell proliferation or necrosis (Hiraishi *et al.*, 1993).

Counting of immunostained cells and areas of diffuse matrix staining was performed over 3 consecutive sections and 5 random areas (at times 25 magnification) for each patient sample. For the cell numbers allocated these were entered into Excel worksheets and where appropriate statistical analysis was performed by means of the Mann-Whitney U test for non-parametric data. Where the areas on the histological sections were assessed for background immuno-reactivity this was done by allocating the degree of immuno-reactivity for that particular field area, namely as: +++ diffuse strong reactivity; ++ strong immunoreactivity restricted to cellular areas; - no immunoreactivity.

2.3

CELL CULTURE FOR APOPTOSIS ANALYSIS BY FLOW CYTOMETRY

The sterile tissue sample was transported in Hanks medium supplemented with 2% foetal calf serum (FCS) and antibiotics. As soon as possible after surgery (within 2 hours) the tissue sample was washed three times with Dulbecco's calcium and magnesium free phosphate bufferered saline (PBS, Gibco). The tissue was then cut into $1mm^3$ fragments in a large petri dish containing Dulbecco's PBS. The tissue fragments were transferred to a pre-weighed universal container, spun down at 2000 revolutions per minute (RPM) for 5 minutes at room temp and the supernatant discarded. The tissue was then washed 3 times with PBS, the final wash carefully removed and the weight of tissue noted. 2 to 4 g of wet tissue was used per 25ml of collagenase / Deoxyribonuclease 1 (DNase). Tissue fragments were washed into a 100ml Erlenmeyer flask (conical flask) with the dissociation mixture. The tissue was digested for 3.5 hours at 37^0C in a shaking water bath with 25ml Hanks balanced salt solution containing 0.1% collagenase and 0.01% DNase (DN-25, Sigma).

Cells and debris were removed by transferring the sample to a universal container and centrifuged at 400 × g at room temperature for 10 minutes, supernatant then discarded. The cells and tissue washed into the same conical flask used for the first incubation with 50ml Dulbecco's PBS containing 0.05% Typsin (Type 3, Sigma) and 0.02% EDTA and a further incubation at 37^0C in a shaking water bath carried out

The cells were separated from the undigested residue by allowing the residue to settle for 5 minutes then filtering the supernatant through sterile gauze, the remaining residue then washed with DMEM + 10% FCS and then filtered through the gauze. After dilution with DMEM+ 10% FCS, 1ml samples of 1×10^6 cells were plated in $35mm^2$ Nunc petri dishes and placed into a CO_2 incubator at 5% CO_2, 37^0C in a moist atmosphere.

The palmar fascia cells were much slower to grow, taking up to a month to reach a state where the cells had covered most of the petri dish base. Cells were always harvested just before they completely covered the petri dish base, to avoid the subsequent effect of contact inhibition on growth (sub-confluence). The fascia lata fibroblast cultures were quicker but still not as fast at replicating as the Dupuytren's cells. The 6 individual palmar fascia cells cultures were added together into 2 cultures just prior to analysis in order to give sufficient cells for FACS analysis. This was not necessary for the fascia lata cultures

Once sub-confluence was reached, half the cultures of cells from Dupuytren's tissue, palmar fascia tissue and fascia tissue had soluble methyl prednisilone (10^{-3} M) added. 14 hours later all these cell samples were harvested.

An apoptosis detection kit (R & D systems) was used to quantitatively determine the percentage of cells undergoing apoptosis by virtue of their ability to bind annexin V and exclude propidium iodide. The test is based on the observation that soon after the initiation of apoptosis, most cell types translocate phosphatidylserine from the inner surface of the plasma membrane to the cell

surface (Martin *et al.*, 1995). Once on the cell surface, phosphatidylserine can be easily detected by staining for Annexin V, which has a strong, natural affinity for phosphatidylserine (Andree *et al.,* 1990).

Harvested cells were collected by trypsinisation and suspended in 1x binding buffer (R & D annexin V kit) at a concentration of 1x 10^6 cells/ml.

Harvesting by trypsinisation of cell monolayers

1 Decant off medium (Dulbecoo's modification of Eagle's medium + 10 % foetal calf serum and antibiotics).

2 Wash cells with phoshate buffered saline (PBS) without Calcium and Magnesium - Decant off.

3 Add 0.02% EDTA solution sufficient to cover cells and leave at room temperature for 30 seconds and decant off.

4 Add 0.02% EDTA / 0.05% trypsin solution, sufficient to cover cells, leave at room temperatures for 30 seconds and decant off

5 Incubate cells at 37^0C until cells become detached.

6 As soon as cells become detached, resuspend in medium and harvest the cells by spinning at room temperature, 1000 revolutions per minute for 10 minutes on a MSE super minor centrifuge.

7 Wash cells, resuspend in medium or buffer, making sure any clumps are broken up.

The cells were washed with buffer solution and a trypan blue exclusion test used to check the percentage of alive cells at this concentration.

100 *u*l of these cells (1 x 10^5 cells) were transferred to a 5ml tube. 10 *u*l of fluorescein-conjugated annexin V and 10 *u*l of propidium iodide (PI) reagent were added, and the mixture was gently vortexed and then incubated for 15 minutes at 20-25^{0}C (room temperature) in the dark. Following this incubation, and without further washing the cells of excess reagents, 400 *u*l of 1x binding buffer were added. Three additional control tubes were also set up: a) unstained cells; b) cells stained with annexin V-fluorescein only; c) cells stained with PI only (Table 3).

.

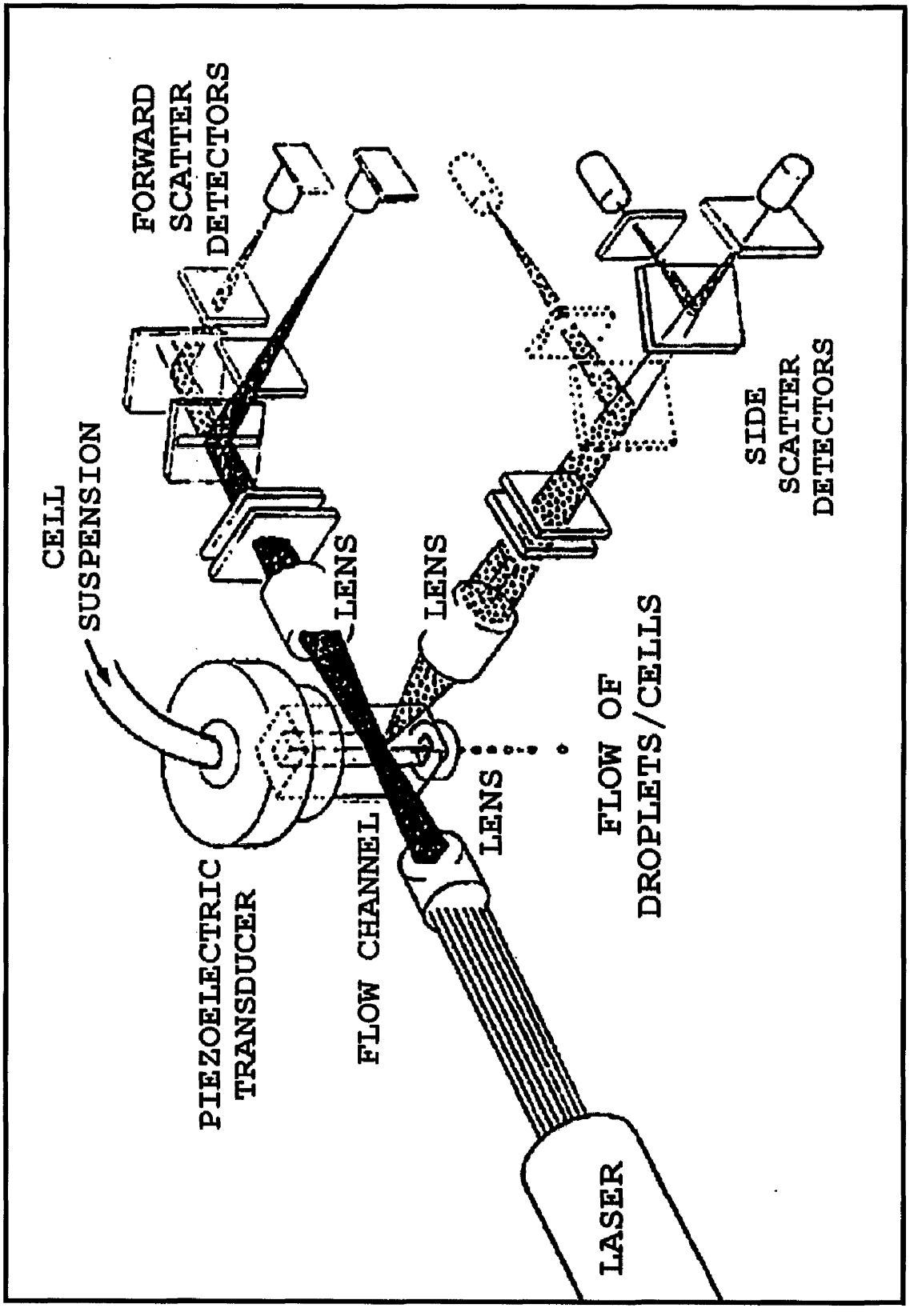
Table 3

Tubes	(Sample 100*u*l)	PI (*u*l)	AnnexinV (*u*l)	Buffer (*u*l)
1	cells	—	—	20
2	cells	—	—	20
3	cells	10	—	10
4	cells	10	—	10
5	cells	—	10	10
6	cells	—	10	10
7	cells	10	10	—
8	cells	10	10	—
9	Steroid treated cells	10	10	—
10	Steroid treated cells	10	10	—

The level of fluorescence was measured with the Lysis II programme on a Becton Dickinson FACScan flow cytometer (Figure 10). Dedicated software permitted statistical analysis of the data obtained. Purely annexin V binding cells were determined by gating of these cells and the ratio compared with and without steroid addition and taken as a level of apoptosis. This was expressed as a percentage of the total cell number. Statistical analysis was performed by the Mann-Whitney U test to compare the effect of steroids on the apoptosis ratio.

Figure 10

Diagrammatic sketch of a flow cyometer. The flow cytometer was equipped with single 15mW argon laser emitting light at 488nm. The FITC detector detects fluorescence data generated from the fluorescein-labelled annexin V labelled cells (usually FL1) and propidium iodide labelled cells are monitored by the detector reserved for phycoerythrin emission (usually FL2). The FITC emission light was measured by filtering through a narrow-band green filter (530 nm).


FORWARD SCATTER DETECTORS
CELL SUSPENSION
LENS
LENS
SIDE SCATTER DETECTORS
PIEZOELECTRIC TRANSDUCER
FLOW CHANNEL
LENS
FLOW OF DROPLETS/CELLS
LASER

2.4

STEROIDS

The steroid used in the patients receiving preoperative palmar injections was depo-medrone 40 mg in a 1ml volume. For the culture experiments this steroid preparation would not have been miscible with the medium to give a clear view of the cells. Therefore, solu-medrone (Upjohn) was used at an equivalent therapeutic concentration for this steroid type of 10^{-3} M. This concentration of 10^{-3} M had induced observable changes consistent with apoptosis in previous cell culture studies at this department. The solu-medrone had induced characteristic changes including intense cell surface activity, cell shrinkage and subsequent condensation of chromatin in a crescent shaped manner along the nuclear envelope. 49.651mg were dissolved in 1 ml of medium to give a 0.1 M solution and this was further diluted to give 10^{-3} M solution.

2.5

MATERIALS

Crude bacterial Collagenase Type 1, Sigma Chemical Company

Nunc Petri dishes, Gibco Europe

Trypsin, Sigma

Content Trypsin 1.0mg/tabley

Calcium Chloride 0.6mg/tablet

Tris Buffer

One tablet, dissolved in 1ml-deionised water yields 1mg/ml trypsin, 4mM $CaCl_2$ in 200mM Tris, pH 7.7 at 25^0C

BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium), Sigma

One tablet, dissolved in 10ml of deionised water

Fast Red TR/Napthol AS-MX Phosphate (4-Chloro-2-methylbenzenediazonium /3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate), Sigma

One tablet dissolved in 1ml of deionised water

Microscope

Axioplan 2 microscope, Carl Zeiss Ltd

SLR 35 mm Camera, Yashica

Dulbecco's modified Eagles culture medium

Culture Medium

Dulbecco's modified Eagles medium (DMEM, Gibco)

Foetal calf serum (FCS, Heat and pH inactivated, Gibco)

100 units / ml : 100 μl /ml Penicillin : Streptomycin (Gibco)

DMEM	500ml
FCS	50ml
Penicillin : Streptomycin	5ml 10000 units / ml : 10000 mg / ml

Stored sterile at 4 °C for no more than 1 month.

Dissociation Medium

0.1% collagenase (Type 1, Sigma)

0.01% Deoxyribonuclease 1 (DN – 25, Sigma)

29mM Hepes (Gibco)

100 units / ml : 100μl /ml Penicillin : Streptomycin (Gibco)

Hanks balanced salt solution (HBSS, Gibco)

	100ml
Collagenase	0.1g
Deoxyribonuclease	0.01g
Hepes	2ml 1M HEPES
Penicillin : Streptomycin	1ml 10000 units/ml : 10000mg /ml
HBSS	10ml (10 x Concentration)

Make to 100ml with deionised water and sterilise by filtration through a 0.2μm pore size filter.

Trypsin / EDTA

0.05% Trypsin (Type III, Sigma)

0.02% Ethylenediaminetetra-acetic acid (Disodium salt, EDTA)

Phosphate buffered saline (PBS, Gibco)

	1 x
Trypsin	0.1g
EDTA	0.04g
PBS	20 ml (10 x concentration)

Make to 200ml with deionised water and sterilise by filtration through a 0.2μm pore filter and store in deep freeze.

Hanks Transport Medium

Sodium bicarbonate (Gibco)

Hanks balanced salt solution (HBSS, Gibco)

Foetal calf serum (FCS, Gibco)

250 units / ml : 250μg / ml Penicillin : Streptomycin (Gibco)

Sodium bicarbonate	70mg
HBSS	20ml 10 x conentration
FCS	4ml (heat and pH inactivated)*
Penicillin : Streptomycin	5ml 100000 / ml : 100000mg / ml

*Heat to inactivate complement. pH to inactivate alpha-2-macroglobulin, this is necessary for collagenase estimations as α-2-macroglobulin binds irreversibly with collagenase

CHAPTER 3

RESULTS

3.1 Immunohistochemistry - Pro-inflammatory cytokines in Dupuytren's disease and the effect after steroids

3.2 Immunohistochemistry - matrix and adhesion factors and the effect of steroids

3.2 Immunohistochemistry - apoptosis and proliferation and the effect of steroids

3.3 Flow Cytometry Analysis - apoptosis in cell cultures and the effect of steroids

3.1

Immunohistochemistry for Pro-Inflammatory Cytokines

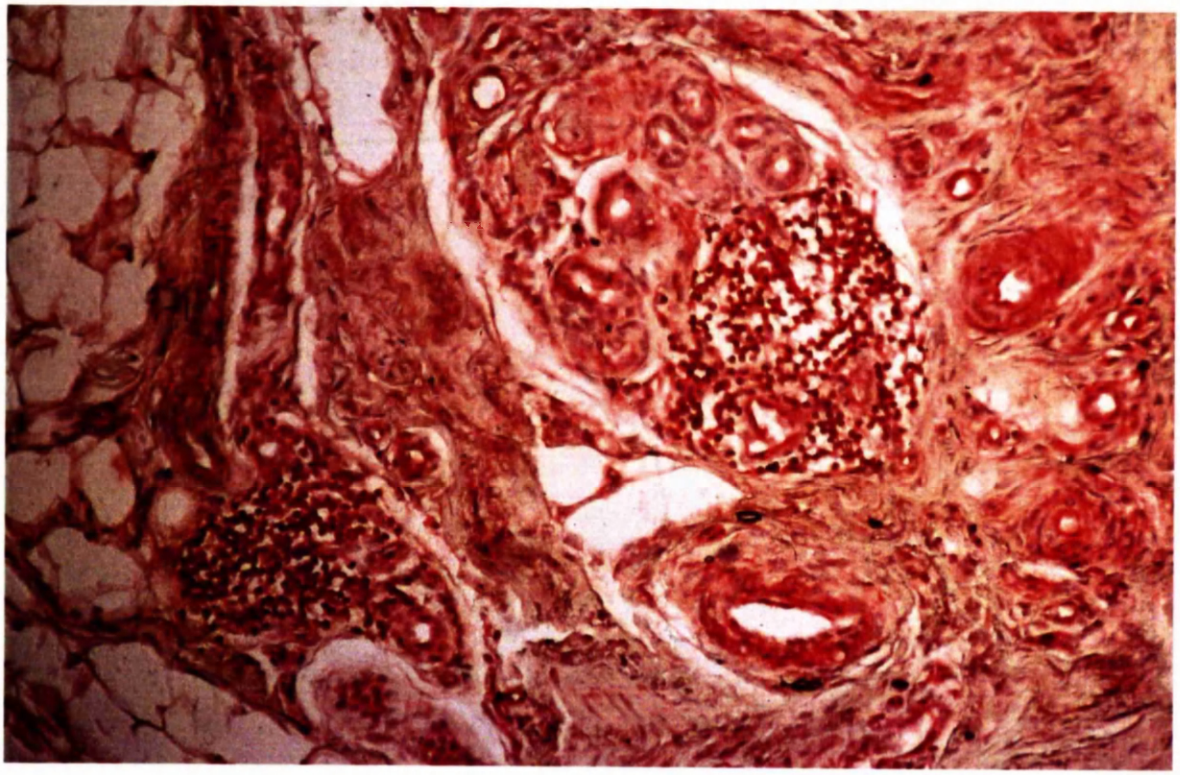
The results of immunological studies on Dupuytrens control tissue samples were analysed according to the histological areas of: (1) fibrofatty tissue surrounding the Dupuytren's nodule, (2) the nodule itself and (3) cord tissue.

Histology of Fibrofatty Tissue Surrounding The Nodules

This perinodular tissue histologically demonstrated the presence of a chronic inflammatory reaction. The unaffected subcutaneous fat gave way to vascular connective tissue containing many small blood vessels and an inflammatory infiltrate composed chiefly of lymphocytes and occassional mast cells. The smaller blood vessels lay within a stroma of disorientated and loosely woven collagenous connective tissue. Within this stroma there were well-defined bundles of arterioles and venules in close proximity to collections of lymphocytes (Figure 11). These clusters of lymphocytes occurred only within the perinodular tissue and did not occur within the nodules or in the cord tissue. There was little evidence of other types of inflammatory cells in these tissues other than occasional macrophages and mast cells. The arteriolar walls were often thickened with smooth muscle hypertrophy and the capillary endothelium was frequently swollen.

Figure 11

Section of Dupuytren's tissue stained by immuno-histochemistry for TNF-alpha antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). The general histology of the perinodular tissue (x 25 magnification) is demonstrated. The unaffected subcutaneous fat gives way to vascular connective tissue containing many small blood vessels and inflammatory cells. 2 pockets of lymphocytes can be seen. The arterioles walls were thickened.



Immunohistochemistry of Fibrofatty Tissue Surrounding The Nodules

IL-1, TNF-alpha and IL-8 were all localised in similar areas of endothelium and in inflammatory cells (Figures 12,13, 14). Staining of endothelium was strong in some areas but weak in others. In general, those microvessels, which lay in close proximity to lymphoid follicles, stained positively for all three cytokines, whereas staining was absent in blood vessels with no adjacent inflammatory cells, despite these vessels often being narrowed by smooth muscle proliferation. Most of the positive staining cells were present around the margins of the lymphoid follicles, with some identified within the small blood vessels. Small numbers of positively staining lymphocytes were present within the connective tissue close to the blood vessels. Occasionally, the extracellular matrix showed slight staining for all three cytokines but in general was negative for IL-1, TNF-alpha and Il-8 in this area.

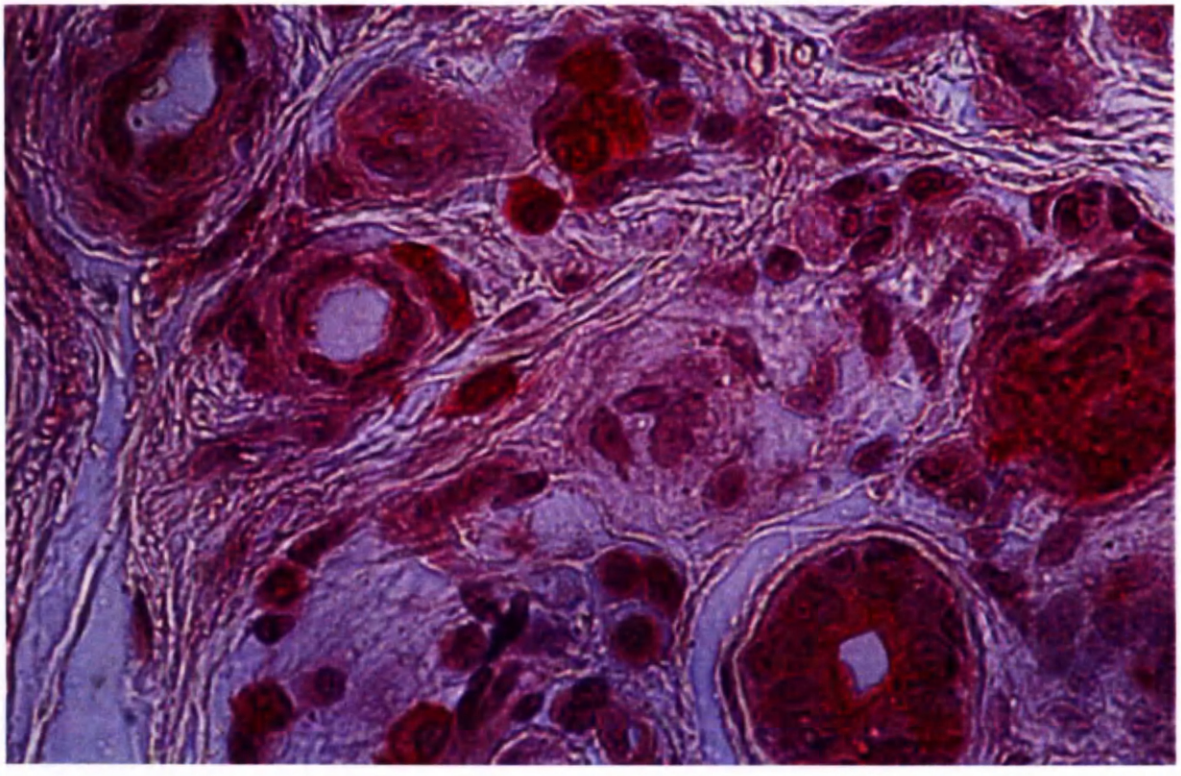
IL-4 stained very positively in inflammatory cells in general and lymphocytes in particular (Figure 15). Interleukin-6 was present in some small capillaries but in general the main blood vessels were negative (Figure 15). bFGF was strongly staining in small and slightly larger blood vessel's endothelium (Figure 16).

