THE ROLE OF FIBRIN IN SKIN FIBROSIS

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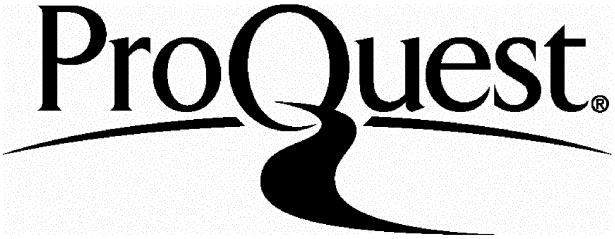

ABSTRACT

Patients with chronic venous insufficiency develop characteristic skin changes on the lower leg, known as lipodermatosclerosis. There is limited information regarding the cellular and molecular mechanisms that regulate the development of this fibrotic condition even though it often proceeds to leg ulceration. Chronic venous hypertension causes extravasation of plasma fibrinogen from the cutaneous vasculature resulting in extensive fibrin deposition. Patients with lipodermatosclerosis show reduced fibrinolytic activity leading to a persistence of fibrin in the skin. Although fibrin deposition is an important part of normal tissue repair, it is also associated with many fibrotic conditions. This thesis addresses the hypothesis that reduced fibrinolysis and the persistence of fibrin matrix are associated with enhanced accumulation of collagen by dermal fibroblasts, resulting in the development of fibrotic skin disorders such as lipodermatosclerosis. The aims of this thesis were to characterise the fibrotic skin changes related to lipodermatosclerosis and to investigate the role of fibrin matrix on procollagen production by dermal fibroblasts in normal and reduced fibrinolytic conditions.

Skin affected by lipodermatosclerosis showed extensive fibrin deposition and increased procollagen gene expression in the dermal and subcutaneous layers compared with control skin. The role of fibrin on procollagen production by dermal fibroblasts was assessed *in vitro* and *in vivo* using novel strategies. Procollagen production by dermal fibroblasts was significantly increased when grown in fibrin gels compared with monolayer cultures. Furthermore, multiple, combined subcutaneous injections of fibrinogen and thrombin in Balb-c mice resulted in a significantly increased level of collagen deposition, compared with PBS-treated or untreated control skin. Under reduced fibrinolytic conditions, dermal fibroblasts grown in fibrin gels produced a further significantly increased level of procollagen compared with control cultures. This was confirmed *in vivo*, as multiple subcutaneous injections of fibrinogen and thrombin in tissue plasminogen activator-deficient mice resulted in a significantly increased level of collagen deposition compared with the skin of wild-type mice. Enhanced collagen accumulation was found to be due to increased procollagen gene expression and decreased collagen degradation through reduced activation of matrix metalloproteases.

In conclusion, this thesis has shown that reduced fibrinolysis and the persistence of a fibrin matrix results in an excessive accumulation of collagen in both *in vitro* and *in vivo* systems. These events may occur during the development of LDS as well as other fibrotic disorders where extensive fibrin matrix deposition is a key feature.

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"*Il-neputi huwa tabib*"

Nannu Mike

ABBREVIATIONS

α_2-AP	Alpha 2-antiplasmin
Ab	Antibody
APMA	Aminophenylmercuric acid
BSA	Bovine serum albumin
CEAP	Classification Etiology Anatomy Pathophysiology
COL1A1	Procollagen type 1 α-chain
CRR	Centre for respiratory research
CTGF	Connective tissue growth factor
CVI	Chronic venous insufficiency
DAB	3-3'diaminobenzidine
dd water	Double-distilled water
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's modified eagle medium
DPA	2,4-dinitrophenyl quencher
DVT	Deep vein thrombosis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPR-1	Effector cell protease receptor-1
EtOH	Ethanol
FCS	Foetal calf serum
FDP	Fibrin degradation product
FITC	Fluorescein isothiocyanate
H & E	Haemotoxylin and eosin
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HSF	Human skin fibroblasts
ICAM	Intracellular adhesion molecule
ISH	*In situ* hybridisation
LDS	Lipodermatosclerosis
MAPK/ERK	Mitogen activated protein kinase/extracellular signal related kinase

MCA	7-methoxycoumarin-4-yl acetate
MCT	Mast cell tryptase
MDCK	Madin-Darby Canine Kidney
MFR	Mitogenic fibrinogen receptor
mmHg	Millimetres of mercury
MMP	Matrix metalloproteinase
MMP-1*i*	Matrix metalloproteinase-1 inhibitor
MOPS	3-(N-morpholinopropanosulfonic acid)
MSB	Martius scarlet blue
MWt	Molecular weight
NaOH	Sodium hydroxide
NBD-Cl	7-chloro-4nitrobenzo-2-oxa 1,3-diazole
NBT/BCIP	Nitrotetrazolium blue chloride/ 5-bromo-4-chloro-3-indolyl phosphate
OHpro	Hydroxyproline
PAI	Plasminogen activator inhibtor
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCP	Procollagen C proteinase
PDGF	Platelet derived growth factor
RER	Rough endoplasmic reticulum
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SSC	Saline sodium citrate
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
tPA	Tissue-type plaminogen activator
uPA	Urokinase-like plasminogen activator
uPAR	Urokinase-like plasminogen activator receptor
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor

Chapter 1

REVIEW OF THE LITERATURE

Section 1: Lipodermatosclerosis

1.1 Chronic venous disease and lipodermatosclerosis

Chronic venous disease affects 10-20% of the adult population in the United Kingdom and so has great social and economic significance (Callam et al., 1985). Complications of venous disease include deep vein thrombosis, varicose veins, lipodermatosclerosis and ulcer formation. Affected individuals develop raised venous pressure upon exercise, which is transmitted to the cutaneous microvasculature (Coleridge-Smith, 1996), resulting in a series of changes that lead to lipodermatosclerosis (LDS). LDS refers to pre-ulcer skin changes that occur on the distal leg and is characterised by skin thickening and hyperpigmentation. The disease usually manifests over many years and often proceeds to ulcer formation, which in turn, affects 1% of the adult population (Callam et al., 1985).

Treatment of chronic venous disease has relied on established principles of compression bandaging and limb elevation, originating from the time of Hippocrates more than 2500 years ago (Coleridge-Smith, 1996). This treatment is expensive and time-consuming and not always successful. Furthermore, drug therapy and surgery have been shown to have some but limited benefit. The underlying disorders leading to chronic venous insufficiency in the lower leg are well established, however, the manner in which subsequent skin changes develop and proceed to ulceration is not known.

1.2 Aetiology of chronic venous disease

Venous insufficiency is not a condition limited to the elderly, since greater than 40% of patients develop venous ulcers before the age of 50 years (Falanga, 1993). Approximately 50% of all venous ulcers are present for periods greater than nine months and two-thirds are recurrent. Venous disease consumes a considerable amount of resources from healthcare systems annually with the cost of treatment estimated to be between £2000 and £4000 per patient per year, for each of the 150,000 patients with

a venous leg ulcer (Coleridge-Smith,1996). The total cost of treatment is in excess of £800 million per annum, approximately 2% of the total annual healthcare budget in the UK (Coleridge-Smith, 1996).

1.3 Pathophysiology of venous disease

The superficial blood system of the leg is composed of the long and short saphenous veins (Figure 1.1). The long saphenous vein ascends the leg to join the femoral vein in the thigh via the medial malleolus and the short saphenous vein inserts into the popliteal vein, near the popliteal fossa at the back of the knee via the lateral malleolus. Both of these superficial veins have numerous communicating tributaries that connect them to deep veins beneath the fascia. The deep, communicating and superficial venous systems are equipped with valves that prevent retrograde blood flow. In the standing position, the venous pressure in the deep veins is high, approximately 80mmHg. During exercise, the muscles of the calf contract leading to increased deep vein pressure (200mg Hg) and the emptying of the blood from the leg towards the heart. Flow of blood out of the deep system results in a subsequent decrease in deep vein pressure (10mm Hg) that drives flow from the superficial venous system (with a pressure of 90mmHg) to the deep venous system. The valves of the lower leg rely on the pressure differential in order to direct blood flow to the heart (Browse et al., 1977).

In patients with venous disease, the predicted fall in deep vein pressure on exercise does not occur and the pressure in the superficial veins remains high (90mm Hg). This is termed 'ambulatory venous hypertension' even though the blood pressure in the superficial venous system never increases beyond the maximal 'standing pressure' (90mm Hg). Venous hypertension occurs due to a number of abnormalities such as damaged communicating or superficial vein valves, incompetent deep vein valves, deep vein occlusion or thrombosis, and calf muscle dysfunction singularly or in combination (Falanga, 1993). The severity of the calf pump abnormality varies between patients, but the most common contributors to calf muscle failure are superficial vein regurgitation and deep vein obstruction.

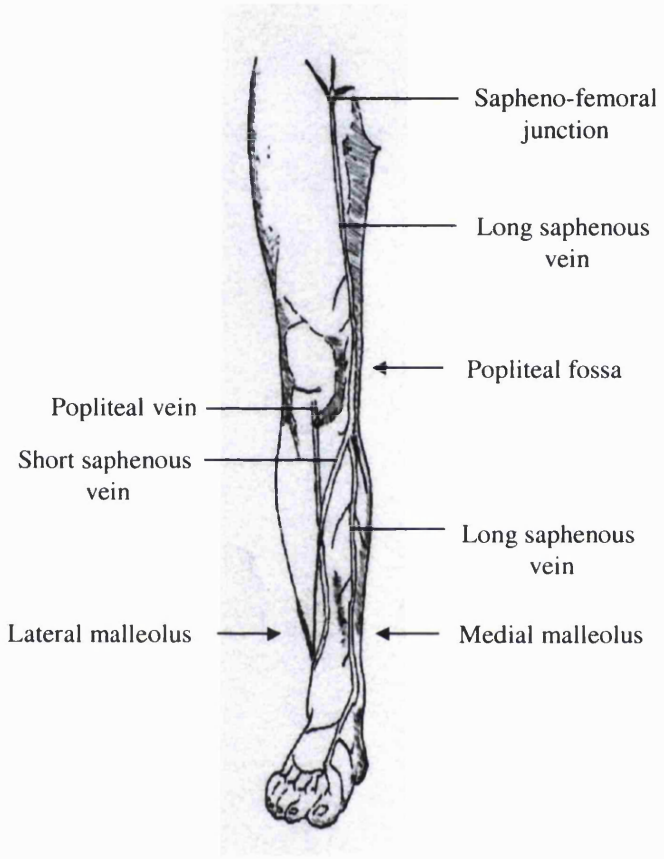


Figure 1.1. Schematic diagram of the superficial venous system of the leg.
The superficial venous system in the leg comprises of long and short saphenous veins. The long saphenous vein emanates from the sapheno-femoral junction at the groin and descends to the medial malleolus of the distal limb. The short saphenous vein starts at the popliteal fossa on the dorsal aspect of the knee and descends to the lateral aspect of the malleolus.