Histology And Immunohistochemistry of Nodules

The nodules were highly cellular and composed of fibroblasts arranged in tightly packed whorls containing substantial numbers of capillaries. The nodules again stained positive for IL-1, TNF-alpha and IL-8, this time demonstrating both

Figure 12

Section of Dupuytren's tissue stained by immuno-histochemistry for TNF-alpha antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). TNF-alpha distribution demonstrated in the perinodular of Dupuytren's tissue (x 40 magnification). There is strong staining of the endothelium around the margin and of individual inflammatory cells.



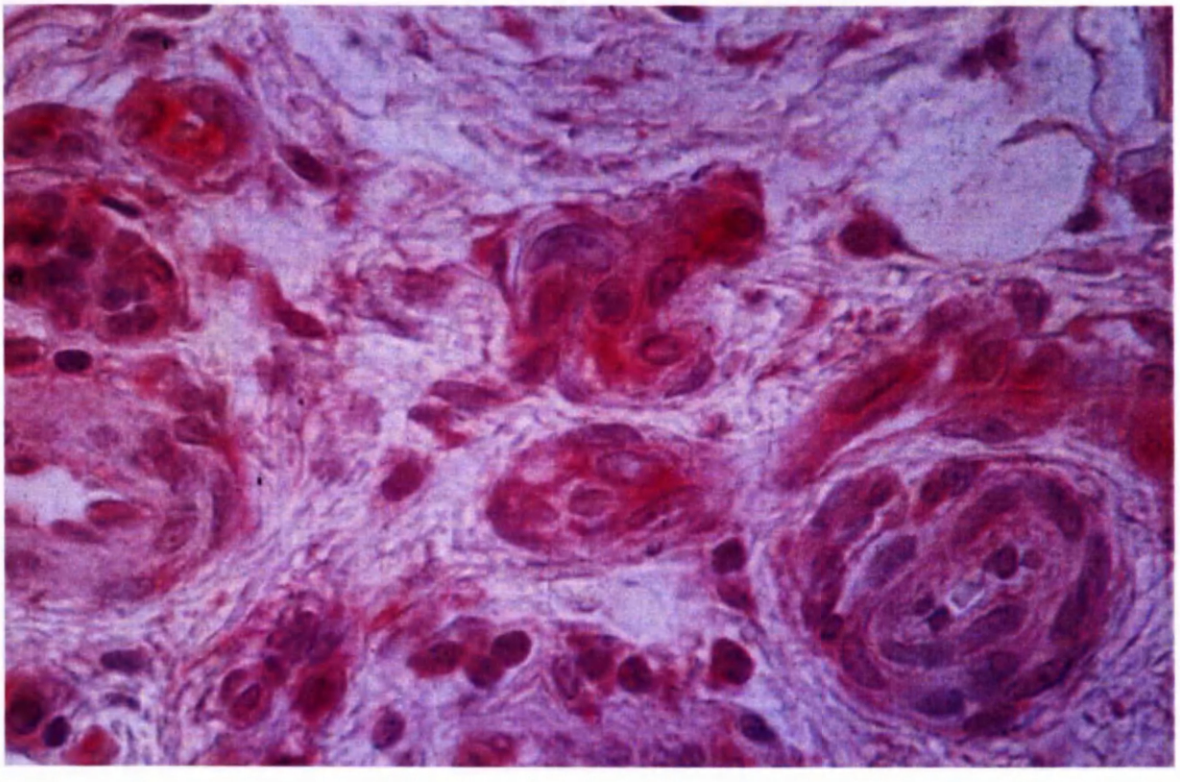
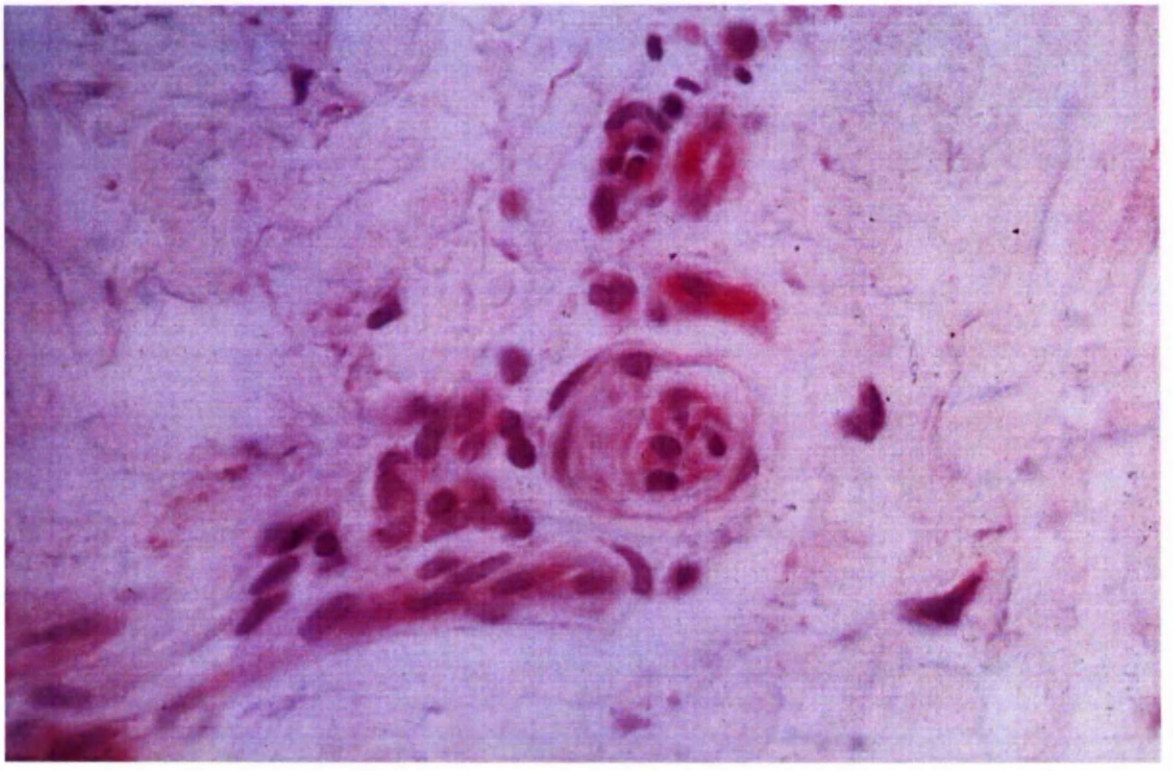


Figure 13

Section of Dupuytren's tissue stained by immuno-histochemistry for IL-1 antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). IL-1 demonstrated in the perinodular area (x 40 magnification). There is strong staining of the endothelium around the margin and some staining of individual inflammatory cells.



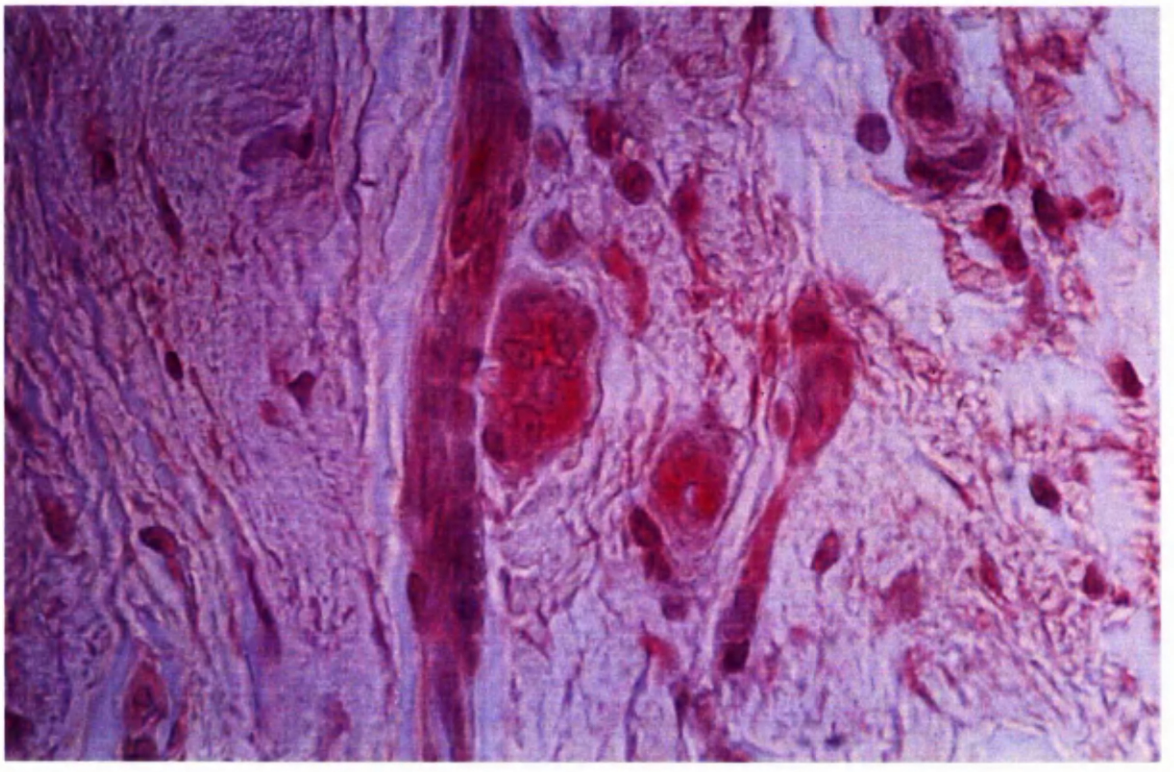


Figure 14

Section of Dupuytren's tissue stained by immuno-histochemistry for IL-8 antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). IL-8 demonstrated in the peri-nodular area (x 10 magnification). There is strong staining of the endothelium around the margin but in general, the distribution was not as widespread as for TNF-alpha and IL-1.

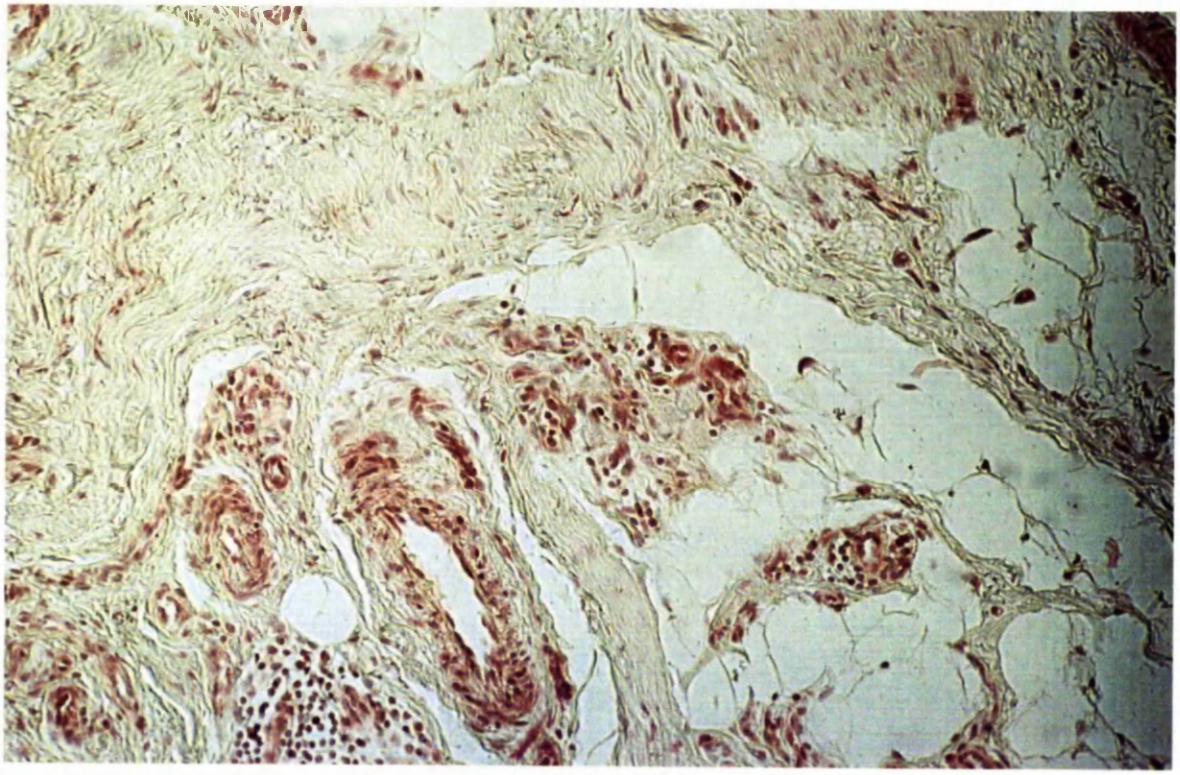
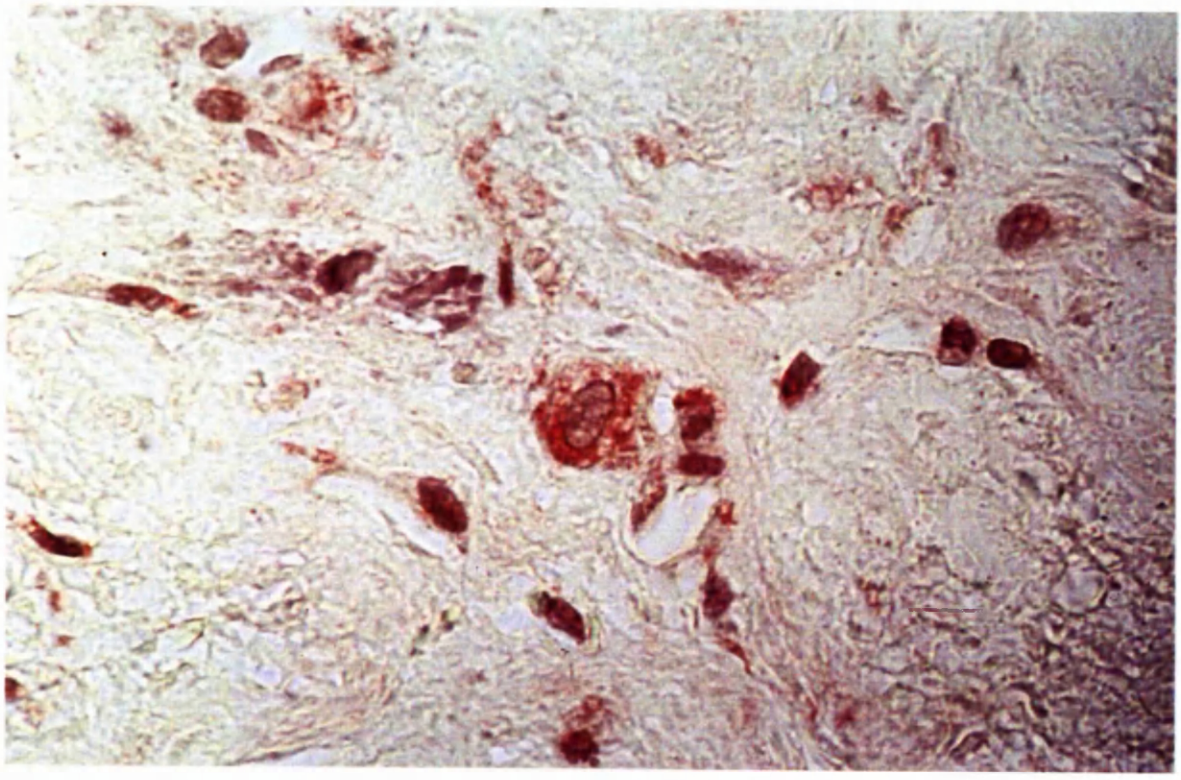


Figure 15

Section of Dupuytren's tissue stained by immuno-histochemistry for IL-4 and IL-6 antibodies and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). IL-4 and IL-6 demonstrated in the perinodular area. Top figure demonstrates IL-4 stained quite positively for inflammatory cells in general (x 25 magnification). Bottom figure demonstrates IL-6 was present in the small capillaries of the fibro-fatty area of the tissue (x 10 magnification).



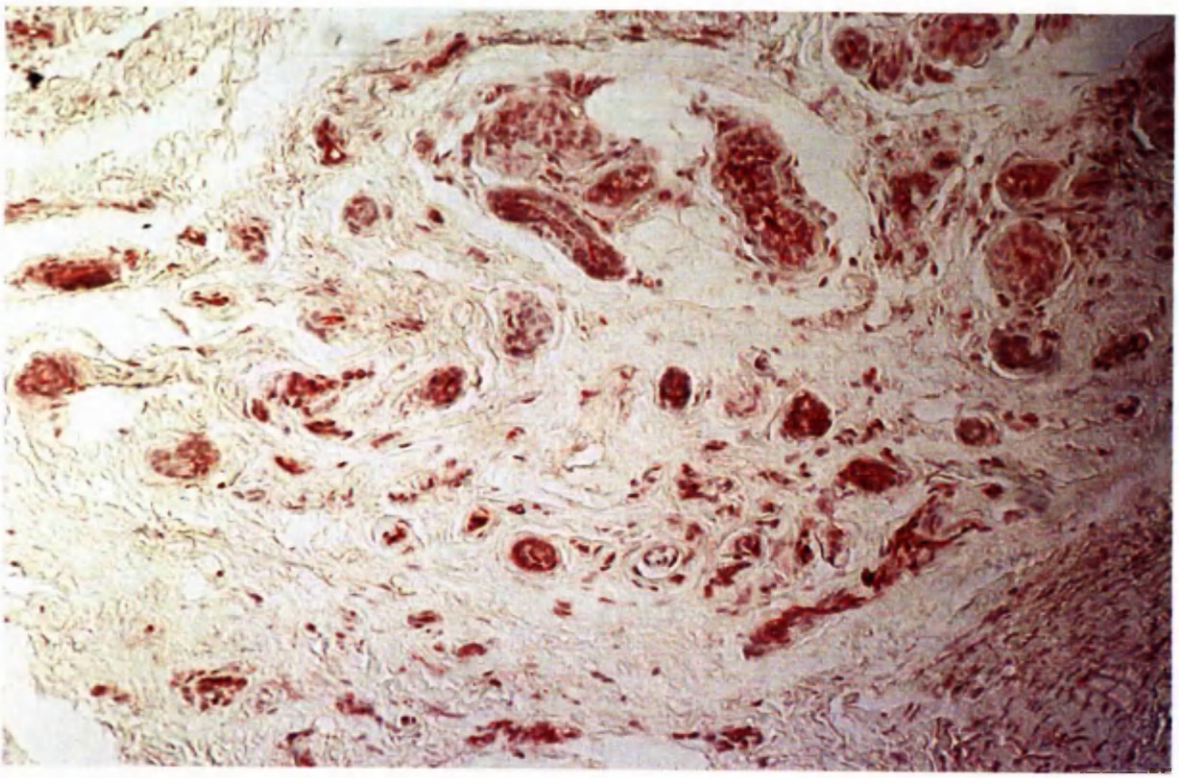
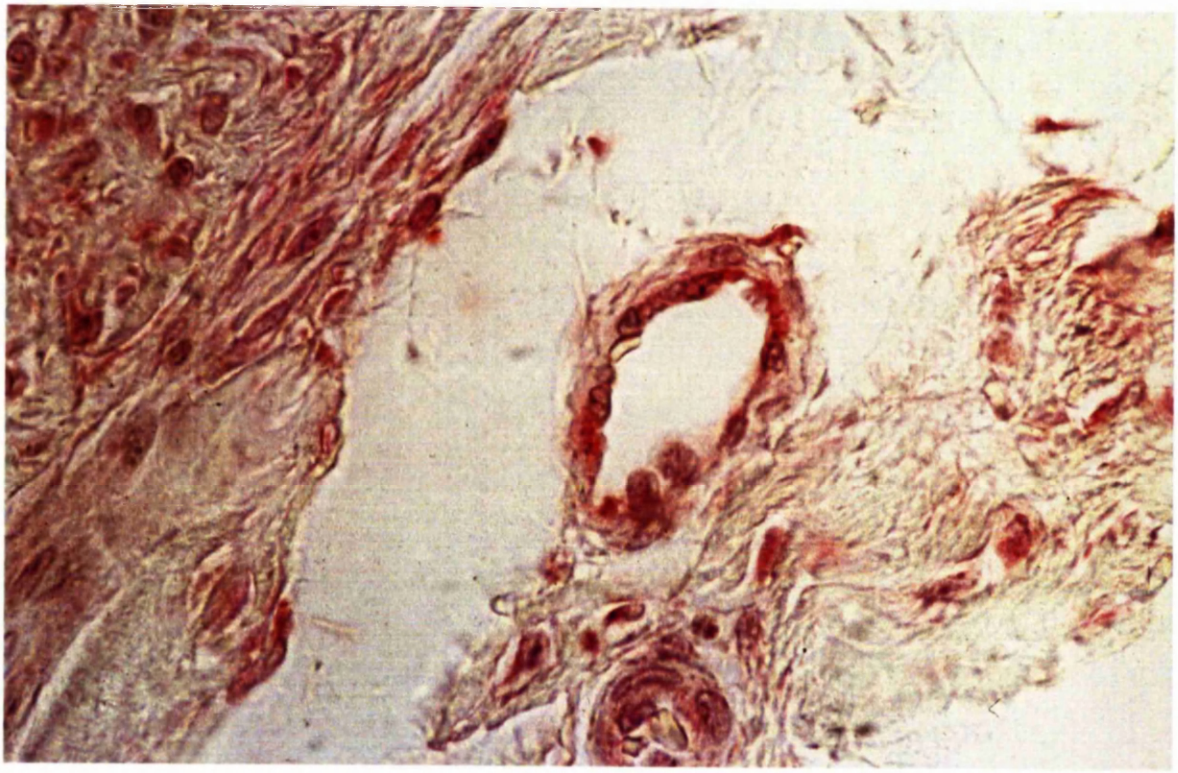


Figure 16

Section of Dupuytren's tissue stained by immuno-histochemistry for bFGF antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). bFGF demonstrated in the perinodular area (x 25 magnification). bFGF is present in the blood vessel endothelium.



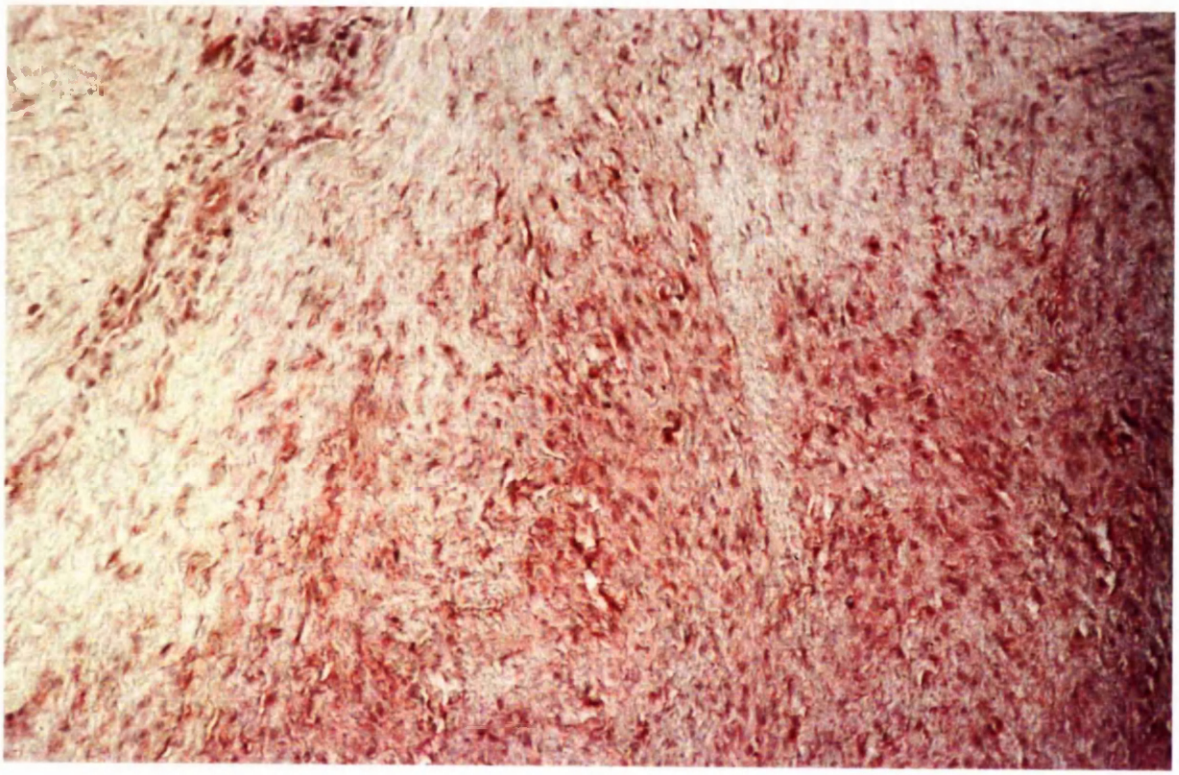
intracellular and intercellular staining (Figures 17,18,19). Again, the distribution was very similar for all three. The fibroblasts, the capillary endothelium and the extracellular matrix all stained strongly. IL-8 distribution was less homogenous than IL-1 and TNF-alpha. Although IL-8 was invariably present in mononuclear inflammatory cells, it was sometimes confined in a few distinct clumps of fibroblasts within the nodule. It was apparent from the sections that each nodule comprised a number of positively staining mini-nodules separated by fibro-cellular septae, which showed no cytokine staining. The capillaries, which passed through these septae, showed positive staining for these pro-inflammatory cytokines. The strongest staining within the nodules was found in the capillary endothelium and lymphocytes and macrophages, which were present in small numbers. Some lymphocytes were visualised within the capillaries and occasionally could be observed adherent to the endothelium while others were present in small cuffs just outside the capillaries. Occasional positively staining macrophages were also observed in the tissues.

IL-4 and IL-6 were scantily present within the actual nodule itself (Figure 20). IL-4 stained positively in occasional inflammatory cells, mainly macrophages, and IL-6 was generally present in some small capillaries. bFGF stained in some Dupuytren's fibroblasts, mainly in the centre of the nodules (Figure 21).

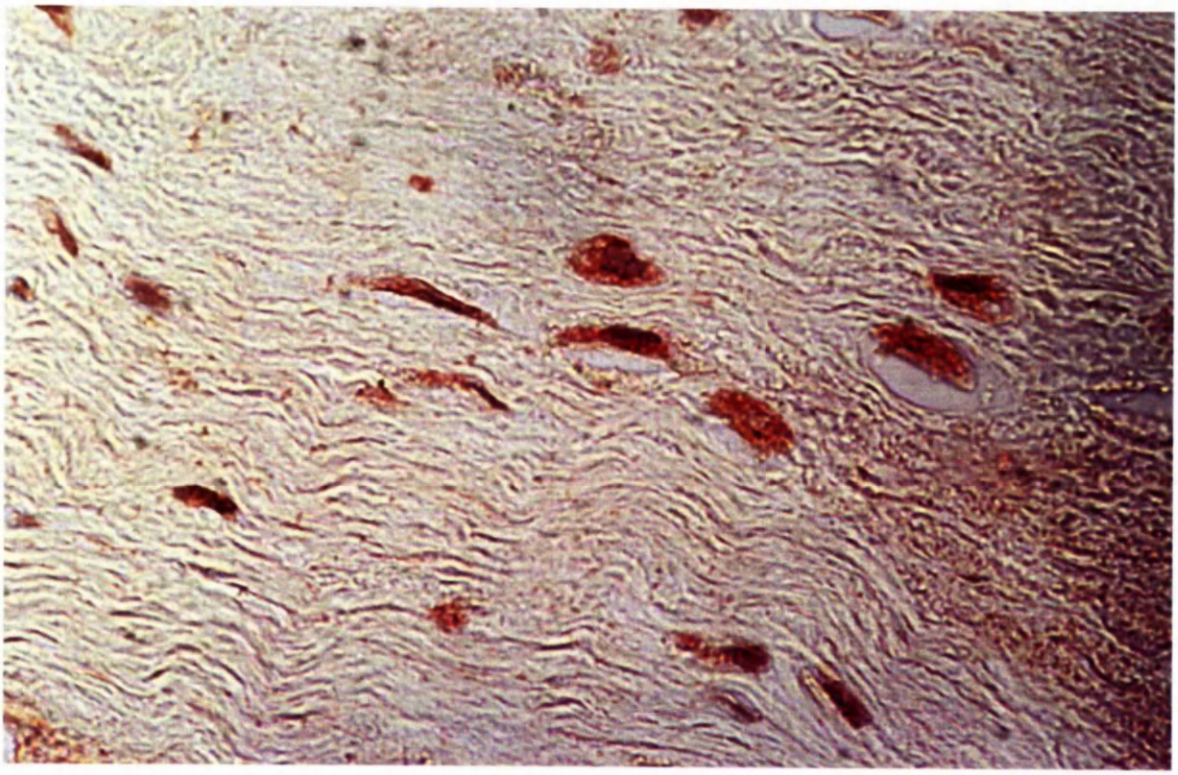
Figure 17

Section of Dupuytren's tissue stained by immuno-histochemistry for TNF-alpha and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). TNF-alpha demonstrated in the nodule. TNF-alpha is present with both intracellular and intercellular staining with a diffuse pattern present within defined areas of the nodule. (a) The staining was quite homogenous within the mini-nodules in the larger nodule (x 25 Magnification). (b) Some individual fibroblasts also stained strongly for TNF-alpha (x 40 Magnification). (c) TNF-alpha stained strongest for sweat glands and blood vessels towards the edge of the nodule (x 10 Magnification).

17 (a)



17 (b)



17 (c)

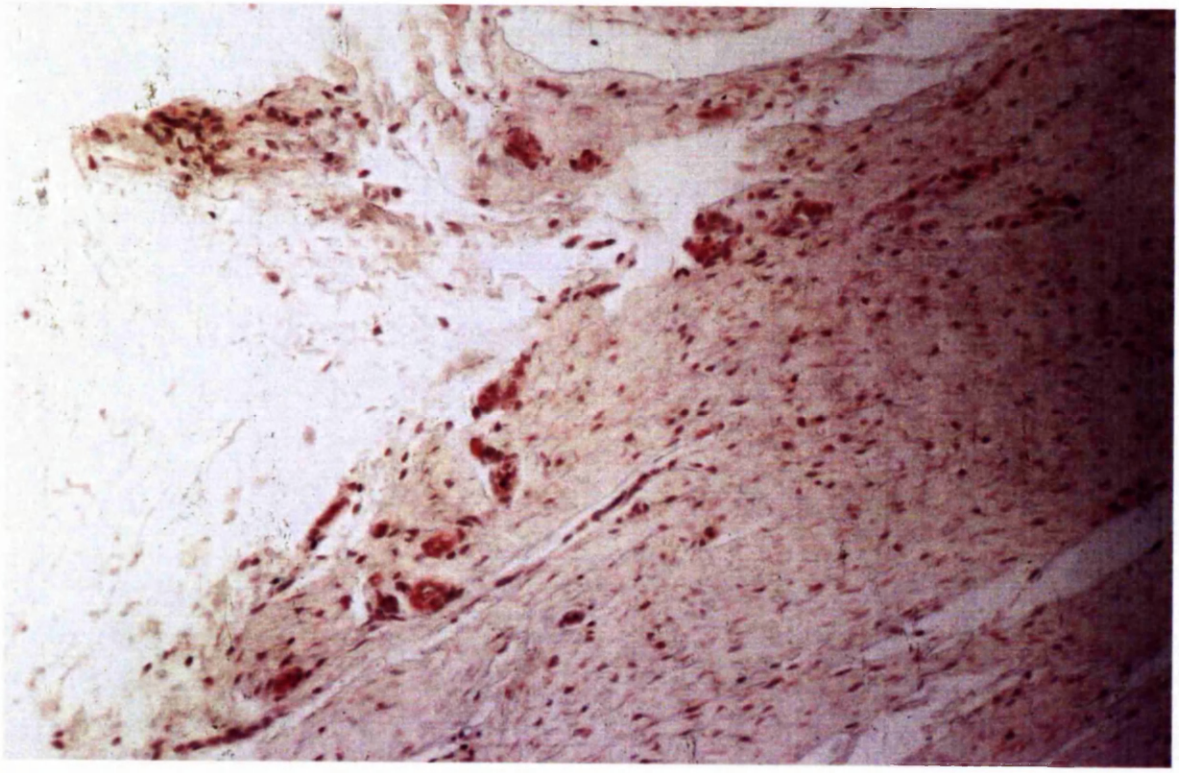
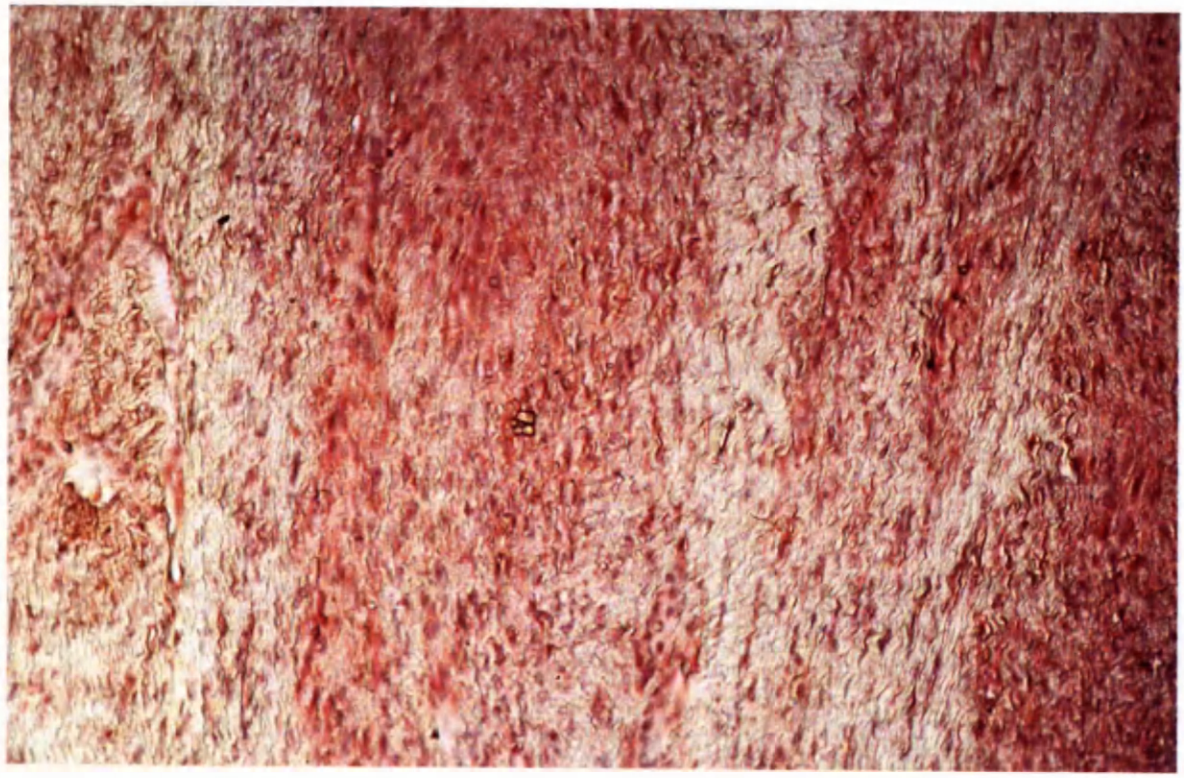
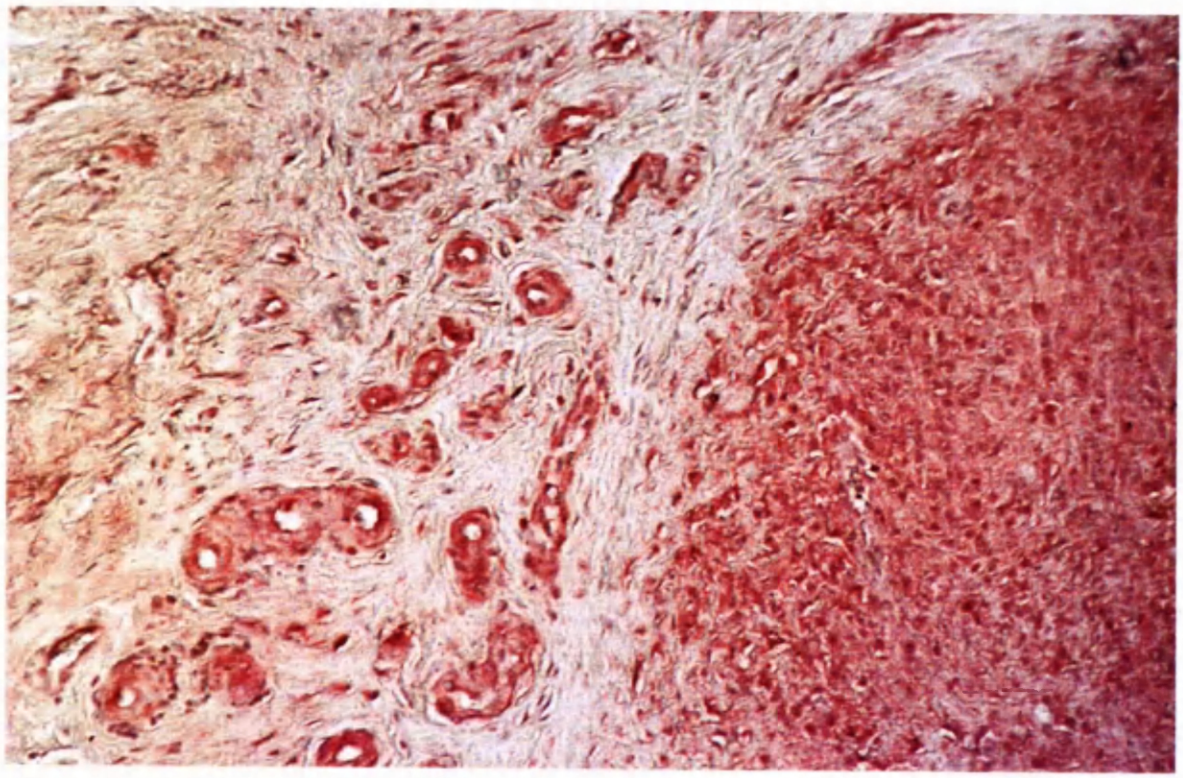


Figure 18

Section of Dupuytren's tissue stained by immuno-histochemistry for IL-1 antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). IL-1 demonstrated in the nodule. (a) and (b) IL-1 is present with both intracellular and intercellular staining, often with a diffuse pattern present, or within well defined areas of the nodule and is present in the blood vessels passing through the nodule (x 25 Magnification). IL-1 positively stained the blood vessels penetrating via septa into the nodule (x 25 magnification). (c) IL-1 stained positively for the occasional inflammatory cell present within the nodule (x 40 Magnification).