Venous hypertension in the distal limb on exercise is believed to lead to raised intraluminal pressure in the delicate capillary bed of the skin. The raised pressure in the microvasculature is thought to cause chronic leakage of plasma proteins and extravasation of red blood cells into the surrounding interstitium (Leach and Browse, 1986). These consequences of venous hypertension lead to LDS skin changes, characterised by a thickened and highly pigmented region of skin around the medial malleolus (Figure 1.2) and often progresses to chronic venous ulcer formation (Figure 1.3), generally initiated by minor trauma (Falanga, 1993). Although the pathophysiological changes that accompany macrovasculature dysfunction are well documented (Browse, 1988), the cellular and molecular mechanisms leading to LDS and subsequent ulceration of the leg are not known.

Despite the high incidence and the important socio-economic aspects of chronic venous insufficiency, there have been few studies examining the association between the underlying pathology with subsequent skin changes and progression to leg ulcer formation. Falanga (1993) confirmed that LDS was frequently observed around venous ulcers, with skin thickening of the distal leg in 21 out of 22 patients examined with active venous ulcers. In addition, 94% of patients with clinical evidence of deep vein thrombosis and/or varicose veins have LDS. However, 36% of patients with venous ulcers caused by venous insufficiency due to conditions such as rheumatoid arthritis showed LDS skin changes (Gross et al., 1993).

The treatment of chronic venous leg ulceration has not changed significantly for decades. The conventional treatment for venous ulceration is leg elevation in combination with compression bandaging, which decreases local oedema and prevents venous hypertension. Elevation of the distal leg is effective but often impractical and difficult to enforce on patients unless in the hospital setting. However, the use of graded compression stockings has proved to be significant in the healing of venous ulcers (Kirsner et al., 1993). Surgery to correct the impaired venous system has been successful in the treatment of LDS and venous ulcers, when the cause is purely due to superficial vein incompetence. The procedure involves the ligation of the sapheno-femoral junction at the groin followed by stripping of the long saphenous vein

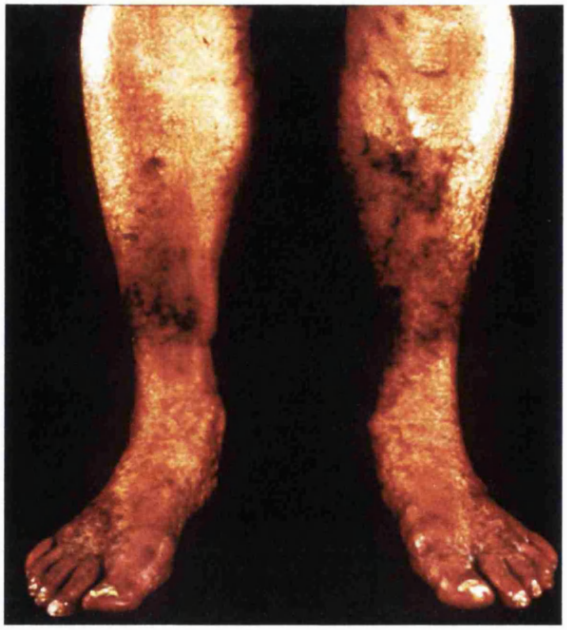


Figure 1.2. Lipodermatosclerosis
Lipodermatosclerosis is characterised by thickened and hyperpigmented skin of the distal limb.

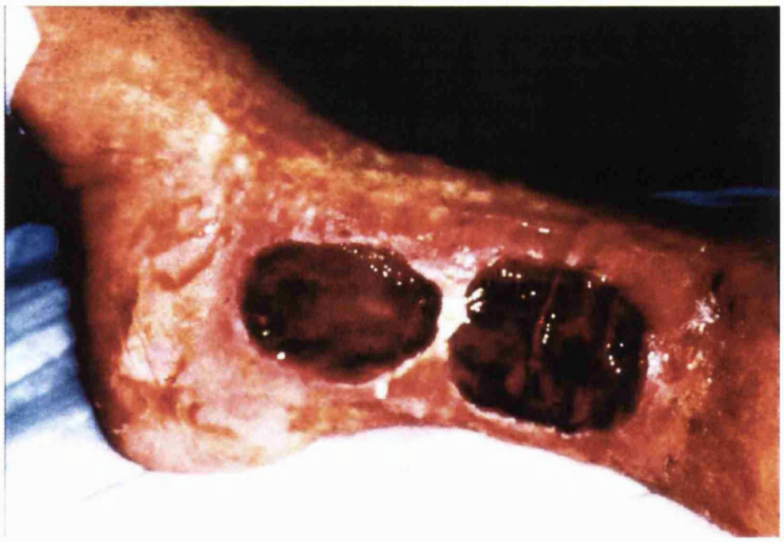


Figure 1.3. Chronic venous ulceration
Chronic venous ulceration usually occurs at the medial malleolus of the distal limb. The ulcer is surrounded by thickened and pigmented lipodermatosclerotic skin.