18 (a)





18 (b)

18 (c)

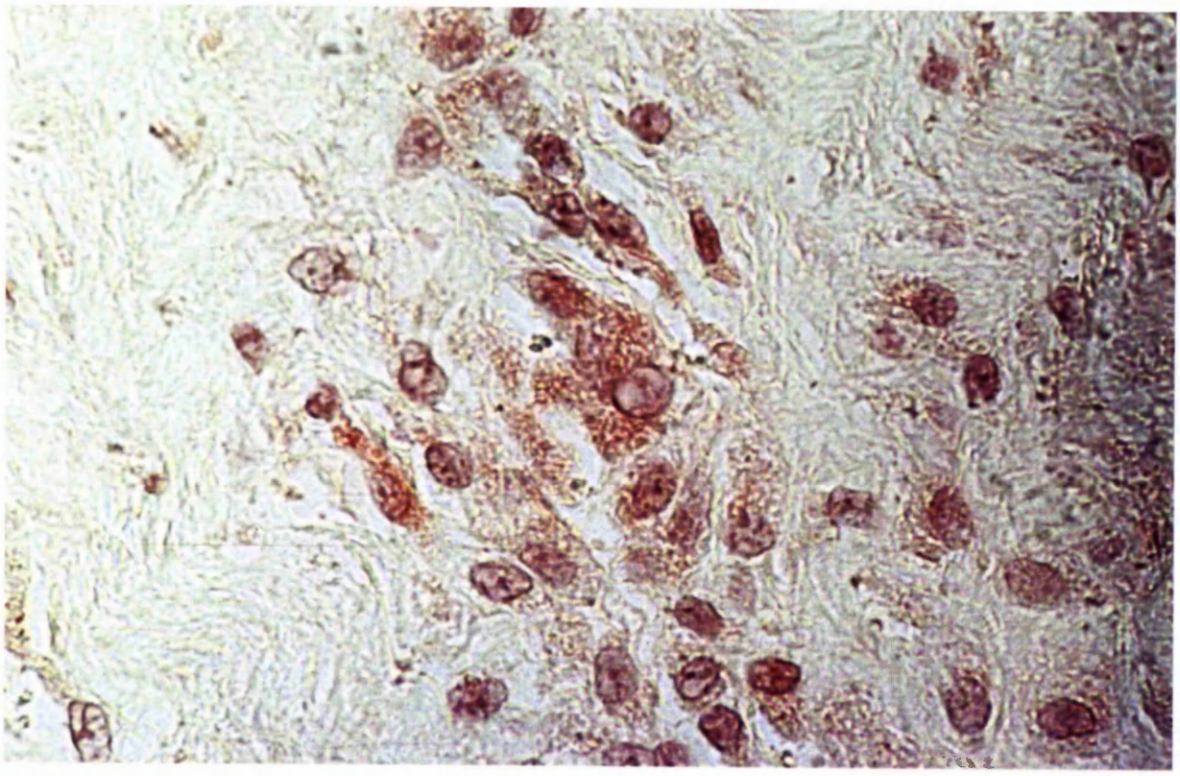
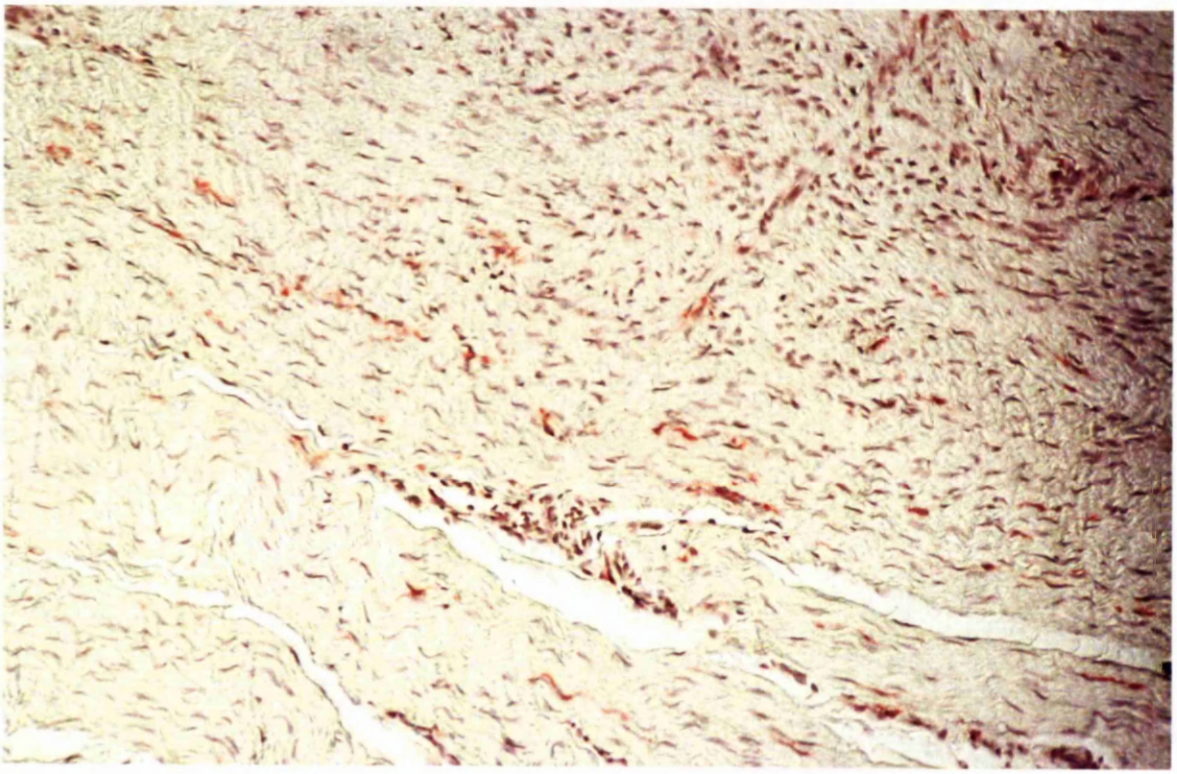


Figure 19

Section of Dupuytren's tissue stained by immuno-histochemistry for IL-8 antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). IL-8 demonstrated in the nodule edge. IL-8 is present with both intracellular and intercellular staining, but with a more distinct focus of individual areas of positive staining than IL-1 or TNF-alpha. (a) IL-8 stained positively around some individual fibroblast (x 10 magnification) and (b) also the blood vessels towards the edge of the nodule.

19 (a)



19 (b)

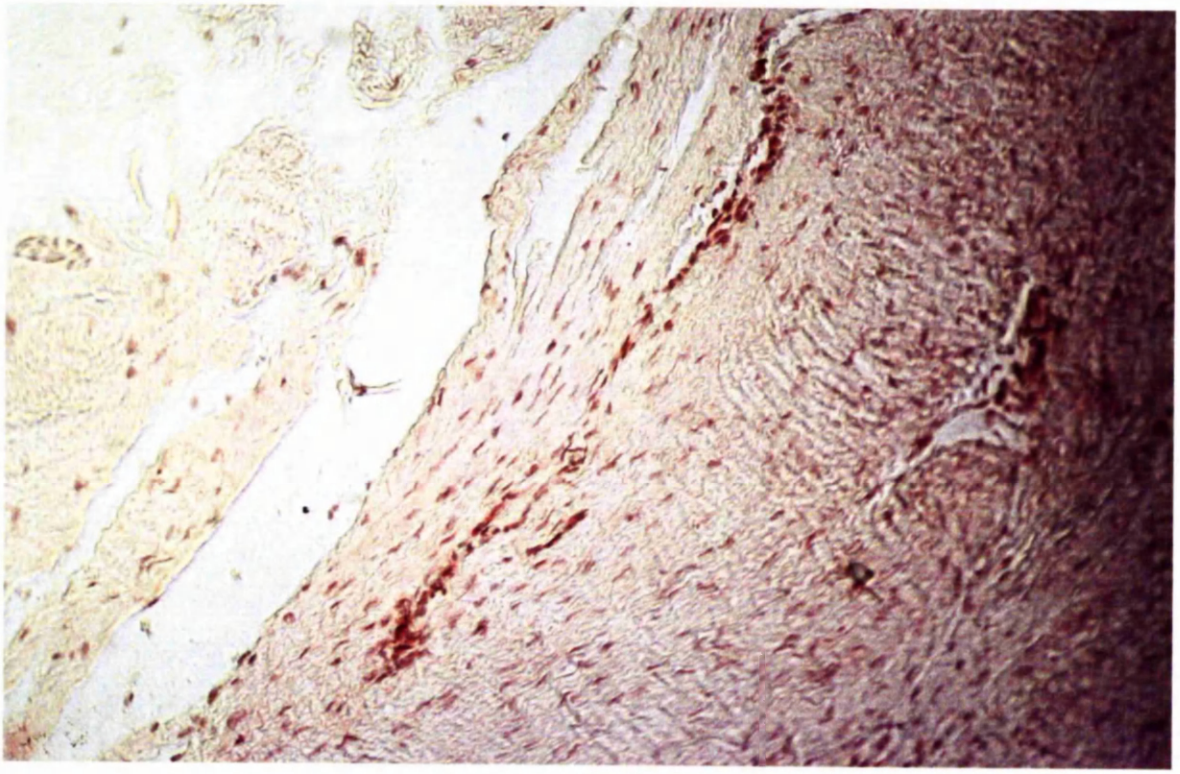
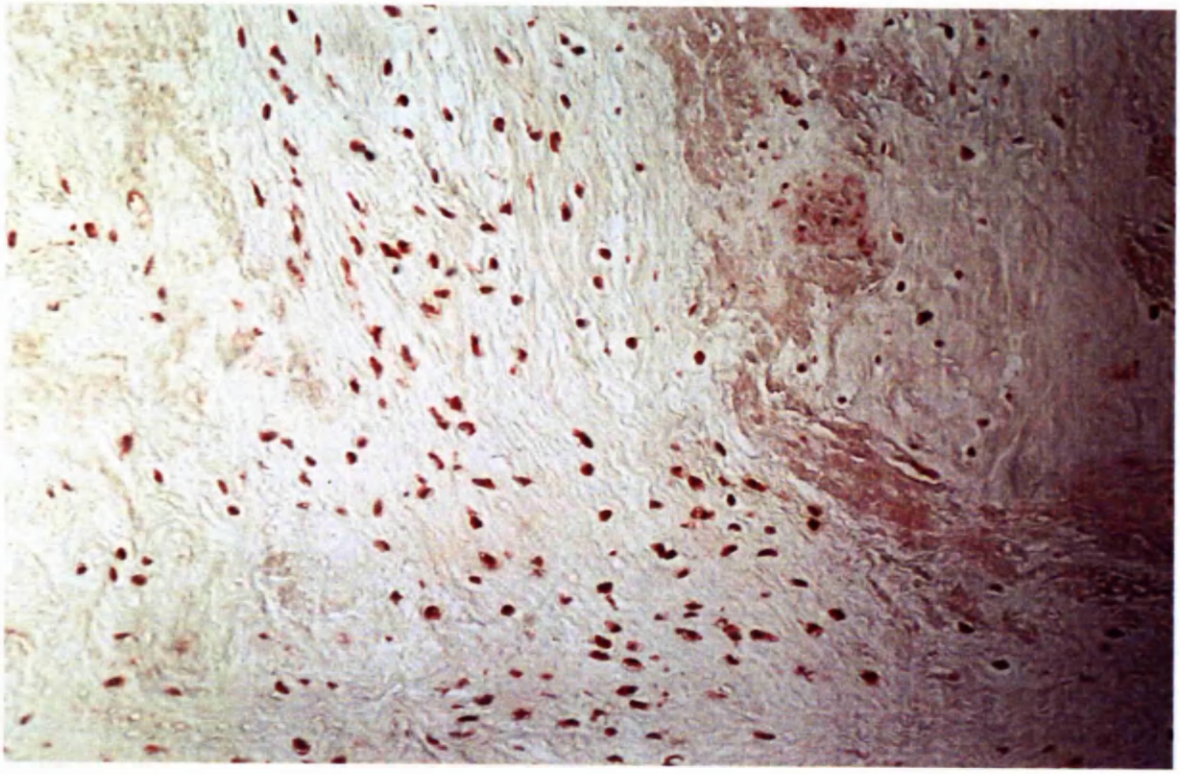
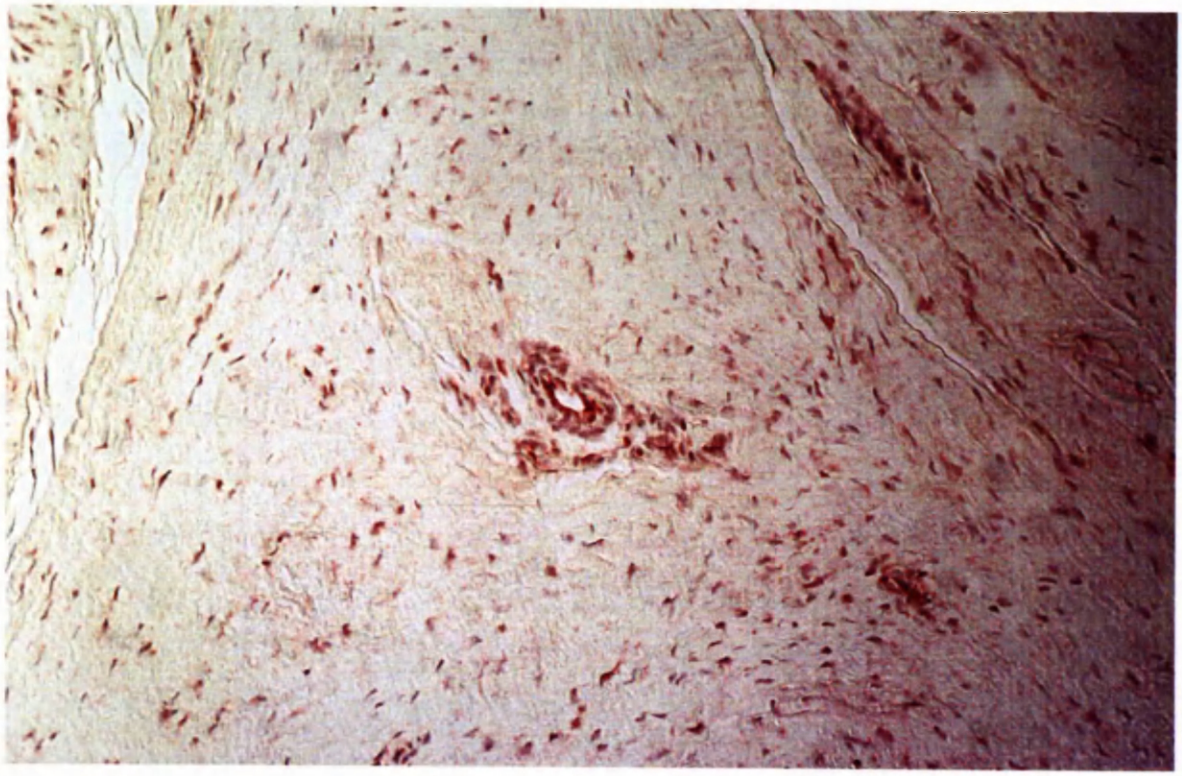


Figure 20

Section of Dupuytren's tissue stained by immuno-histochemistry for IL-4 and IL-6 antibodies and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). IL-4 and IL-6 demonstrated in the nodule. (a) IL-4 stained the fibroblasts and occasional inflammatory cell (x10 magnification) but (b) IL-6 was generally more present in the smaller blood vessels in the septae running between and through the nodules.

20(a)

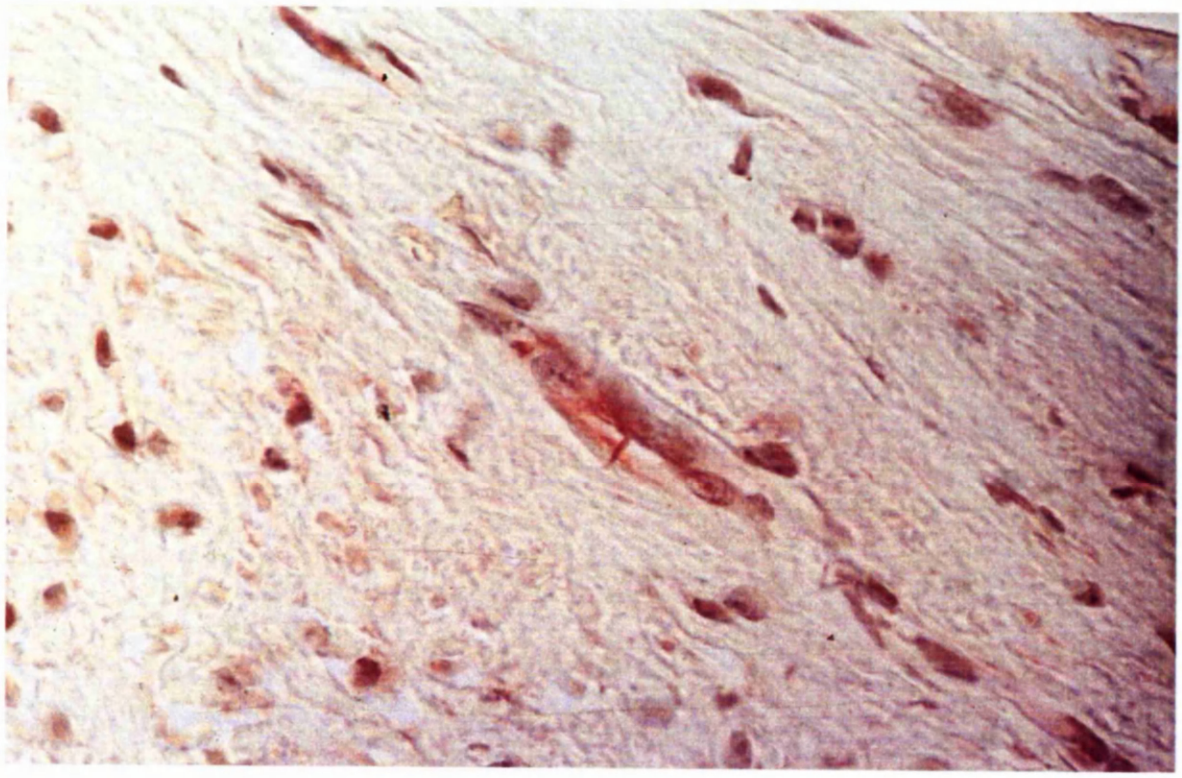




20(b)

Figure 21

Section of Dupuytren's tissue stained by immuno-histochemistry for bFGF antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). bFGF demonstrated in the nodule (x 25 magnification). bFGF stained the occasional fibroblast in the centre of the nodule and was present in some of the blood vessel endothelium.



Histology and Immunohistochemistry of Cord Tissue

The cord tissue was of uniform appearance. They comprised dense longitudinally orientated bundles of collagen interspersed with thin elongated fibroblasts. Very few blood vessels were present either within the cord or in the adjacent tissues. Inflammatory cells were conspicuous by their absence. Staining for pro-inflammatory cytokines was absent within the cords.

Histology and Immunohistochemistry of Control Palmar Fascia Tissue

This tissue was almost identical histologically to cord tissue (Figure 22) and was almost completely devoid of any immuno-staining for pro-inflammatory cytokines.

Results of Preoperative Steroid Injections on the Immunohistocemistry of all areas of Dupuytren's tisssue

The pre-operatively treated Dupuytren's tissue showed an almost complete loss of any immuno-histochemical staining for pro-inflammatory cytokines. There was a very weak response for TNF-alpha and IL-8 in a few small perindular areas in 2 specimens only (Figure 23).

Figure 22

Section of Palmar Fascia Tissue stained by van Gieson method for elastin fibres (x 10 magnification). The tissue is very similar to cord tissue with regular fascicles of collagen and a low cell density. All sections were almost completely devoid of positive staining for pro-inflammatory cytokines.

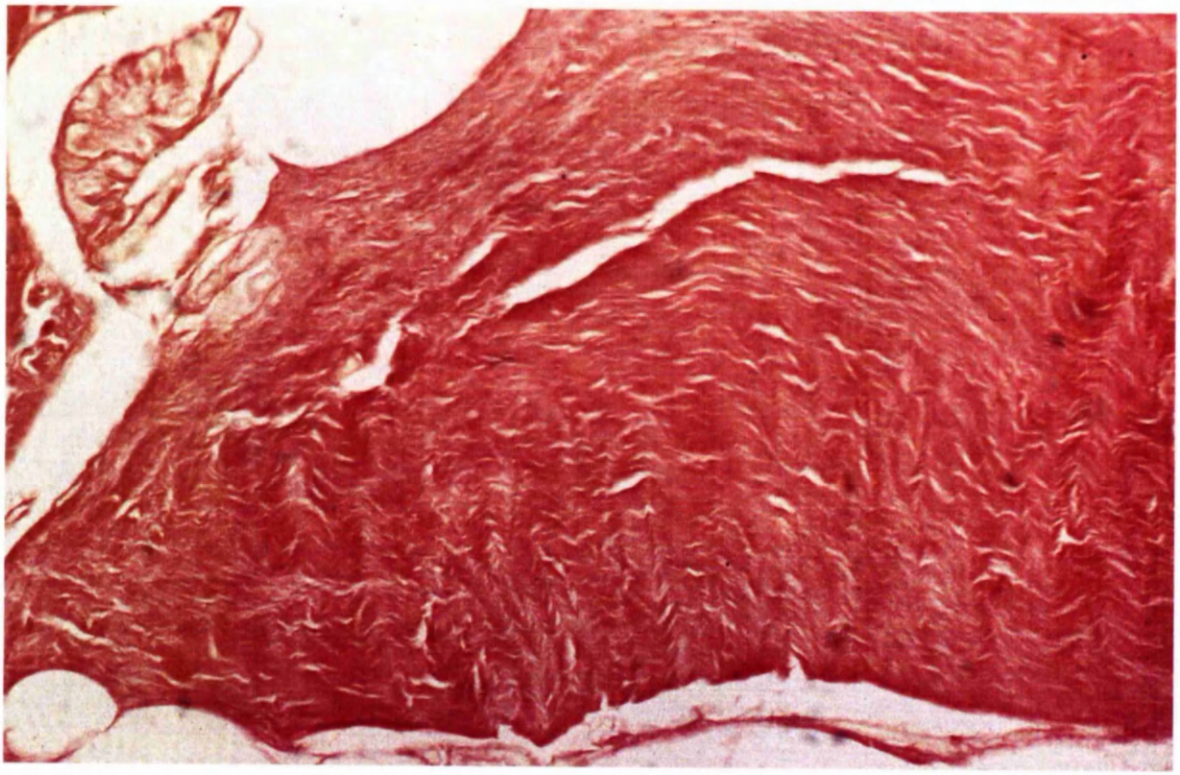


Figure 23

Section of Fibro-fatty area of Dupuytren's tissue preoperatively treated with steroids. Stained by immuno-histochemistry for IL-8 antibody and visualised with Fast Red TR/Napthol AS-MX Phosphate (Intense red stain positive). Only one section demonstrated a very weak staining for IL-8 around inflammatory cells (x 25 Magnification).

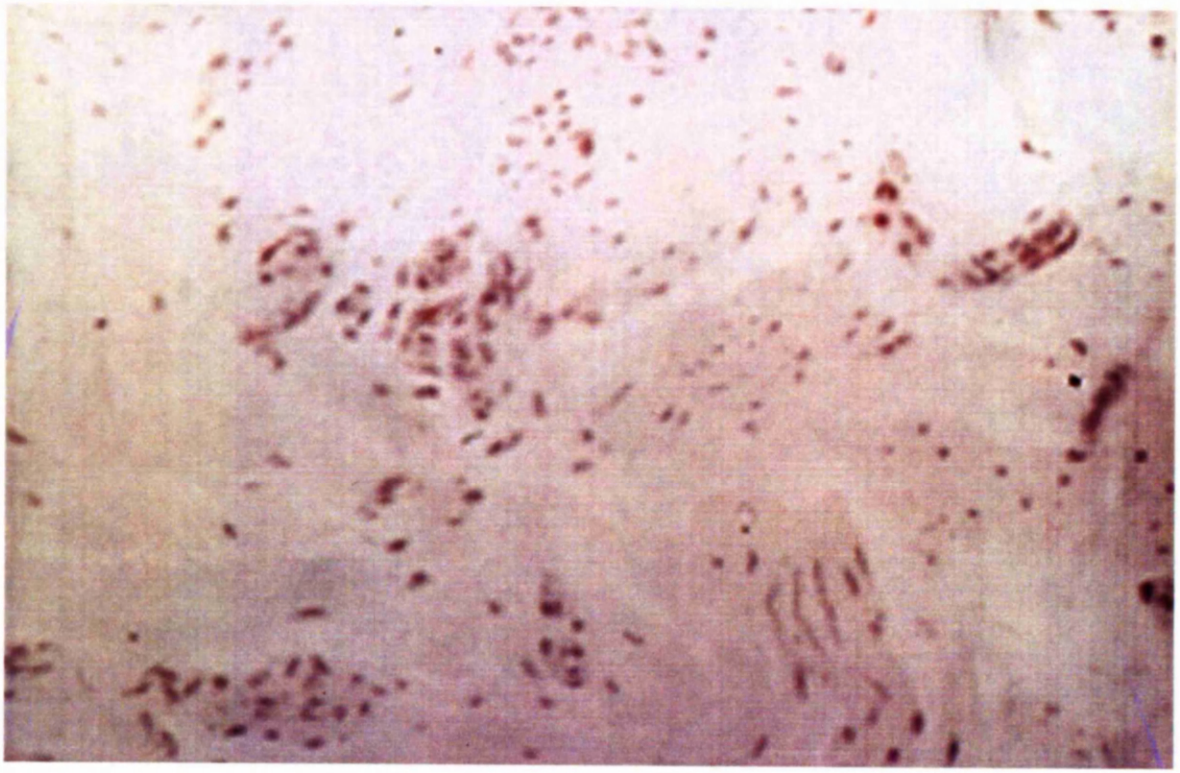


TABLE 4

Immuno-Histochemistry Staining for Il-1, Il-4, Il-6, Il-8, TNF-alpha, bFBF in Sections of Dupuytren's Tissue, with and without Pre-Operative Steroid Treatment, and in Sections of Palmar Fascia

	IL-1	IL-4	IL-6	IL-8	TNF-alpha	bFGF
CONTROL DUPUYTREN'S TISSUE (N=10)	++	++	++	++	++	++
STEROID TREATED DUPUYTREN'S TISSUE (N=10)	-	-	-	-	-	-
PALMAR FASCIA TISSUE (N=4)	-	-	-	-	-	-

Presence of Il-1, Il-4, Il-6, Il-8, TNF-alpha, bFBF in nodular tissue and fibrofatty tissue surrounding the nodules in both control and pre-operatively steroid treated Dupuytren's tissue, and for palmar fascia sections, analysed by immuno-histochemistry, and summarised in Table 4. The results represent the average of 5 independent fields at times 25 magnification. As the areas on the histological sections were assessed for background immunoreactivity this was done by allocating the degree of immunoreactivity for that particular field area, namely as: ++ diffuse strong reactivity; + strong immunoreactivity restricted to cellular areas; - no immunoreactivity.

3.2

Matrix and Adhesion Factors in Dupuytren's Tissue and The Effects of Pre-operative Steroids Injections

Fibrofatty tissue surrounding the nodules

The endothelium of the majority of blood vessels in this perinodular area had considerable positive staining for VCAM-1 (Figure 24). Very few cells stained positively for VCAM-1 other than these endothelial cells. CS1 sequence of fibronectin stained positively for some of these blood vessels, both on the endothelium and around the arterioles in the loosely woven collagenous connective tissue (Figure 25). $\alpha 4\beta 1$ integrin expressing cells were abundant in the blood vessels and in the peri-vascular areas corresponding to areas where VCAM-1 stained positively for the endothelium of blood vessels (Figure 26). Finally TGF-beta 1 and 2 stained positively around the inflammatory cells in a perivascular relationship (Figure 27) that appeared to be in a similar distribution with the expression of CS1 sequence of fibronectin (Figure 28).

Nodules

There was little in the way of VCAM-1 expression within the actual nodule, other than by the some of the larger capillary vessels penetrating between the nodules. CS1 sequence of fibronectin stained quite positively for many of these blood vessels passing in between the smaller nodules. In addition, there was positive staining around the active myofibroblasts, principally at the periphery of

Figure 24

Section of Dupuytren's tissue stained by immuno-histochemistry for VCAM-1 and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium). Top left section (x 10 magnification) stained endothelial cells positively (dark purple) in the blood vessels at the peri-nodular and peripheral nodule tissue. The other sections, at higher magnification (Top right and bottom left at x 25 magnification and bottom right at x 40 magnification), demonstrated endothelial staining for VCAM-1 in the blood vessel (dark purple) at the peripheral nodular areas and fibro-fatty tissue (counter-stain neutral red).

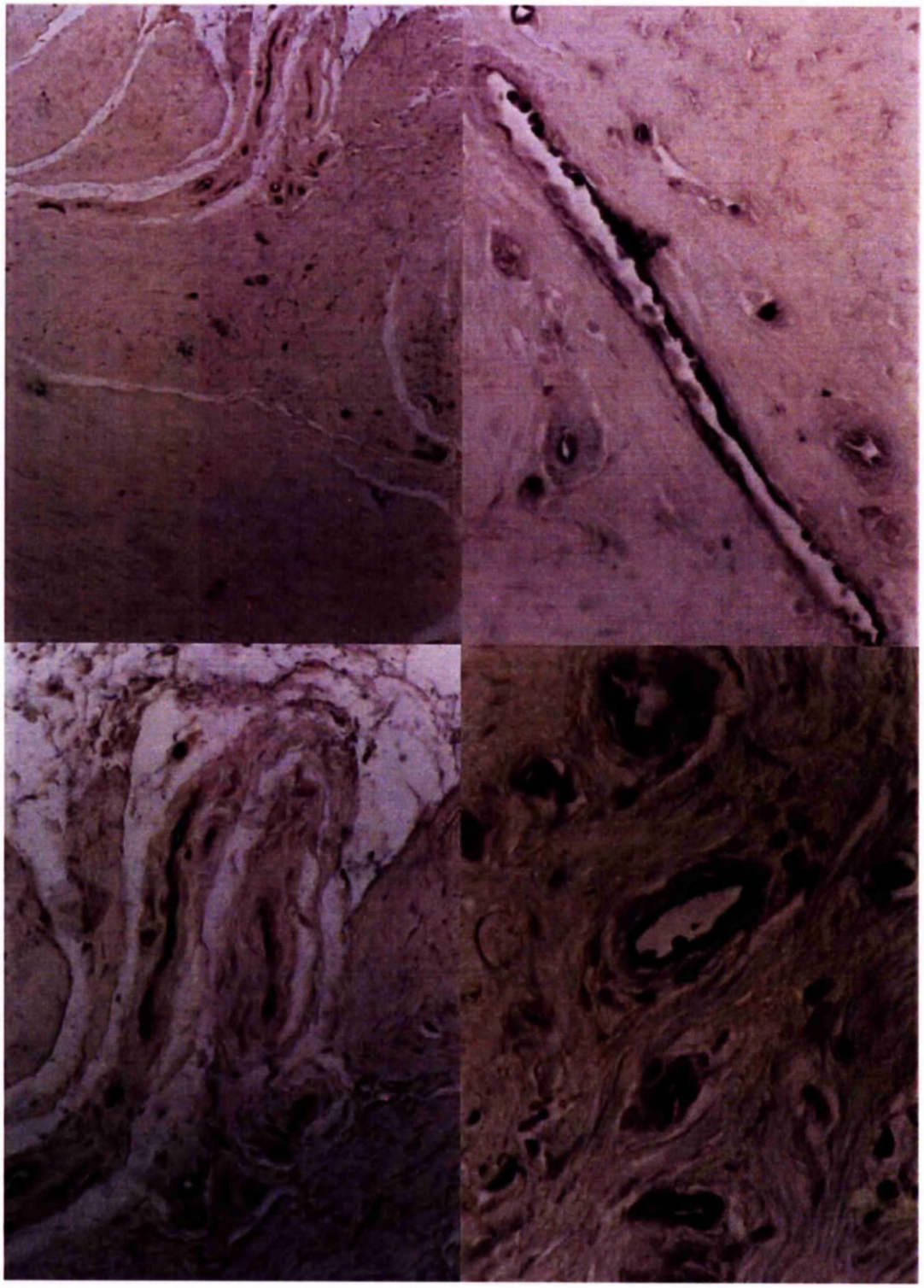
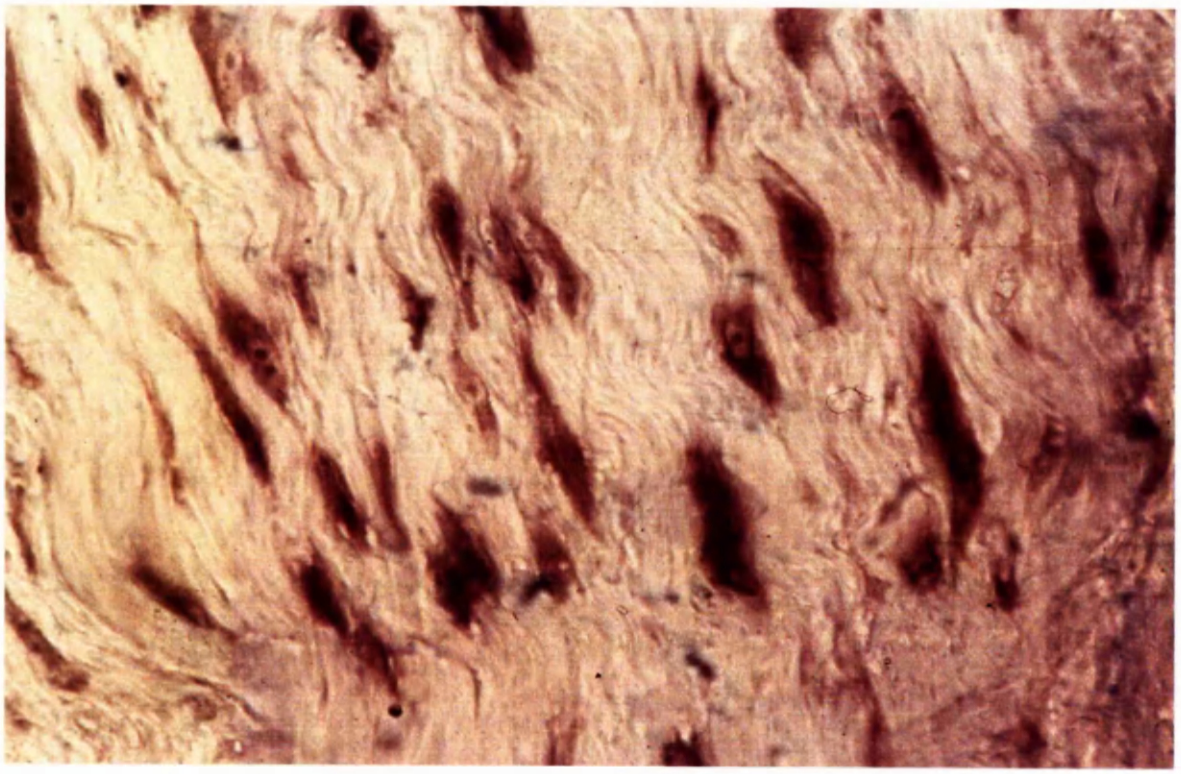


Figure 25

Section of nodule area of Dupuytren's tissue stained by immuno-histochemistry for CS1 sequence of fibronectin and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) with an intense purple stain (counter-stain neutral red). The bottom section demonstrated positive staining around the blood vessels at the periphery of active nodules and around the myofibroblasts themselves (x 25 magnification). The top section shows a closer view of the staining around and of the myofibroblasts themselves (x 40 magnification).



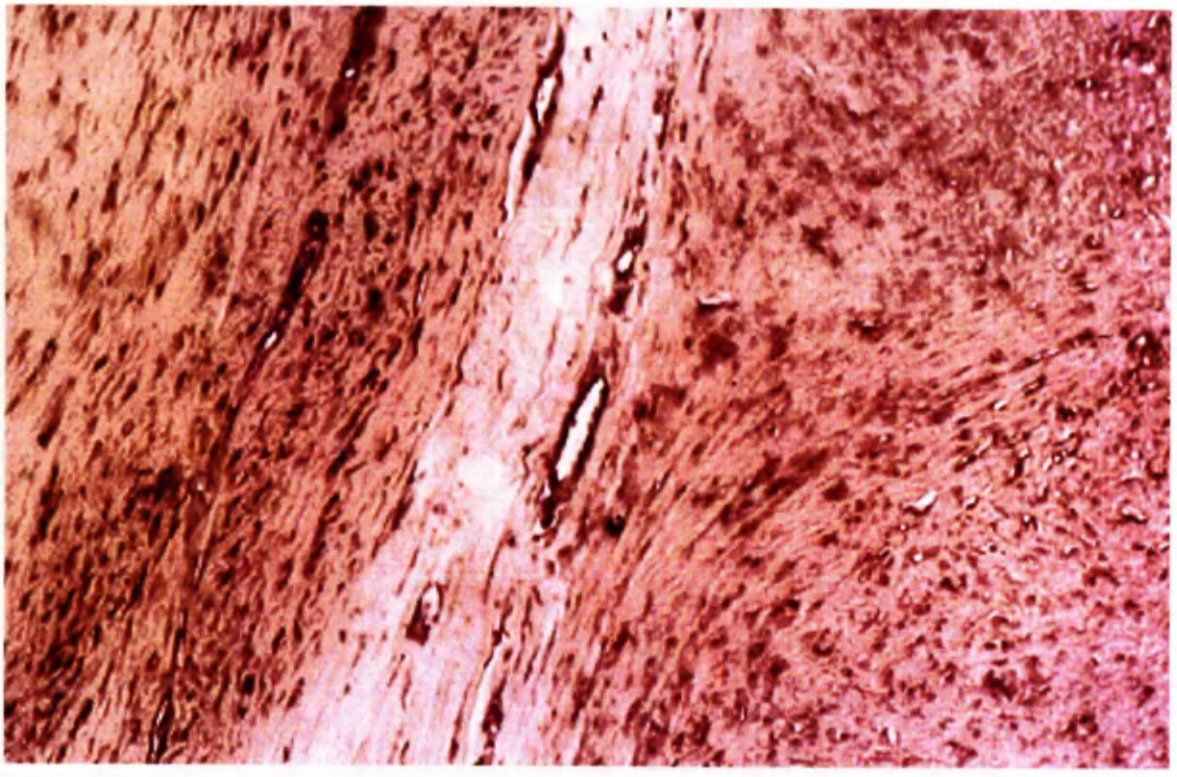


Figure 26

Section of Dupuytren's tissue from around the edge of the nodule, stained by immuno-histochemistry for $\alpha 4\beta 1$ integrin antibody and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) with an intense purple stain. The sections, all at x 40 magnifications, demonstrated positively (dark purple) staining inflammatory cells around the blood vessels at the peri-nodular and peripheral nodule tissue (counter-stain neutral red).

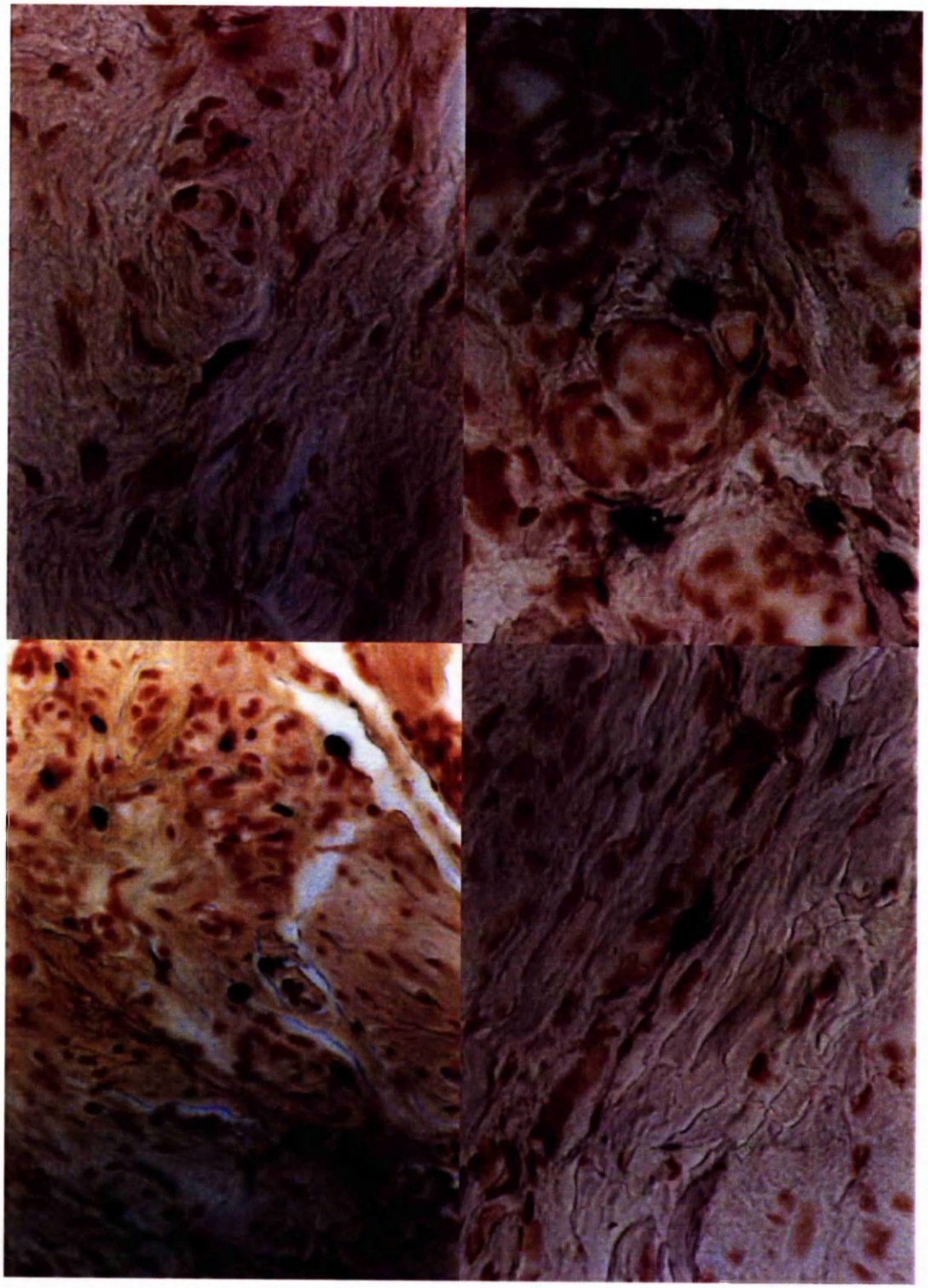
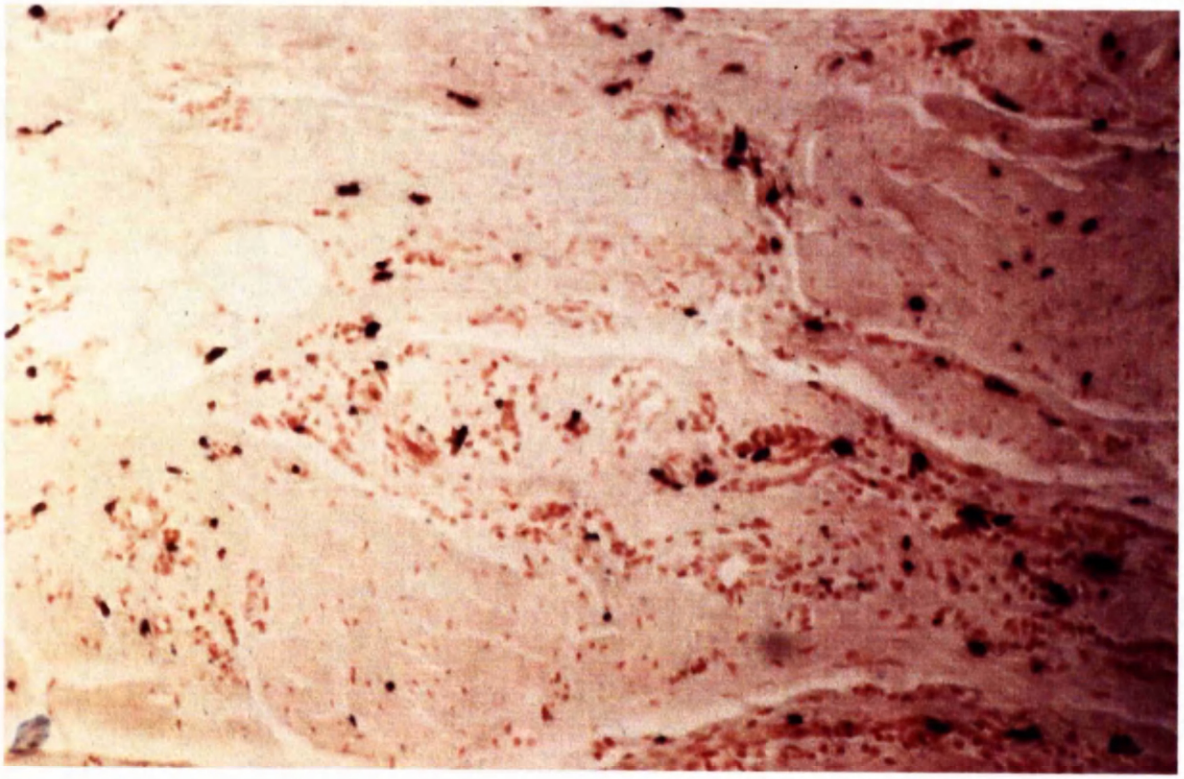


Figure 27

Section of Dupuytren's tissue stained by immuno-histochemistry for TGF-beta 1& 2 antibody and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) with an intense purple stain. TGF-beta 1& 2 demonstrated at the edge of the nodule (Top section x 25 magnification and bottom x 10 magnification). Individual cells stained rather than a diffuse background of the extracellular matrix (counter-stain neutral red) at these particular areas.