(Coleridge-Smith, 1996). However, surgery is expensive and painful and is further limited by surgeon availability.

1.4 Classification of venous disease

Porter and Moneta (1995) originally suggested that limbs with chronic disease should be classified according to a number of characteristic features such as clinical appearance, cause, anatomic distribution and pathophysiological condition. They subsequently published a classification table (Table 1.1) developed by an international consensus conference on chronic venous disease held under the auspices of the American Venous Forum (Porter and Moneta, 1995). This method of classifying chronic venous disease was designed to provide the additional details necessary to accurately compare limbs in medical and surgical treatment trials.

Class 0	No visible or palpable signs of venous disease
Class 1	Telangiectases, reticular veins, malleolar flare
Class 2	Varicose veins
Class 3	Varicose veins and oedema without skin changes
Class 4	Skin changes ascribed to venous disease – LIPODERMATOSCLEROSIS (pigmentation, induration, atrophie blanche, oedema)
Class 5	Skin changes as defined above with healed ulceration
Class 6	Skin changes as defined above with active ulceration

Table 1.1 Clinical classification of chronic lower extremity venous disease (modified from Porter and Moneta, 1995).

They suggested that there was a progression of changes in the clinical appearance of the disease from class 1 (no sign of venous disease) to class 6 (active ulceration). Patients initially develop swollen blood vessels (telangiectases) in the skin, often with oedema around the calf and lower leg. Patients with more severe venous insufficiency may develop superficial varicose veins, due to irreversible damage and scarring of the

valves, possibly caused by deep vein thromboses (Class 2 and 3). If patients with LDS are not treated with compression bandaging, the skin begins to break down and ulcer formation occurs (class 6). Even after ulcer healing (class 5), recurrence is an extremely common problem (Callam et al., 1985).

Patients in each class are further divided by etiology (congenital, primary or secondary), anatomic distribution (superficial, deep or perforator, alone or in combination) and pathophysiological dysfunction (reflux or obstruction, alone or in combination). This CEAP system enables uniform disease diagnosis and presentation of results of treatment in identified subsets of patients with impaired venous function.

1.5 Clinical characterisation of LDS

Clinically, LDS skin feels hard and is sharply demarcated from the proximal skin. Skin thickening around the ankle causes oedema in the upper calf resulting in the characteristic LDS appearance, termed 'champagne bottle leg' (Browse, 1983). Occasionally, LDS skin is complicated by atrophie blanche, which manifests as calcification and hyalination of the dermal blood vessels, resulting in scarring (Browse, 1983).

It has been suggested that the degree of skin thickening in patients with LDS is directly related to venous ulcer formation (Kirsner et al., 1993). Furthermore, Nemeth and others (1989) reported that the degree of skin thickening surrounding an ulcer correlated with delayed healing. Therefore, the cellular and molecular mechanisms that cause LDS are likely to contribute to the progressive development of an ulcer, as well as impairing the healing process. There is clearly an association between the two conditions although LDS represents a fibrotic accumulation of connective tissue, whereas ulceration manifests as tissue breakdown. The 'trigger' that initiates the conversion from one disease process to a paradoxical process remains unclear.

1.6 Histological characterisation

The proposed histological characteristics of LDS are confused, due to reluctance to biopsy non-ulcerated areas of skin leading to problems in classifying the clinical

progression of the disease. Kirsner and colleagues (1993) reported that 50% of biopsy sites in patients with LDS did not heal and chronic ulcers developed if left untreated, a concern also expressed previously by Burnand and colleagues (1976). Furthermore, there are no suitable animal models that truly demonstrate the skin changes associated with chronic venous insufficiency.

Lipodermatosclerosis was first described as 'hypodermitis sclerodermaformis' by Hurietz and colleagues in 1955. Since then, LDS has also been known as 'sclerosing panniculitis' and 'liposclerosis' in view of the scleroderma-like hardening of the skin on the lower legs (Jorizzo et al., 1991; Browse et al., 1977). Kirsner and colleagues popularised the term 'lipodermatosclerosis' in 1993, however, this term does not denote the venous aetiology of the disease (Fisher, 2000).

Although a comprehensive histological evaluation of LDS skin is lacking, it is agreed that the predominant change in the skin occurs in the subcutaneous fat layer (Browse and Burnand., 1982; Alegre et al., 1988; Jorizzo et al.,1991). Jorizzo and colleagues (1991) suggested that there were early, intermediate and advanced changes in LDS-affected skin. The early lesions showed a lymphocytic infiltrate in the subcutaneous septa with areas of ischaemic necrosis. In the intermediate group, the septa were markedly thickened by fibroplasia with mixed inflammatory cell infiltrate. In addition, zones of sclerosis were found within the fat layer, characterised by linearly arranged collagen bundles. The most advanced cases showed pronounced sclerosis within the fat with diminished or no inflammatory cell infiltrate. Kirsner and colleagues (1993) also noted changes in the fat layer and described LDS as having two stages, acute and chronic skin changes. The acute phase was similar to the early and intermediate skin changes of Jorizzo's group, whereas, the chronic stage consisted of fibrotic skin changes, characterised by excessive accumulation of extracellular matrix in the deep dermal and subcutaneous layers of the skin. However, these proposed fibrotic skin changes have not been well characterised, and better histopathological examination may provide novel insight into cellular and molecular mechanisms that perpetuate the development of LDS.