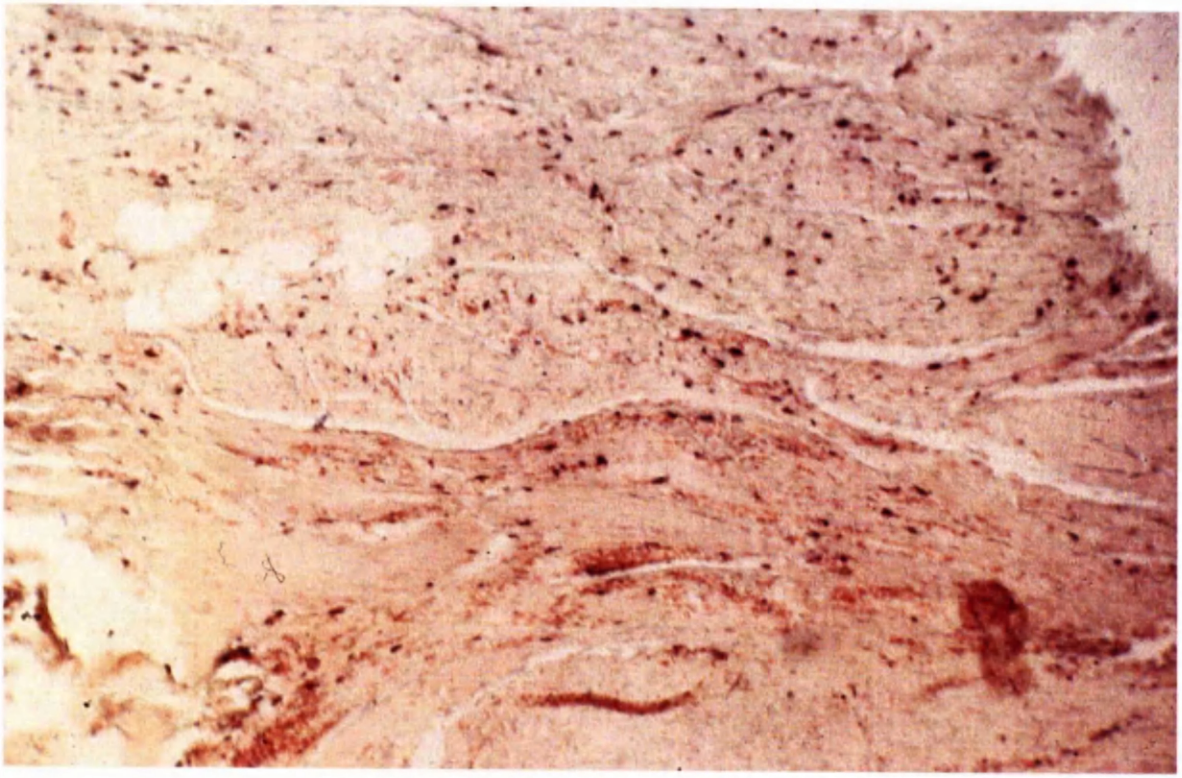
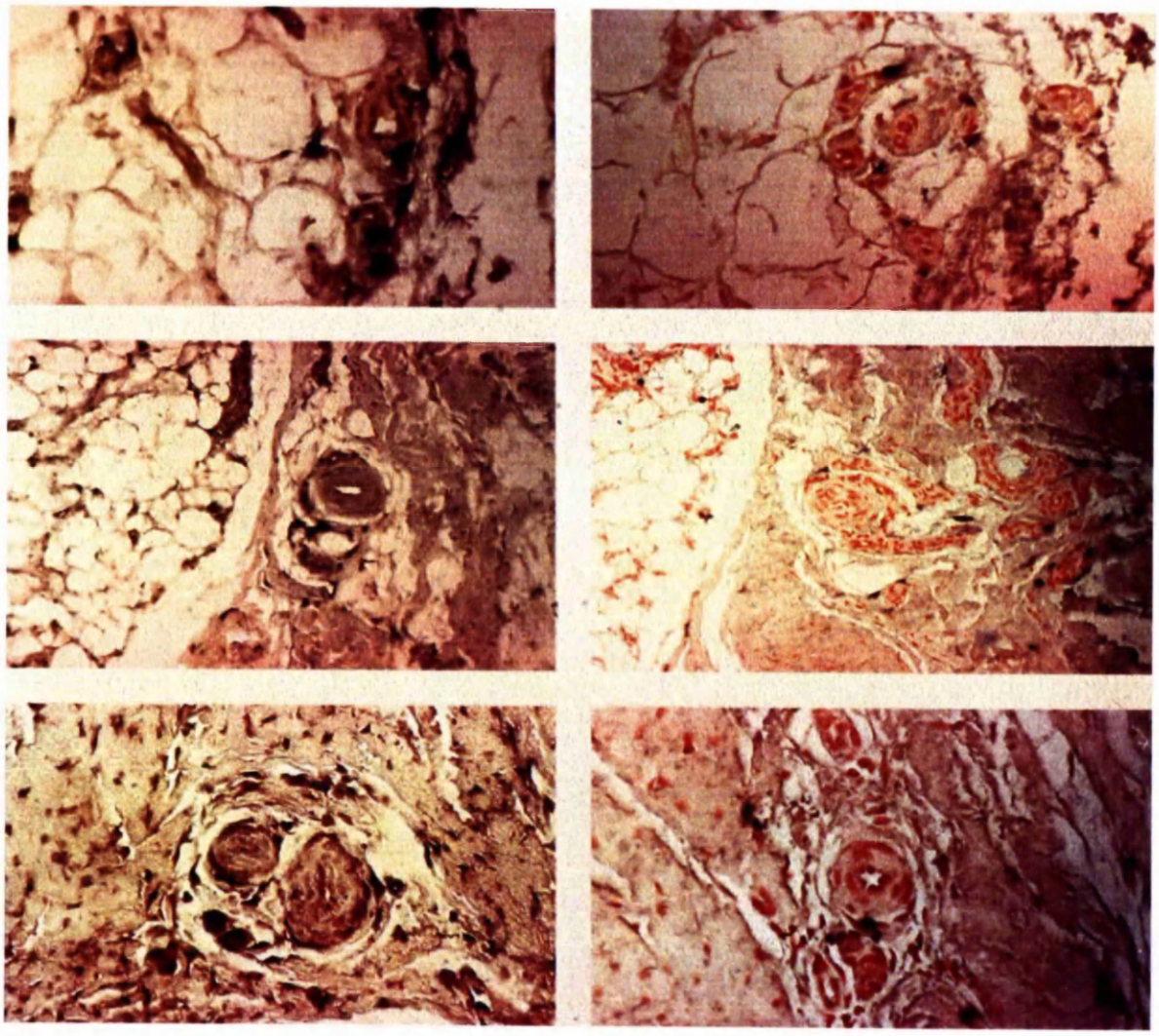


Figure 28

Sequential sections of Dupuytren's tissue stained by immuno-histochemistry for TGF-beta 1& 2 antibodies (left 3 figures) and CS1 sequence of fibronectin antibody (right 3 figures) and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) with an intense purple stain. There was a similar distribution of intensity of staining demonstrated, particularly around the blood vessels in the peri-nodular tissue (x 10 magnification, counterstain neutral red).



the nodules but also within small-localised swirls within some nodules (Figure 25). $\alpha 4\beta 1$ integrin expressing cells were relatively rarer within the nodule, mainly presenting as localised clumps within the nodule periphery. TGF-beta 1,2 stained positively around some myofibroblasts in the nodule, and again this was similar to the distribution of the CS1-sequence of fibronectin.

Cord tissue

The cord tissue was notably absent of any VCAM-1 expression. Similarly, there was no staining for $\alpha 4\beta 1$ integrin, and only very sparsely for CS1 sequence of fibronectin or TGF-beta 1,2.

Control Palmar Fascia Tissue

The endothelium of palmar fascia blood vessels did not express VCAM-1 to any great extent. There were only a very few scattered cells expressing $\alpha 4\beta 1$ and these appeared relatively randomly distributed throughout the tissue sections. CS1 sequence of fibronectin and TGF-beta 1,2 showed only limited and random positive staining.

Steroids

The steroid-treated Dupuytren's tissue did not express VCAM-1 strongly in the endothelium of the blood vessels and was much more akin to the palmar fascia immuno-staining pattern (Figure 29). $\alpha 4\beta 1$ expressing cells were still present in a similar pattern of distribution but in reduced amounts. There was a generalised

reduction in CS1 sequence of fibronectin distribution, as also there was for TGF-beta 1,2 expression (Table 5).

Figure 29

Sections of Dupuytren's tissue preoperatively treated with an injection of methyl-prednisilone. Sections stained by immuno-histochemistry for VCAM-1 antibody and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) with an intense purple stain. The area is towards the periphery of the nodule and the surrounding fibro-fatty tissue, which would normally stain very positively for VCAM-1. The VCAM-1 expression has been reduced, as shown by the lack of purple staining of the endothelium of the blood vessels (x 10 magnification, counter-stain neutral red).

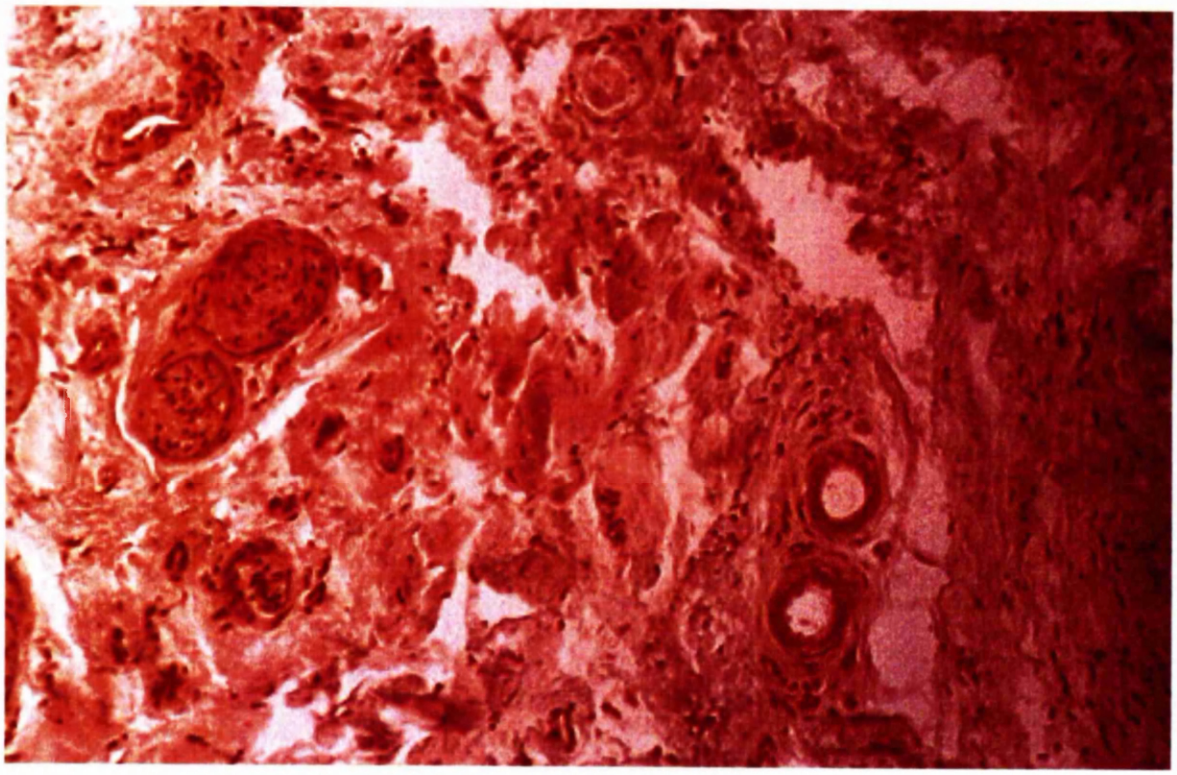


TABLE 5

Immunohistochemistry Staining for VCAM-1, $\alpha 4\beta 1$, TGF-β and CS1-Fibronectin Antigens in Sections of Dupuytren's Tissue, with and without Pre-Operative Steroid Treatment, and in Sections of Palmar Fascia

CELLULAR COUNTS PER FIELD AREA +/- 1 STANDARD DEVIATION

	VCAM-1	$\alpha 4\beta 1$	TGF-beta	CS1 Fibronectin
CONTROL DUPUYTREN'S TISSUE (N=16)	2.8 +/-2.7	2 +/-2.6	1.5 +/- 2.7	3.8 +/-8.7
STEROID TREATED DUPUYTREN'S TISSUE (N=16)	* 0.6 +/- 0.9	* 0.9 +/- 1.7	~ 1 +/- 1.8	* 1.3 +/-1.8
PALMAR FASCIA TISSUE (N=4)	* 0.3 +/-0.6	~ 0.1 +/- 0.4	* 0.3 +/- 0.6	~ 0.2 +/- 0.5

Mann-Whitney U test **Level of Significance** ~ p<0.05; * p<0.02; # p<0.01

VCAM-1, $\alpha 4\beta 1$, TGF-beta and CS1 sequence of fibronectin in the nodular tissue and fibrofatty tissue surrounding the nodules on both control and steroid treated Dupuytren's tissue and for palmar fascia sections, was analysed by immuno-histochemistry, and summarised in Table 5. Counts were made from five fields

(×25 magnification) from comparable areas of the nodule periphery. Three consecutive slide sections were counted for each patient sample.

3.3

Immunohistochemistry For Apoptosis And Proliferation In Sections Of Dupuytren's Tissue, With And Without Pre-Operative Steroid Treatment

In sections of the control Dupuytren's tissue, the cells staining positively for the Ki67 proliferation marker were predominately fibroblasts and were located at the periphery of many of the active nodules (Figure 30). In particular, the density was increased in the vascular perinodular fibrofatty layers. There were a few positively stained cells within the centre of nodules and very few within the cords leading away from the nodules. The Lewis Y marker for apoptosis stained in a similar pattern for fibroblasts in control Dupuytren's tissue to the Ki67 positive cells, but in marginally reduced frequency. A few macrophages and occasional lymphocyte also stained positively for Lewis Y.

In the steroid treated Dupuytren's tissue the frequency of Ki67 expressing cells was generally decreased, and in particular the peripheral tissue normally associated with proliferation of fibroblasts showed the greatest reduction in expression of Ki67. Although there was an increase in the number of Lewis Y expressing fibroblasts at the periphery of the steroid treated Dupuytren's tissue, the cells demonstrating the most marked increase in Lewis Y expression were macrophages. There was also a slight increase in the relative numbers of lymphocytes staining positively for Lewis Y. The changes in frequency of total cell markers are summarised (Table 6).

Figure 30

Sections of Dupuytren's tissue stained by immuno-histochemistry for Ki67 and Lewis Y antibodies and visualised with BCIP/NBT (5-Bromo-4-Chloro-Indolyl Phosphate / Nitro Blue Tetrazolium) with an intense purple stain. The top 2 sections show Dupuytren's tissue untreated with steroid and stained for Ki67 antigen. A large number of cells positively stained at the periphery of active Dupuytren's nodules (×10 magnification, counter-stain neutral red). The bottom two sections show Dupuytren's tissue treated pre-operatively with steroid and stained for Lewis Y marker. The positively stained Lewis Y cells demonstrated were both macrophages and fibroblasts (×40 magnification on left and ×10 magnification on right, counter-stain neutral red).

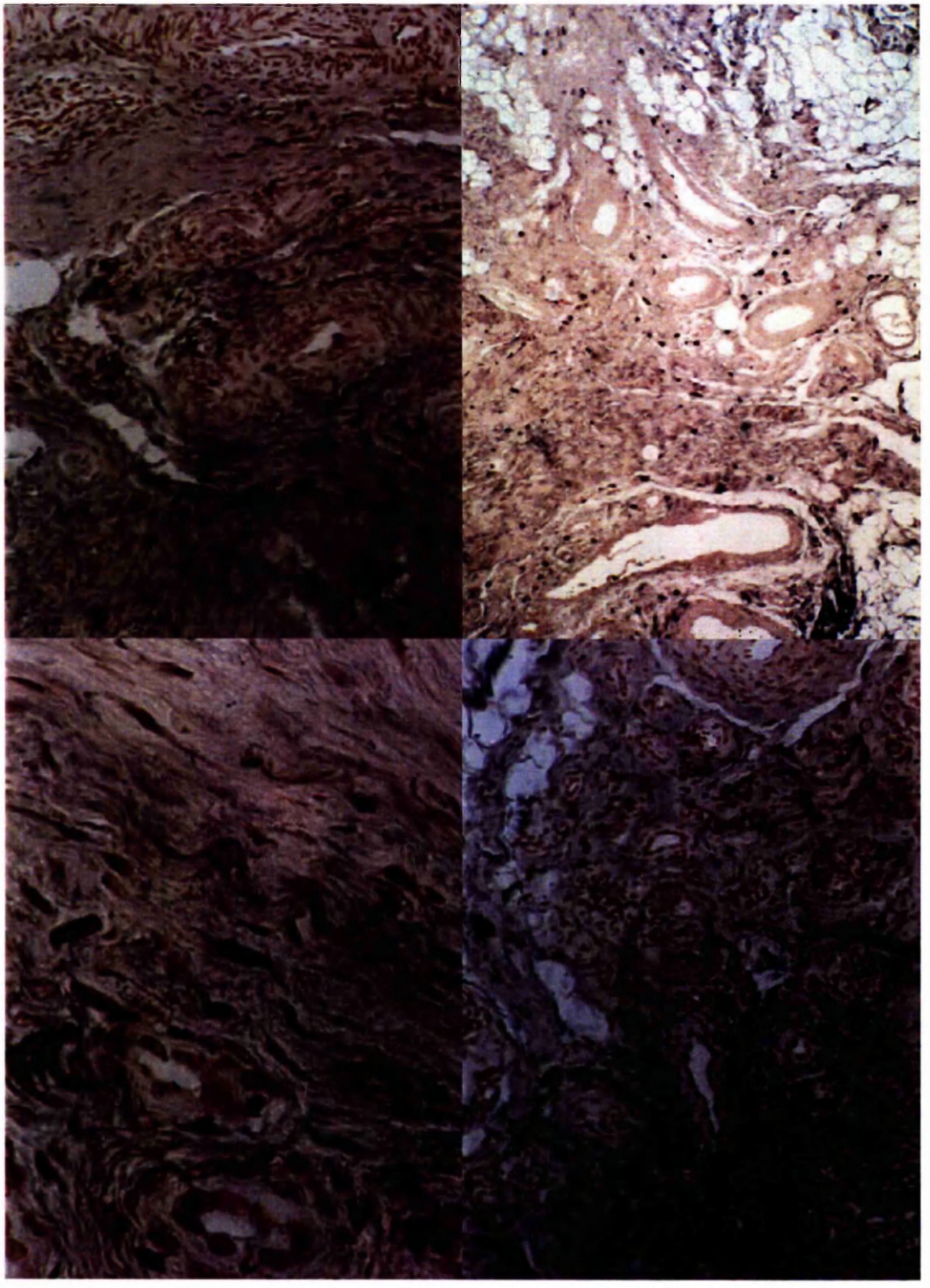


TABLE 6

Immunohistochemistry Staining for Lewis Y and Ki67 Antigens in Sections of Dupuytren's Tissue, with and without Pre-Operative Steroid Treatment

CELLULAR COUNTS
PER FIELD AREA

	Lewis Y	Ki67
CONTROL DUPUYTREN		
TISSUE (N=16)	1.0 +/- 1.5	1.6 +/-2.1*
STEROID TREATED	1.7 +/- 2.1	1.1 +/- 1.7*
DUPUYTREN TISSUE (N=16)		

Mann-Whitney U test

Level of Significance
* P <0.05

Lewis Y and Ki67 antigens in the nodular tissue and fibro-fatty tissue surrounding the nodules on both control and steroid treated Dupuytren's tissue were analysed by immuno-histochemistry, and summarised in Table 6. Counts were made from five fields (x 25 magnification) from comparative areas of the nodule periphery. Three consecutive slide sections were counted for each patient sample.

3.4

Flow Cytometry Analysis for Annexin V Binding of Cells from Dupuytren's Tissue Cultures, Palmar Fascia Tissue Cultures and Fascia Lata Tissue Cultures

Steroids produced different effects on Annexin V binding for the three groups of cells analysed by flow cytometry. These groups were cells from (1) Dupuytren's cell cultures, from (2) palmar fascia cell cultures and from (3) fascia lata cultures. In the first group of 6 separate Dupuytren's cell cultures there was an increase in the percentage of cells purely binding Annexin V from 1.7% to 18% and this was statistically significant ($p<0.02$) (Table 7). In the second group of palmar fascia cells, the 6 individual cultures had been added together into 2 groups in order to give sufficient cells for FACS analysis. Following the addition of steroid, Annexin V binding altered from 4.3% to 3.9% (Table 8). In the last group of fascia lata cells, steroid added to the cultures altered average Annexin V binding from 4.3% to 5.3% (Table 9). Thus, only the Dupuytren's cells had a statistically significant increase in Annexin V binding on the addition of steroid (Table 7).