1.7 Theories relating to the development of venous ulceration

The cellular and molecular mechanisms leading to the development of LDS are not clear. Several theories have been proposed to explain the development of venous ulcers however, these do not address the issue of pre-ulcer LDS skin changes that occur initially. The main theories are discussed below and their role in LDS progression will be summarised.

The 'fibrin cuff' theory

Browse and Burnand (1982) first reported the presence of pericapillary fibrin cuffs in tissue sections of LDS and this has since been confirmed (Claudy et al.,1991). Burnand and co-workers (1982) related pericapillary fibrin deposition to the degree of venous hypertension. Jarrett and others (1976) first reported that vein walls and peripheral blood of patients with LDS and venous ulcers showed reduced fibrinolytic activity. Reduced levels of fibrin degradation products suggest that fibrinolysis is significantly impaired in patients with this condition (Falanga et al., 1991; Margolis et al., 1996; Rogers et al., 1999). Fibrin injected subcutaneously into the upper arm of patients with LDS was found to be degraded slower than in patients with no evidence of venous insufficiency (Leach and Browse, 1986). Moreover, biopsy specimens of LDS skin have decreased tissue fibrinolytic activity suggesting that fibrin deposition persists in the skin of patients with LDS (Peschen et al., 2000). Based on their preliminary evidence, Browse and Burnand (1982) proposed the fibrin cuff theory to account for events leading to venous ulcer formation. They suggested that fibrinogen leaks from damaged capillaries and polymerises to form fibrin. This pericapillary fibrin cuff forms a barrier, impeding the exchange of oxygen and other nutrients causing tissue hypoxia which ultimately leads to ulceration. However, no explanation for the role of pericapillary fibrin cuffs in LDS pathogenesis was proposed in these studies.

The 'white cell trapping' theory

In 1987, Moyses and colleagues reported a reduction in the number of leukocytes leaving the venous system of the distal leg of healthy individuals, during a period of experimental venous hypertension. Subsequently, Thomas and Dormandy (1988) showed a significant decline in the number of leukocytes in the circulation leaving the

legs of patients with chronic venous insufficiency and concluded that leucocytes were sequestered in the lower limb. Furthermore, the number of visible capillaries was reduced following episodes of venous hypertension, suggesting that blood flow in capillaries was impaired (Coleridge-Smith et al., 1988). This evidence led Coleridge-Smith and colleagues to propose the "white cell trapping" theory. They suggested that raised venous pressure resulted in decreased blood flow, and hence increased leucocyte margination, activation and adhesion. The group further proposed that enhanced leukocyte-endothelial interactions may mediate the release of inflammatory growth factors and cytokines, as well as proteases and toxic free radicals, and may perpetuate the formation of venous ulcers. Additional studies showed an increased inflammatory cell infiltrate in skin from patients with LDS (Thomas and Dormandy, 1988; Scott et al., 1991; Saharay et al., 1997) as well as upregulation of cell adhesion molecules ICAM-1, V-CAM-1 and E-selectin (Weyl et al., 1996). Peschen et al., (1999) confirmed these observations and further demonstrated that levels of cell adhesion molecules and their ligands were elevated on endothelial cells and infiltrating leukocytes in LDS skin. However, these studies provided no evidence to support a role for 'white cell trapping' in the development of LDS but merely a plausible explanation for the formation of chronic ulcers.

The 'growth factor trapping' theory

Falanga and Eaglstein (1993) proposed an alternative mechanism for the development of chronic venous ulcers, known as the 'growth factor trap' hypothesis. It proposes that the pericapillary cuff composed of fibrin, fibronectin and collagen, traps growth factors, so that they are unavailable to the repair process, leading to ulceration and delayed healing. Higley et al. (1995) compared the distribution of fibrin cuffs, procollagen and transforming growth factor β-1 and 2 (TGF-β1 and TGF-β2) within venous ulcers and normal healing wounds by immunohistochemistry. Positive TGF-β1 staining was demonstrated in perivascular fibrin cuffs but TGF-β1 and 2 was absent from the provisional matrix of the ulcer base compared with normal wounds. They concluded that TGF-β1 was abnormally trapped in the perivascular fibrin cuff preventing granulation tissue formation. However, Pappas and colleagues (1999)

demonstrated increased levels of TGF-β1 in LDS skin compared with control skin with positive staining of both fibroblasts and macrophages. The group concluded that macrophages secrete high levels of TGF-β1 which stimulates the up-regulation of matrix protein gene expression by dermal fibroblasts, and suggested that this mechanism may play a role in the development of fibrosis in LDS.

The theories which have been described are primarily related to venous ulcer formation and do not address the mechanisms leading to initial LDS development, in particular, the fibrotic skin changes that are commonly observed and thought to be directly related to ulcer formation. Furthermore, much of the evidence for the theories comes from examining LDS skin removed from around active venous ulcers rather than LDS skin from pre-ulcerated skin, which may be more informative.

1.8 Summary

A key feature of LDS is fibrosis of the dermal and subcutaneous layers of the skin on the distal leg. There is no clear molecular or cellular cause of the pathogenic skin changes observed. Several theories have been proposed to explain the development of skin changes resulting in ulceration due to chronic venous insufficiency, however, they do not address mechanisms that mediate the fibrotic changes in the dermal and subcutaneous layers of the skin.

Excessive fibrin deposition in the dermis is a prominent characteristic of LDS skin, and is associated with locally and systemically reduced fibrinolysis in these patients. The deposition of fibrin in the interstitium is an integral part of the wound repair process that occurs following local tissue injury. Fibrin is gradually remodelled and replaced by collagenous matrix. Indeed, collagen is deposited in intimate association with pre-existing fibrin (Dvorak, 1986). However, fibrin accumulation and its persistence is also associated with many disease processes, such as fibrotic and tumorigenic disorders (Dvorak, 1986; Brown et al., 1989). Disruption of the fibrinolytic pathways may have severe consequences, particularly with regard to collagen accumulation and neovascularisation, which ultimately results in impaired tissue structure and function. Therefore, the following section will describe fibrin matrix assembly, degradation and

remodelling during tissue repair. The potential pathophysiological roles that chronic and persistent fibrin deposition may play in LDS development will be discussed.

Section 2: Fibrin remodelling and its role in tissue repair

1.9 Overview of tissue repair

Tissue damage following injury such as trauma, inflammation and radiation, results in haemorrhage or leakage of blood from damaged blood vessels. Extravasated blood or plasma clots rapidly via activation of the coagulation cascade, the end result being a fibrin matrix that prevents further blood loss and serves as a provisional stroma into which initially, inflammatory cells migrate, phagocytose debris and locally degrade the matrix. Subsequently, endothelial cells and fibroblasts penetrate the provisional matrix, proliferate and through the synthesis and deposition of extracellular matrix components (ECM) such as collagen, fibronectin and proteoglycans, remodel the fibrinous scaffold into a fibrous matrix. At this stage, tissue is cellular, oedematous and highly vascular, and known as 'granulation tissue'. These events are orchestrated by growth factors and cytokines released predominantly by platelets and inflammatory cells. Over time, the new tissue matures with ECM remodeling and a decrease in cellularity and vascularisation. The end result of the cutaneous wound healing process is an avascular and acellular scar that is composed mainly of dense parallel-arranged collagen bundles.

1.10 Fibrinogen synthesis and structure

Fibrinogen, the 340kDa precursor of fibrin, is primarily synthesised by hepatocytes and is present in the blood at a concentration of 9μM (for review see Herrick et al., 1999). It is comprised of two symmetric half molecules, each consisting of one set of three different polypeptide chains termed Aα, Bβ and γ (Mossesson, 1990, Figure 1.4). The molecule is highly heterogeneous due to alternative splicing, extensive post-translational modification and proteolytic degradation. Furthermore, there is considerable polymorphic variation in the Aα and Bβ chains, resulting in an individual possessing over one million non-identical forms of fibrinogen in their blood (Henschen-Edman, 1995).

During episodes of inflammation, the synthesis of fibrinogen is dramatically enhanced, an effect thought to be mediated by interleukin-6 (IL-6), glucocorticoids and oncostatin M (Guadiz et al., 1997). IL-6 and glucocorticoid response elements have been identified in all three fibrinogen chain genes, whereas the oncostatin M response elements have not been mapped.

Each of the three polypeptide chains of the fibrinogen molecule is encoded by a separate gene located on chromosome 4. The predominant Aα chain of circulating fibrinogen contains approximately 610 amino acid residues (70kDa), the Bβ chain consists of 461 amino acids (56kDa) and the most common form of the γ chain, designated γ_A consists of 411 residues (48kDa). The fibrinogen molecule has three distinct domains consisting of two terminal 'D' domains (67kDa), each linked to a central 'E' domain (33kDa) by a triple-stranded array of the polypeptide chains, conformationally existing as α helical coils. The three chains and the two halves of the fibrinogen molecule are held together by a series of 29 disulphide bonds, with all 58 cysteine residues participating in those interactions (Mossesson, 1997).

1.11 Fibrin matrix assembly

The formation of a provisional fibrin matrix initially involves the activation of the coagulation cascade following plasma or blood product extravasation. This generates active thrombin, which converts fibrinogen into fibrin monomers. These spontaneously aggregate and combine with other ECM molecules to assemble an insoluble matrix.

1.12.1 The coagulation cascade pathways

The conversion of circulating zymogens of the coagulation cascade into active serine proteases involves three overlapping pathways, the extrinsic, intrinsic and common pathways (Figure 1.5).

The extrinsic pathway

The extrinsic pathway is initiated by tissue factor on the surface of activated platelets and cells that constitutively express the protease (fibroblasts, smooth muscle cells, mesothelial cells and endothelial cells) (O'Brien, 1989). The interaction between tissue factor and factor VII in the presence of calcium ions, facilitates the conversion of inactive factor VII to the active serine protease factor VIIa. Factor VIIa/tissue factor complex converts factor X to its active conformation, factor Xa.