TABLE 7

Flow Cytometry for Annexin V in Cell Cultures from Dupuytren's Tissue, With and Without The Addition of Steroid to The Culture Medium

Percentage of Annexin V positively staining cells in gated area

DUPUYTREN CELL CULTURES	CONTROL %	STEROID %
1A	1.3	25
1B	2.8	20.8
2A	0.2	1.5
2B	0.5	11.6
3A	3.4	10.1
3B	1.5	8.8
4A	8.8	37
4B	8.8	36
5A	7.9	16.6
5B	2.8	20
6A	5	13
6B	3.3	7
AVERAGE	**3.9**	***17.3**
+/- 1 Standard Deviation	**+/-3.1**	**+/-11.0**

Mann-Whitney U test

Level of Significance
*** p<0.002**

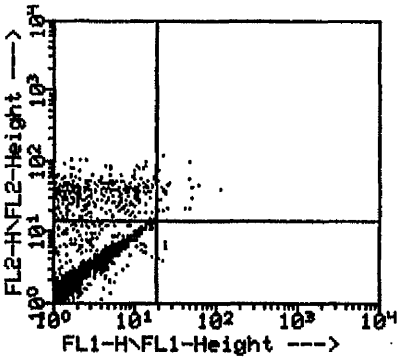
The percentage of cultured Dupuytren's cells expressing purely Annexin V binding on flow cytometry analysis. Each culture was independently reassessed by analysing a further sample (A and B). The effect of the addition of a solution with solu-medrone (10^{-3} M) on Annexin V binding is expressed in the second

column. In all cases, there was an increase in Annexin V binding and this was statistically significant as analysed by Mann-Whitney U test.

Figure 31

Illustrative Figures Of Annexin V Binding By Flow Cytometry For Cells From Pre-Operatively Untreated Dupuytren's Tissue Cultures, With And Without The Addition Of Steroid To The Culture Medium

Typical Flow cytometry analysis of Dupuytren's sample. The left two graphs demonstrate 2 samples of pre-operatively untreated Dupuytren's cells. The right two graphs demonstrate the same cells samples treated with solu-medrone 10^{-3} M added to the culture medium. Pure Annexin V binding is measured in the lower right quadrant as in the superior graphs. Where the pattern for cellular staining did not lend itself for quadrant division, the area was gated for the Annexin V binding cells as seen in the lower graphs (R3), and the reading of pure Annexin V binding compared. As can be seen there was an increase in Annexin V binding after the addition of steroid.



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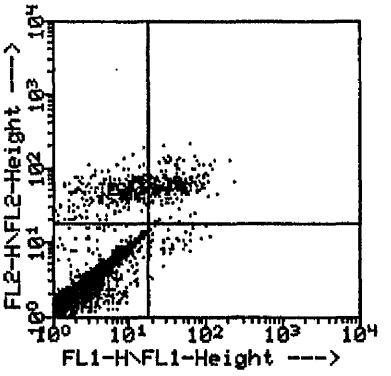
2 UR	26	1.08
3 LL	1965	81.64
4 LR	5	0.21



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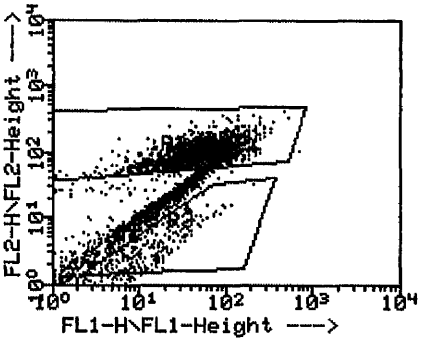
2 UR	251	8.65	
3 LL	2269	78.21	
4 LR	43	1.48	



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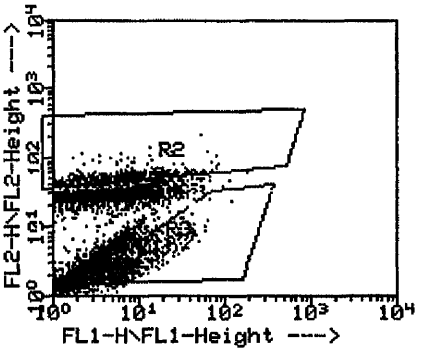
2 R2	2218	44.36
3 R3	250	5.00



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Rgn	Events	% Gated
1 R1	1772	35.44
2 R2	2443	48.86
3 R3	652	13.04

TABLE 8

Flow Cytometry for Annexin V in Cell Cultures from Palmar Fascia Tissue, With and Without The Addition of Steroid to The Culture Medium

Percentage of Annexin V positively staining cells in gated area

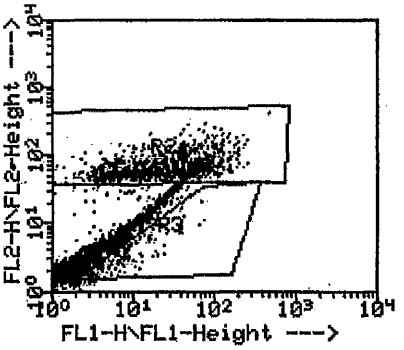
PALMAR FASCIA CELL CULTURES	CONTROL %	STEROID %
1	8.5	3.9
2	4.0	5.6
AVERAGE	6.25	4.75

The percentage of cultured Palmar Fascia cells expressing purely Annexin V binding on flow cytometry analysis. The effect of the addition of a solution with solu-medrone (10^{-3} M) on Annexin V binding by individual cells is expressed in the second column.

Figure 32

Illustrative Figures Of Annexin V Binding By Flow Cytometry For Cells From Palmar Fascia Tissue Cultures, With And Without The Addition Of Steroid To The Culture Medium

Typical Flow cytometry analysis of pooled samples for palmar fascia cultures. The left two graphs demonstrate 2 samples of untreated Palmar Fascia cells. The right two graphs demonstrate the same cells samples treated with solu-medrone 10^{-3} M added to the culture medium. Pure Annexin V binding is measured by gating for these cells (R3 in upper graphs and R2 in lower graphs). There was no consistent change in the Annexin V binding.



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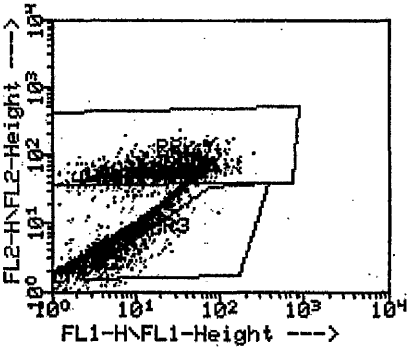
Rgn	Events	% Gated	%
1 R1	3691	100.00	
2 R2	1059	28.69	
3 R3	151	4.09	



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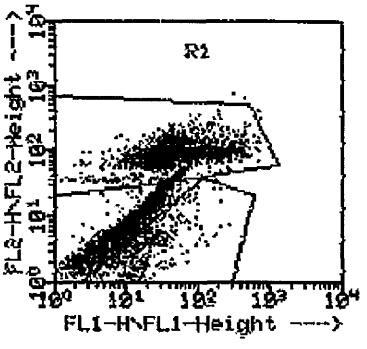
Total= 5000 Gated=

Rgn	Events	% Gated
1 R1	3357	100.00
2 R2	1490	44.38
3 R3	187	5.57



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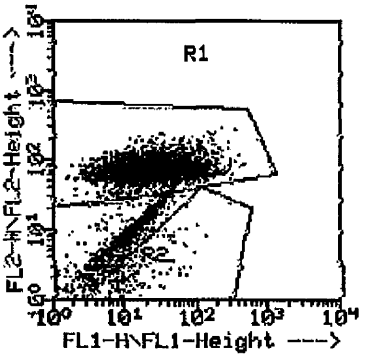
Total= 5000 Gated=

Rgn	Events	% Gated
1 R1	2069	41.38
2 R2	426	8.52



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Rgn	Events	% Gated	%
1 R1	3824	76.48	
2 R2	195	3.90	

TABLE 9

Flow Cytometry for Annexin V in Cell Cultures from Fascia Lata Tissue. With and Without The Addition of Steroid to The Culture Medium

Percentage of Annexin V positively staining cells in gated area

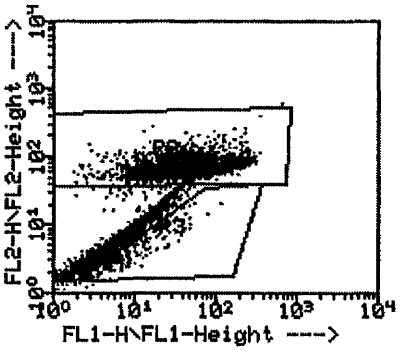
FASCIA LATA CELL CULTURES	CONTROL %	STEROID %
1	4.2	6.8
2	14.6	2.2
AVERAGE	**9.4**	**4.5**

The percentage of cultured Fascia Lata cells expressing purely Annexin V binding on flow cytometry analysis. The effect of the addition of a solution with solu-medrone (10^{-3} M) on Annexin V binding by individual cells is expressed in the second column.

Figure 33

Illustrative Figures Of Annexin V Binding By Flow Cytometry For Cells From Fascia Lata Tissue Cultures, With And Without The Addition Of Steroid To The Culture Medium

Typical Flow cytometry analysis of Fascia lata samples. The left two graphs demonstrate 2 samples of untreated Tensor Fascia Lata cells. The right two graphs demonstrate the same cells samples treated with solu-medrone 10^{-3} M added to the culture medium. Pure Annexin V binding is measured by gating for these cells (R3 area in graphs). There was no consistent change in the Annexin V binding.



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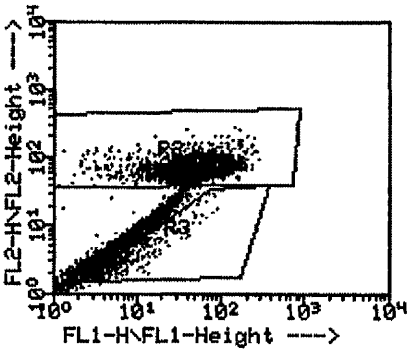
Rgn	Events	% Gated	%
1 R1	4557	100.00	
2 R2	2496	54.77	
3 R3	190	4.17	



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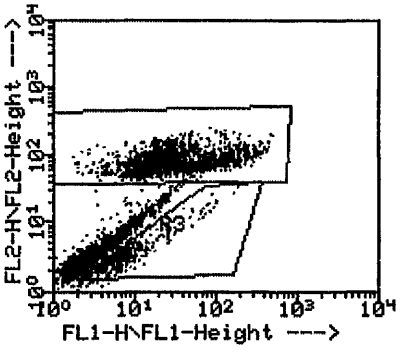
Rgn	Events	% Gated
1 R1	4624	100.00
2 R2	2145	46.39
3 R3	315	6.81



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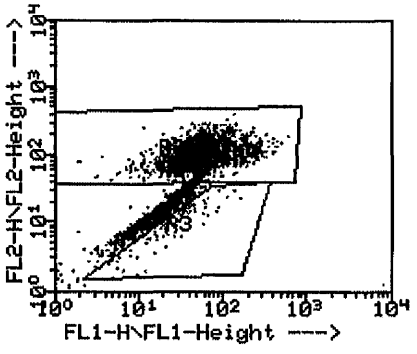
Rgn	Events	% Gated	%
1 R1	4011	100.00	
2 R2	1846	46.02	
3 R3	585	14.58	



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Rgn	Events	% Gated
1 R1	4331	100.00
2 R2	2467	56.96
3 R3	97	2.24

CHAPTER 4

DISCUSSION

4.1 Dupuytren's Disease - A chronic inflammatory condition

4.2 Effect of steroids on transendothelial migration and extracellular matrix constitution

4.3 Steroid Induced Apoptosis in Dupuytren's disease

4.1

Dupuytren's Disease - A Chronic Inflammatory Condition

From these immuno-histology results looking at the pro-inflammatory cytokines, it is apparent that the mononuclear cells, endothelium and nodular fibroblasts in Dupuytren's disease express IL-1, TNF-alpha and IL-8. A possible hypothesis is that if this IL-8 production is a sustained production, then it will lead to a chronic inflammatory cell infiltration of the palmar fascia tissues by virtue of its leukocyte chemoattractant and angiogenesis promoting properties. The fibrogenic cytokines produced by both the inflammatory cells and the fibroblasts would lead to fibroblast proliferation and ultimately fibrosis and to the condition known as Dupuytren's Disease.

Evidence to support this hypothesis can be based on the spectrum of clinical conditions associated with Dupuytren's disease and on the biological effects of the associated growth factors. In all the medical disorders associated with Dupuytren's disease, peripheral blood monocytes produce increased amounts of TNF-alpha or IL-1 (Table 10). These pro-inflammatory cytokines promote the adherence of circulating inflammatory cells to endothelium, facilitate their migration into the Dupuytrens tissues and may induce a complex cascade of cytokine interactions leading to fibroblast proliferation and ultimate fibrosis.

Trauma appears to be the only acute condition, which may be triggering the onset of Dupuytren's contracture. This may be only in genetically susceptible individuals. Within a few months following a carpal tunnel decompression or

Colles fracture Dupuytren's nodules can appear. In this situation, after injury, the acute phase response will produce large numbers of activated monocytes and lymphocytes circulating through the microvessels of the palmar fascia. Factors that increased the endothelial stickiness for these inflammatory cells would increase their transmigration into the palmar fascial tissues. If the production of pro-inflammatory cytokines is increased, for whatever reason, then both the circulating inflammatory cells would be activated and the endothelium would increase adhesion molecule expression. In acute trauma the production of such pro-inflammatory cytokines as TNF-alpha and IL-1 will be greater than in chronic conditions, perhaps explaining the rapid onset here in those genetically susceptible patients.

Elevated circulating levels of pro-inflammatory cytokines are found in chronic conditions associated with Dupuytren's contracture (Table 10). It may be theorised that people who develop Dupuytren's contracture, have cells in their palmar tissue, which respond to these elevated cytokine levels by producing their own local inflammatory mediators in a sustained response and fail to switch off by the normal physiological feedback mechanisms. This would ensure the endothelium wall remained activated to attract in further inflammatory cells and refuel the cycle.

For example, in hepatic cirrhosis, associated with Dupuytren's contracture, the peripheral blood monocytes produce increased amounts of TNF-alpha (McLain and Cohen, 1989), associated with further acute peaks with episodes of endotoxaemia, independent of sepsis (Bigatello *et al.*, 1987)

Dupuytren's disease was reported to develop within one month of immobilisation in bed for pulmonary tuberculosis (Hueston, 1991). During this phase of treatment the mycobacteria were being destroyed, and this has been shown to be associated with the peripheral blood monocytes producing increased amounts of TNF-alpha. (Ogawa *et al.*, 1991). The baseline of circulating TNF-alpha is also elevated in tuberculosis perhaps priming the system (Kaplan, 1994). TGF-beta production by blood monocytes from patients with active tuberculosis is also enhanced (Toossi *et al.*, 1995).

Spontaneous release of TNF-alpha and IL-1 from peripheral blood monocytes is much increased in HIV infection (Roux-Lombard *et al.*, 1989), particularly in the later clinical stages of Acquired Immunity Disorder Syndrome (AIDS), when the incidence of Dupuytren's contracture is much increased. Increased soluble TNF receptor expression was noted by monocytes and CD8+ lymphocytes in HIV-1 infected patients (Rautonen *et al.*, 1994; Kalinkovich *et al.*, 1995) and HIV-1 has been noted to induce TNF alpha and IL-1 gene expression in primary human macrophages independent of productive infection (Herbein *et al.*, 1994); macrophages more often stain positive for IL-1 in HIVpositive patients than in controls (Tyor *et al.*, 1993). HIV infection induced TNF alpha release can also stimulate arachidonic acid and subsequent PGE2 release from mononuclear phagocytes (Nokta *et al.*, 1995). HIV infected Kaposi sarcoma cells produce more IL-1 beta, IL-6 and bFGF (Huang *et al.*, 1993).

In vitro, peripheral blood monocytes from patients with epilepsy, when stimulated produce significantly more IL-1 alpha and beta and IL-6 than peripheral blood monocytes from normal control patients. There was no statistical difference in this cytokine production between patients receiving anti-epileptic drugs, though those treated with carbamazepine did have elevated IL-2 production by the blood monocytes (Pacifici *et al.*, 1995).

An elevated Low Density Lipoprotein (LDL) level occurs in Diabetes Mellitus (DM), smoking and hyperproteinaemia. Peripheral blood moncytes are induced to produce IL-1 when there is an elevated level of minimally oxidised LDL (Cathart *et al.*, 1985)

Table 10

Clinical conditions associated with Dupuytren's Disease where there is increased synthesis of pro-inflammatory cytokines by circulating peripheral blood mononuclear cells

Alcoholic Liver Disease	**TNF-alpha** (McLain and Cohen D, 1989)
HIV	**TNF-alpha, IL-alpha** (Roux-Lombard *et al.*, 1989)
Chronic Pulmonary Disease	**IL-1 alpha TNF-alpha** (Strieter *et al.*, 1990)
Tuberculosis	**TNF-alpha** (Ogawa *et al.*, 1991)
Increased LDL (Diabetes Mellitus, Smokers, Hyperlipoproteinaemia)	**IL-1 alpha** (Cathcart *et al.*, 1985; Griffith *et al.*, 1988)
Epilepsy	**IL-1 alpha** (Pacifici *et al.*, 1995)

Effect of Steroids

Steroids reduced the expression of pro-inflammatory cytokines by endothelial cells, fibroblasts, macrophages and lymphocytes. In addition to the effect on the pro-inflammatory cytokines IL-1 and TNF alpha, steroids reduced IL-8 production. By reducing IL-8, there is a reduction in the chemoattractant gradient for inflammatory cells to undergo transendothelial migration.

Criticism could be made of the qualitative assessment of cytokine presence by immuno-histochemistry for these first experiments. However, this assessment has been used before in peer reviewed articles (Magro *et al.*, 1995) for assessing immuno-histochemisty sections. Ideally, automated digitalised assessment of antibody binding in these sections would have given more robust data for analysis but this was not available at this time.

Nevertheless, the reduction in general pro-inflammatory cytokine expression appeared substantial. This may be secondary to a reduction in the total number of inflammatory cells or due to less cytokine production by individual inflammatory cells or fibroblasts. The results demonstrate inflammatory cells are still present, so it is not the complete removal of such cells that is reducing the production of pro-inflammatory cytokines.

The discussion can be divided into the effect of steroids on the orchestration of inflammatory cells trans-endothelial migration mechanism and the subsequent extracellular matrix constitution, and that of direct apoptosis of the fibroblasts

and inflammatory cells constituting Dupuytren's nodules and involved in the process of fibrosis.

4.2

Effect of steroids on transendothelial migration and extracellular matrix constitution

Fibrosis is a process involving mononuclear cells and lymphocyte regulation. Most clinically fibrosing conditions have been demonstrated histologically to contain macrophages, eosinophils or lymphocytes eg. systemic sclerosis (Kraling *et al.*, 1995), renal interstitial fibrosis (Eddy, 1995; Yamate *et al.*, 1995), sarcoidosis (Hunninghake, 1995), idiopathic pulmonary fibrosis (Xing *et al.*, 1996; Groen *et al.*, 1994), reidel's thyroiditis (Heufelder and Hay, 1995) retroperitoneal fibrosis (Hughes and Buckley, 1993; Menke *et al.*, 1996) and Dupuytren's disease (Baird *et al.*, 1993a), and some of these have had impressive clinical results after treatment with steroids.

It would appear that these inflammatory cells infiltrate sites of inflammation after triggering by chemotactic and activating mediators. This is followed by an escalation of cytokines that induce fibroblast and endothelial proliferation with the subsequent deposition of extracellular matrix. In the absence of inhibitory signals the continued production of these mediators sustains the connective tissue accumulation resulting in permanent alteration in tissue structure and function.

Dupuytren's contracture is a chronic inflammatory disorder characterised by palmar nodules of proliferating fibroblasts, which eventually become incorporated into cords of mature scar tissue, producing finger contracture. Inflammatory cells have been found at the periphery of Dupuytren's nodules

(Baird, 1994; Sugden *et al.*, 1993; Andrews *et al.*, 1991). These appear to produce a number of potent growth factors and cytokines (Baird *et al.*, 1993b; Gonzalez *et al.*, 1992; Badalamente *et al.*, 1992). It may well be that it is this drive that stimulates Dupuytren's myofibroblasts to express their contractile fibrosing phenotype (Baird, 1994). To prevent the accumulation of these initiating inflammmatory cells it is necessary to discover the pathway for their migration.

These results have shown that it would be possible for $\alpha 4\beta 1$ carrying inflammmatory cells to adhere to Dupuytren's nodule blood vessels and the peripheral nodular tissue as they express VCAM-1 molecules and the CS1 site of fibronectin. The expression of $\alpha 4\beta 1$ and VCAM has also been studied in two cases of acute and late stage (fibrotic) sympathetic opthalmitis. In the acute stages (similar to the active periphery of Dupuytren's nodules) there is also an increase in $\alpha 4\beta 1$ and VCAM-1 expression and lymphocyte infiltration versus normal eyes. However in the later stages (equivalent perhaps to cord tissue) there is no significant increase in $\alpha 4\beta 1$ or VCAM versus normal iris cilary body and choroid (Kuppner *et al.*, 1993).

In many samples, the immunohistochemistry staining of CS1 fibronectin appeared to have a surface pattern of distribution on the myofibroblasts as well as an extracellular distribution. The expression of fibronectin at the surface of myofibroblasts and in the rough endoplasmic reticulum and Golgi complex of myofibroblasts has been noted previously (Del Cacho *et al.*, 1993; Singer *et al.*, 1984; Eyden, 1993). The surface fibronectin has been found to co-localise with

actin fibres at regions surrounding the nucleus and form a fibronexus, representing an intracellular junction between myofibroblasts. In particular, it provides contact between myofibroblasts and the matrix that mediates continuity between intracellular contractile filaments and extracellular matrix proteins such as collagen (Singer, *et al.*, 1984; Eyden, 1993). The fact that the myofibrolasts in Dupuytren's tissue have intense cell surface staining with antibody confirms the presence of a fibronectin fibril of a fibronexus. This has been previously demonstrated by electron microscopy (Tomasek and Haaksma, 1991).