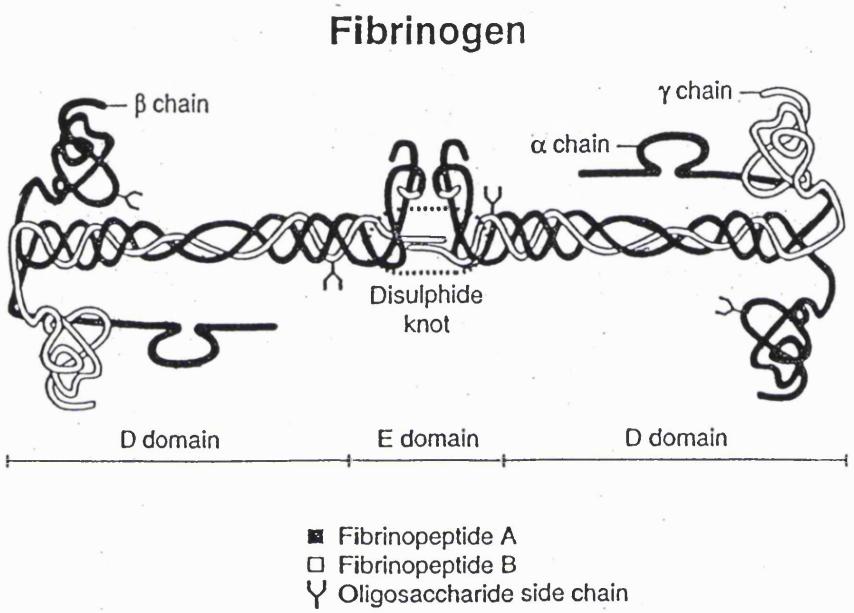


Figure 1.4. Schematic model of the fibrinogen molecule.
Fibrinogen consists of three pairs of polypeptide chains, Aα, Bβ and γ, joined by disulphide bonds to form a symmetric dimeric structure. The NH_2 terminal regions of all six chains from the central domain (E domain) of the molecule containing fibrinopeptide A and B sequences that are cleaved by thrombin during enzymatic conversion to fibrin (adapted from Mosseson, 1990).