In pulmonary fibrosis, there is again a chronic inflammatory disorder but with neutrophils involved in the early alveolitis. Nakoa, demonstrated increased E-selectin expression restricted to the honeycombing regions and that this co-localises with neutrophil distribution (Nakoa *et al.*, 1995). This suggested another mechanism for the accumulation of a subgroup of inflammatory cells involved in an active chronic inflammatory fibrotic disease.

Interestingly, post-irradiation lung injury demonstrates a release of cytokines after in vivo irradiation, which persists after exposure in some patients and initiates the cycle of chronic inflammatory events resulting in fibrosis (Rubin *et al.*, 1995). We have demonstrated TGF-beta has a similar distribution with CS1 fibronectin in Dupuytren's tissue. Ideally, dual staining would have been performed on sections to see if there was precise co-localisation. Also interesting, TGF-beta has enhanced expression in human lung granulomatous inflammation (Roman *et al.*, 1995) and sarcoid granuloma (Limper *et al.*, 1994) compared to normal lung tissue, and has been noted to co-localise with the $\alpha 5\beta 1$

integrin, (ligand for fibronectin) and $\alpha 2\beta 1$ integrin, (ligand for collagen) (Roman *et al.*, 1995).

The advantage of Dupuytren's tissue was its accessibility in large numbers and that it possesses the whole temporal and histological architecture of cells, cytokines and extracellular matrix in one specimen. Thus what we have demonstrated in Dupuytren's disease is a reproducible mechanism for the accumulation of a subgroup of inflammatory cells in the Dupuytren's nodule by their adhesion to the endothelial wall and extravasation into the nodule and it appears these cells may be a potential source of TGF-beta. If the differential expression of these integrins and adhesion molecules involved in inflammatory cell activation and adhesion can be altered then this would represent a therapeutic option (Figure 22a, b).

Figure 34 (a)

The trans-endothelial migration of inflammatory cells expressing VLA4 integrin into the tissues necessitates binding to the VCAM-1 adhesion molecule or CS1 sequence of fibronectin. The inflammatory cells in areas of high CS1 fibronectin presence stimulate pro-inflammatory cytokine and growth factor production, such as TGF-beta 1 & 2.

TRANSENDOTHELIAL MIGRATION

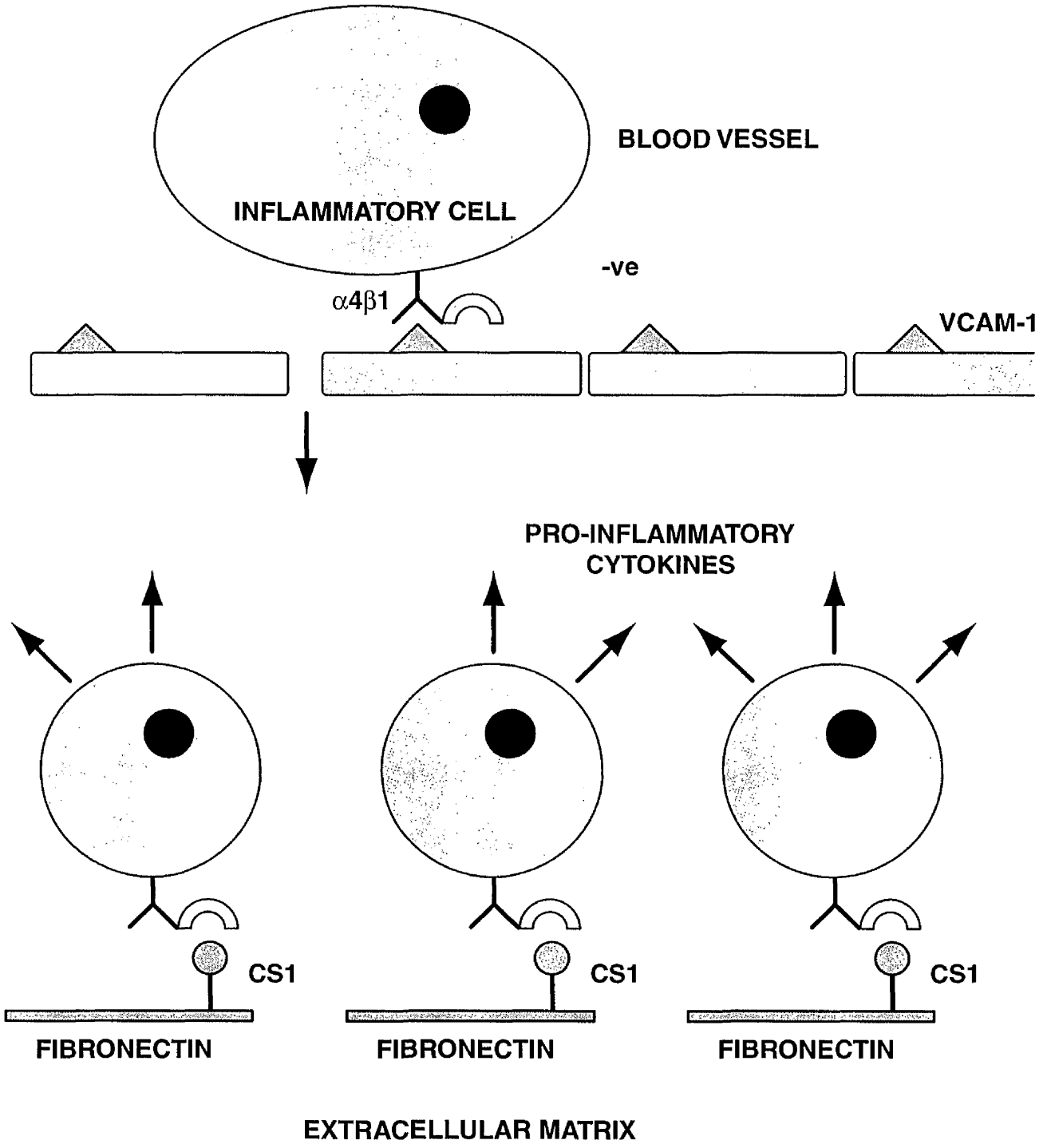
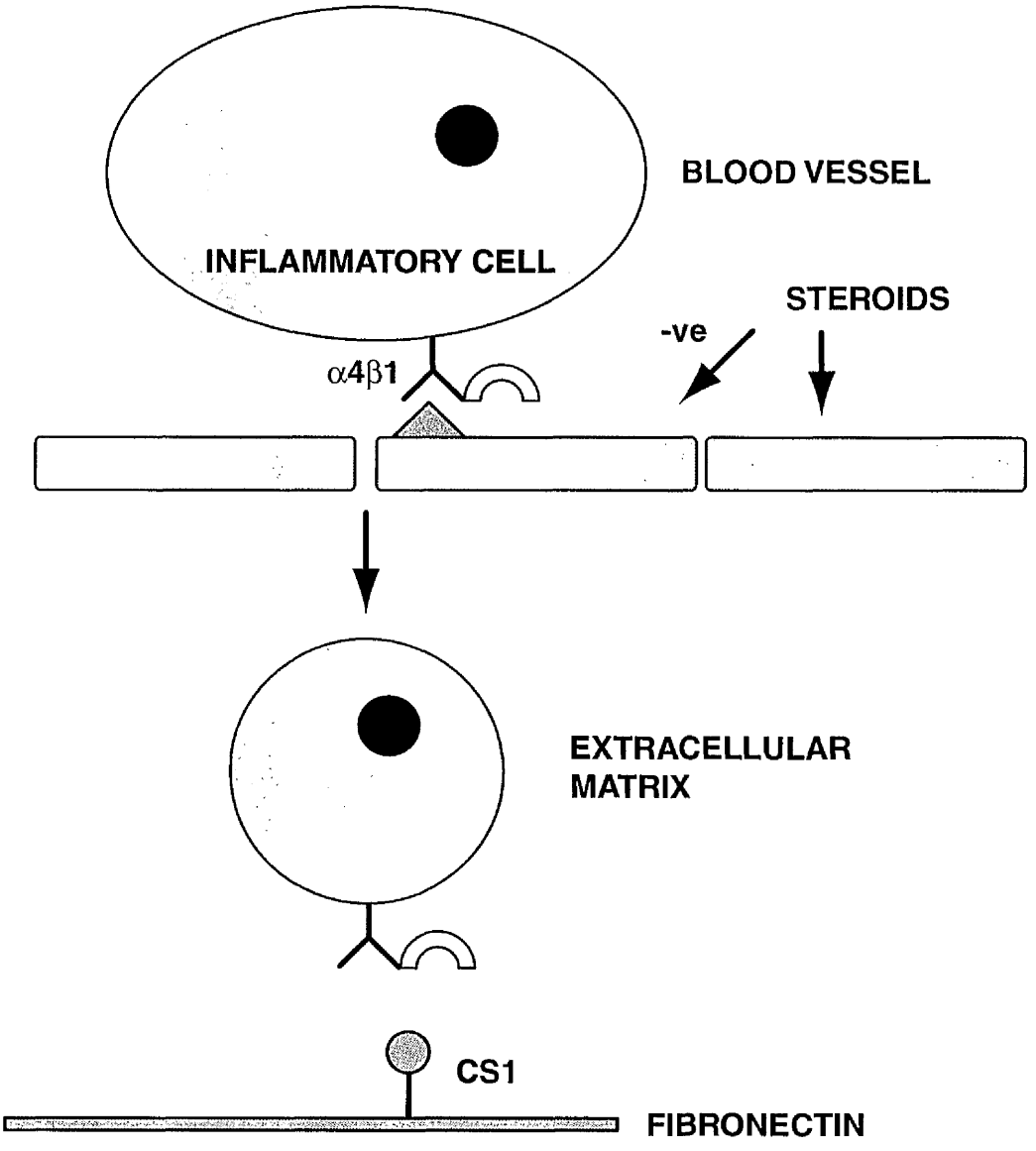


Figure 34 (b)

This process of trans-endothelial migration is influenced by many factors. One of these may be the down regulation of VCAM-1 expression by steroids. The subsequent decrease in inflammatory cell migration and activation by binding to extracellular matrix molecules, such as CS1 sequence of fibronectin, will result in the reduction of pro-inflammatory cytokine synthesis and subsequent fibrosis.

TRANSENDOTHELIAL MIGRATION



Steroids are already known to reduce VCAM-1 expression in cultured aortic cells (Aziz and Wakefield, 1996). We have demonstrated a massive reduction of VCAM-1 expression by Dupuytren's endothelial cells in humans subsequent to hydrocortisone injection treatment. Steroids although reducing VCAM-1 induction did not totally block CS1 fibronectin production and therefore will still allow some cells expressing $\alpha 4\beta 1$ to accumulate in the extracellular matrix. We had noted that some cells expressing $\alpha 4\beta 1$ appeared to be elongated like fibroblasts. These cells were found to express Mac387 and CD68 markers suggesting them to be macrophages behaving like fibroblasts rather than $\alpha 4\beta 1$ expressing myofibroblasts.

The fact that TGF-beta production is not totally blocked by steroid injection is perhaps not surprising. The continued presence of $\alpha 4\beta 1$ expressing inflammatory cells, with which TGF-beta is often closely localised, are a potential source of this potent cytokine. Also other cells than $\alpha 4\beta 1$ expressing cells, Dupuytren's fibroblasts in particular, will produce TGF-beta 1 and 2 (Kloen *et al.*, 1995). The fact that steroids reduced TGF-beta presence agrees with a series of experiments where animal wounds given systemic glucocorticoids and there was a suppression of TGF-beta I and II (Frank *et al.*, 1996). As yet unpublished results by this department have shown that IL-1 alpha stimulated TGF-beta production by Dupuytren's cells in culture is reduced when methylpredisilone is added to the medium. Normal cultured skin cells showed no decline in baseline production of TGF-beta with the addition of methylprednisilone of the same concentration (Baird, Personal communication).

The biological activity of TGF-beta is controlled by their persistence in the extracellular compartment as latent complexes. TGF-beta isoforms are non-covalently associated with a latency-associated peptide (LAP). LAP is required for efficient TGF-beta secretion, preventing TGF-beta binding to ubiquitous cell receptors, and maintaining its availability in a large extracellular reservoir that is readily accessed by activation. The latent TGF-beta is secreted by all cells and is abundant both in circulating forms and bound to the extracellular matrix. The mechanisms of regulation of activation of this latent form of TGF-beta are complex (Bonewald, 1999). A number of molecules are involved in this activation step. Anti-inflammatory molecules such as hydrocortisone inhibit TGF-beta by a mechanism that may include downregulation of transglutaminase expression (Gosiewska *et al.*, 1999). This may be one mechanism steroids inhibit TGF-beta activation in Dupuytren's disease.

Given TGF-beta's critical role in fibrosis this suggests further work is required to modulate its production and activation. The control of inflammatory cell migration and adherence may allow modulation of fibrosis. Other drugs already exist which block CS1 fibronectin or $\alpha 4\beta 1$ itself (Elices, 1995) and once these and drugs which bind to other implicated integrins reach clinical status further immuno-modulation of Dupuytren's disease and fibrosing diseases in general may be possible in the future. Although it would be simplistic to suggest that $\alpha 4\beta 1$ / VCAM-1 transendothelial migration is the sole pathway involved here, it nevertheless demonstrates how one pathway mechanism can be modulated in active chronic inflammatory conditions.

4.3

Steroid-Induced Apoptosis in Dupuytren's disease

Chronic inflammation and the factors that regulate leukocyte survival in this environment are being actively investigated. Chronic inflammation recruits monocytes from the blood into the lesion where they, upon exposure to specific stimuli, differentiate into activated macrophages (Zembala, 1989). These macrophages have a role in exacerbation and resolution of chronic inflammation by phagocytosis and production of cytokines, arachidonic acid metabolites and reactive oxygen species (Zembala, 1989). In the resolution phase, in which healing occurs, the secretion of cytokines decreases and the number of inflammatory cells decreases, particularly monocytes. This is achieved without release of lysosomal enzymes from leukocytes and without further tissue damage and inflammation. The mechanisms involved in this are not fully determined but histological evidence suggests apoptosis (Savill *et al.*, 1990). Survival of leukocytes within a lesion appears to be associated with inflammation continuing. For example, rheumatoid arthritis has been theorised as being a T-cell-mediated disease, the primary defect being lack of apoptosis.

Dupuytren's contracture has been compared histologically to a sarcoma tumour and Azzarone found growth properties of the myofibroblasts derived from Dupuytren's nodules lay between normal fibroblasts and virus-transformed or sarcoma-derived fibroblasts (Azzarone *et al.*, 1983). Actual fibrosarcoma, as with all tumours, represents an imbalance between proliferation and apoptosis of cells. Interestingly, Kihara et al compared infantile fibrosarcoma, which often

undergo a benign course or may even undergo spontaneous regression, with the phenotypically similar adult fibrosarcoma that does not (Kihara *et al.*, 1996). They demonstrated infantile fibrosarcoma had a significantly enhanced apoptosis rate coupled with a lower proliferative index and suggested this was compatible with its benign course. Dupuytren's contracture, although not a malignant tumour, represents a similar imbalance between particular subsets of cells within an inflammatory tissue.

Steroids are the most potent and effective agents in controlling inflammatory diseases (Barnes and Adcock, 1993). Clinically, steroid injections can induce Dupuytren's nodule regression (Ketchum *et al.*, 1972) and histologically this appears to be by apoptosis (Meek *et al.*, 1998). Steroids may induce this apoptosis by a number of methods including the following.

Steroids and modification of cell adherence

Steroids have a multiplicity of effects on the underlying extracellular matrix affecting fibronectin and the balance of various binding sites. For example, steroids alter the distribution of fibronectin, or distribution, type and synthesis of collagen and it's binding properties. This work has demonstrated a down regulation of the fibronectin CS1 domain in Dupuytren's tissue after treatment with steroid and this may play a role in the rate of cellular apoptosis. There is also an increase of collagen type III over type I (Murrell *et al.*, 1989; Brinkley-Parsons *et al.*, 1981). The effect of steroids on this ratio and other matrix

compounds is not precisely known nor how this would affect the binding of cells and their apoptosis rates.

In some cases binding may augment apoptosis depending on the cell type. A study of myeloid leukaemia cell line (M07E), which proliferate in response to human granulocyte/macrophage colony stimulating factor, was undertaken in relation to fibronectin regulation. Fibronectin induced growth suppression in the face of stimulation factors by causing apoptosis. Adding anti $\alpha 5\beta 1$ antibody but not anti $\alpha 4\beta 1$ antibody could reverse this. In addition, fibronectin induced apoptosis was detected in other haemopoeitic cells expressing $\alpha 5\beta 1$ but not in $\alpha 5\beta 1$ negative cells (Sugahara *et al.*, 1994). In this case the fibronectin $\alpha 5\beta 1$ binding site may allow negative regulation.

The basement membrane extracellular matrix induces differentiation and suppresses apoptosis in mammary epithelial cells whereas cells lacking the basement membrane lose their differentiated phenotype and undergo apoptosis (Boudreau *et al.*, 1996).

Various different mechanisms may co-exist for controlling adherence of the monocytes, T cells and myofibroblasts apoptosis. These factors presumably apply in Dupuytren's tissue, controlling the rates of inflammatory cell apoptosis, and as steroids modify the matrix constitution, they alter the rate of apoptosis.

Reduction in inflammatory cell load by apoptosis

Steroids may cause cells to directly undergo apoptosis, alternatively the cells may secondarily undergo apoptosis due to a reduction in pro-inflammatory cytokine production

T cells

The majority of thymocytes are sensitive to the apoptosis inducing activity of glucocorticoids while the majority of mature peripheral T cells are resistant to it (Iwata, 1995). T cell specific growth factor IL-4 specifically rescues T_{H2} cells from steroid induced apoptosis (Zubiaga *et al.*, 1992) whereas IL-2 and IL-1 are ineffective in preventing apoptosis for these cells. However, IL-2 is the relevant rescue factor for glucocorticoid induced apoptosis of T_{H1} cells (Zubiaga *et al.*, 1992). The induction of apoptosis appears to involve protein kinase activation as inhibition blocks the protection effect (Zubiaga *et al.*, 1992).

Dupuytren's tissue contains elevated levels of IL-1, IL-2 and IL-4 (Baird *et al.*, 1993b), which are reduced after steroid injections. In addition, there is immuno-histochemical evidence of T cell apoptosis on injecting steroids. This may be due to the observed reduction in cytokine production or a direct effect. The T cell subgroups that are undergoing apoptosis require to be defined.

Macrophages

A multitude of factors are known to affect macrophage apoptosis in normal physiological resolution of acute inflammation (Tidball and St Pierre, 1996), including nitric oxide (Shimaoka *et al.*, 1995; Messmer *et al.*, 1996), Bordetella pertussis (Khelef and Guiso, 1995), GM-CSF (Gehrmann, 1995) and MC-SF (Munn *et al.*, 1995) or exposure to IFN-γ (Munn *et al.*, 1995). The data here has certainly demonstrated steroids induce macrophage apoptosis in Dupuytren's tissue. Again, this would reduce the pro-inflammatory cytokine drive to the myofibroblasts and possibly contribute to apoptosis of various cells.

Myofibroblasts

Growth factors play a role in controlling apoptosis of fibroblasts. Certainly dexamethasone inhibits IL-1 and TNF production by human lung fibroblasts without affecting IL-1 or TNF receptors (Monick *et al.*, 1994) and could induce self-apoptosis by this downregulation.

A parallel scenario may be seen in the microglia of brain tissue. Apoptosis of microglia is hypothesised as a mechanism of maintaining steady state conditions in vivo. Again, either downregulation of microglial mitogens and survival factors, or directly induced programmed cell death in the microglial cells could be theorised. Certain microglial growth factors induce a mitogenic response, but withdrawal enhances rates of microglial cell death by apoptosis, as assessed by

DNA fragmentation studies (Gehrman, 1995). This suggests a reduction in the concentration of mitogens is one mechanism at work to initiate fibroblast apoptosis.

The effect of steroids may still be partly a direct effect on the fibroblasts. Certainly a direct effect has been demonstrated on rat osteoclasts which underwent morphological changes resembling apoptosis after 2-4 hours of exposure to dexamethasone and corticosterone (Lutton *et al.*, 1996).

The cultures of the Dupuytren's tissue may contain other cells than purely fibroblasts and this may be another criticism of the flow cytometry results. Further antibody labelling of the cultures would prove if there are any significant subgroups of cells other than myofibroblasts. Otherwise, the effect on myofibroblast apoptosis can still be attributed to a reduction in cytokine drive from other cells in the culture.

This study did demonstrate that the steroid drug depomedrone induced apoptosis in Dupuytren's tissue. This has not been reported before. By understanding the factors controlling cell proliferation and apoptosis in this chronic inflammation, possible improved methods of treating such diseases may be attained. Other drugs too can influence apoptosis by modifying the adherence to extracellular matrix molecules or the production of specific pro-inflammatory cytokines. These drugs may well prove to be highly effective treatments for chronic inflammatory disease in general.

In summary, Dupuytren's contracture is a disabling disease of hand function and the treatment of the established contracture is not without morbidity. In order to prevent the progressive contracture, it is necessary to understand its development.

Lastly, I suggest Dupuytren's disease lends itself as a reasonable model for chronic inflammation with its sustained inflammatory cell infiltrate and extracellular matrix synthesis. By understanding the factors controlling inflammatory cell trans-endothelial migration and subsequent stimulation of the extracellular matrix and fibroblast proliferation, with its regulation by apoptosis, improved methods of treating chronic inflammation in such diseases may be attained.

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