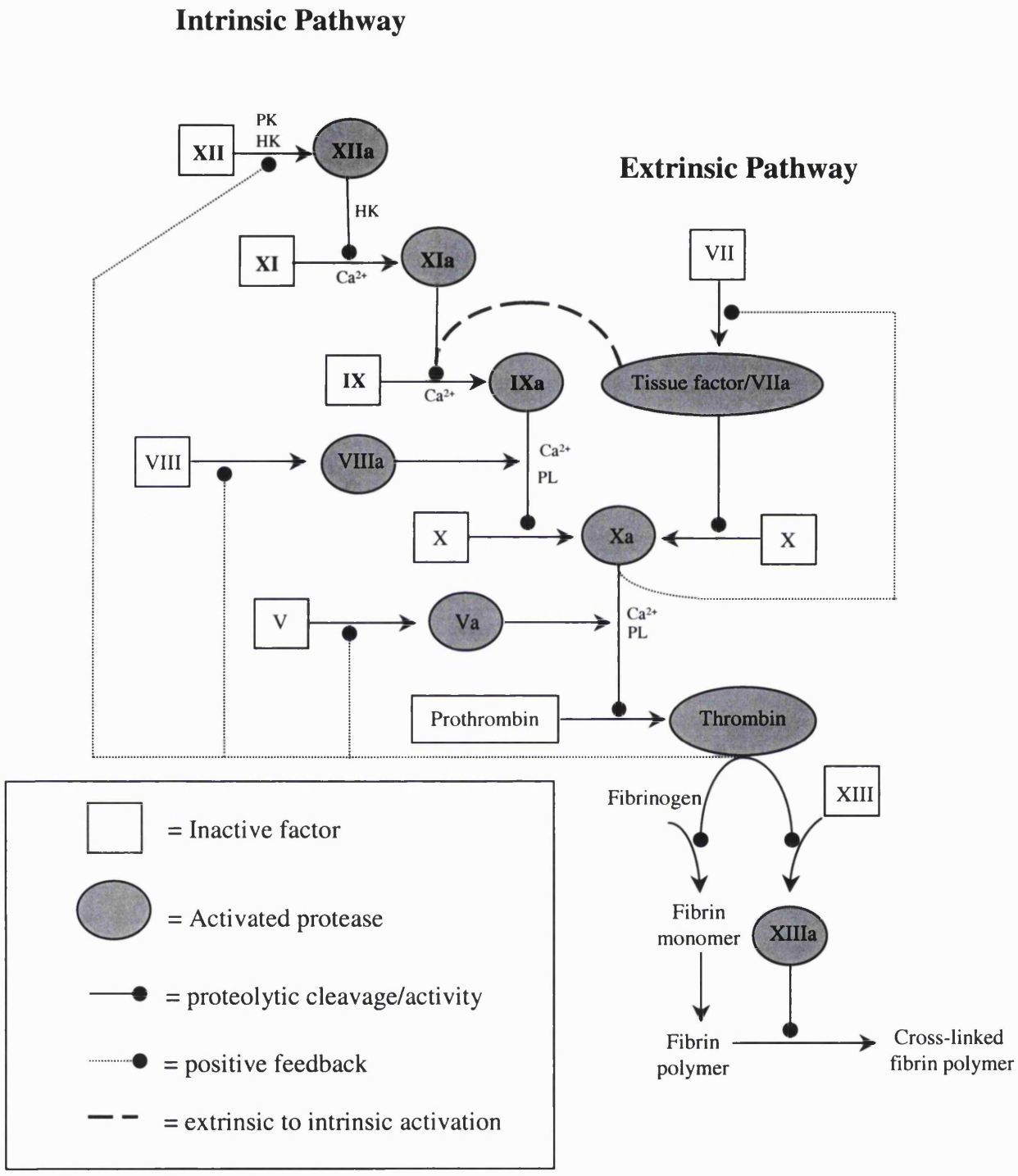


Figure 1.5. The coagulation cascade.
The intrinsic and extrinsic pathways are indicated. The events depicted below factor Xa are designated the 'common pathway', culminating in the formation of cross-linked fibrin. (PK=prekallikrein, HK=HMW kininogen, PL=phospholipids)

The intrinsic pathway

The intrinsic pathway is an alternative process of blood coagulation that occurs when blood comes into contact with artificial or extravascular negatively charged substances such as collagen following damage to blood vessels. The intrinsic cascade can also be triggered by a range of cell types with varying efficiency, including monocytes and macrophages (McGee et al., 1992). The intrinsic pathway comprises of the sequential activation of factor XII, XI and IX with the initial activation step mediated by the auto-activation of factor XII on negatively charged surfaces. Factor XIIa activates both factor XI into factor XIa and prekallikrein into kallikrein on the surface of endothelial cells. Factor XIa activates factor IX into factor IXa. The activated factor IXa associates with the cofactor factor VIIIa in the presence of phospholipids and calcium ions. The proteolytic activity of the complex is enhanced and VIIIa cleaves factor X into factor Xa.

The common pathway

Both the intrinsic and extrinsic pathways lead to the generation of factor Xa. Factor Xa binds effector cell protease receptor-1 (EPR-1) on the surface of platelets and other cell types such as leucocytes, endothelial cells, fibroblasts and smooth muscle cells with high affinity (Blanc-Brude et al., 2001). EPR-1 has been described as a constitutively expressed form of factor Va, and its role is to mediate the procoagulant effects of factor Xa by acting either as a cofactor for the protease, or by providing high affinity binding sites on the cell surface (Bono et al., 1997). The enzyme protein C may act to degrade the coagulation factors Va and VIIIa at this point, thereby inhibiting the coagulation pathway. Protein S serves as a cofactor for activated Protein C in the degradation of these factors.

Factor Xa requires both phospholipid and calcium ions to form a complex with factor Va, known as 'prothrombinase'. Prothrombinase subsequently converts the proenzyme prothrombin to the active serine protease, thrombin (Mann et al., 1990). Thrombin induces its procoagulant functions by activating platelets, inducing their aggregation and degranulation, and by cleaving the blood macromolecule, fibrinogen, to form fibrin monomers.

1.12.2 Fibrinogen cleavage and fibrin formation

Fibrinogen is converted to fibrin at sites of tissue injury by thrombin, resulting in the cleavage of an arginine-glycine peptide bond at the Aα16-17 or Bβ14-15 loci, with the release of fibrinopeptides A and B from the amino-terminal ends of the Aα and Bβ chains respectively. Cleavage of fibrinopeptides A and B exposes binding sites in the amino-terminal regions of the Aα and Bβ chains, promoting the process of self-assembly and polymerisation of the fibrin monomers (Mossesson, 1997). Binding between thrombin and fibrinogen at the cleavage site does not result in the inactivation of the protease and the enzyme retains its coagulant activity (Goldsack et al., 1998). Spontaneous intermolecular cross-linking between monomers occurs to form a stable fibrin clot (Figure 1.6). Polymerisation sites in the central region of the monomer align with complementary sites on alternative fibrin monomers allowing lateral association, with the monomers aligning in to staggered, double-stranded fibrils. An alternative type of fibrin branching structure, termed the 'trimolecular' branch point, forms by the association between three double-stranded fibrin polymers at complementary E domains (Figure 1.7) (reviewed by Mossesson, 1997).

1.12.3 Fibrin matrix cross-linking

The fibrin matrix is normally maintained by non-covalent interactions between monomers but when subjected to forces of stress and strain, it undergoes deformation, sometimes irreversible. Alternatively, in the presence of factor XIIIa, a calcium-dependent transglutaminase that is activated by thrombin, fibrin molecules undergo interchain cross-linking by formation of covalent isopeptide bonds (Credo et al., 1978). Intermolecular cross-linking of γ domains results in bridges between lysine and glutamine residues of adjacent fibrils (Mc Donagh et al., 1971) and intermolecular bonding between α chain residues creates oligomers and large α-chain polymers. Plasma proteins such as fibronectin, vitronectin and glycosaminoglycans become incorporated into the fibrin matrix by factor XIII cross-linking, giving the fibrin clot a high degree of elasticity and strength (Greiling and Clark, 1997). Other substrates of Factor XIII include α_2-antiplasmin, α_2-macroglobulin, von Willebrand factor and factor V of the coagulation system (Paye et al., 1989). Entrapped platelets release their chemoattractant and

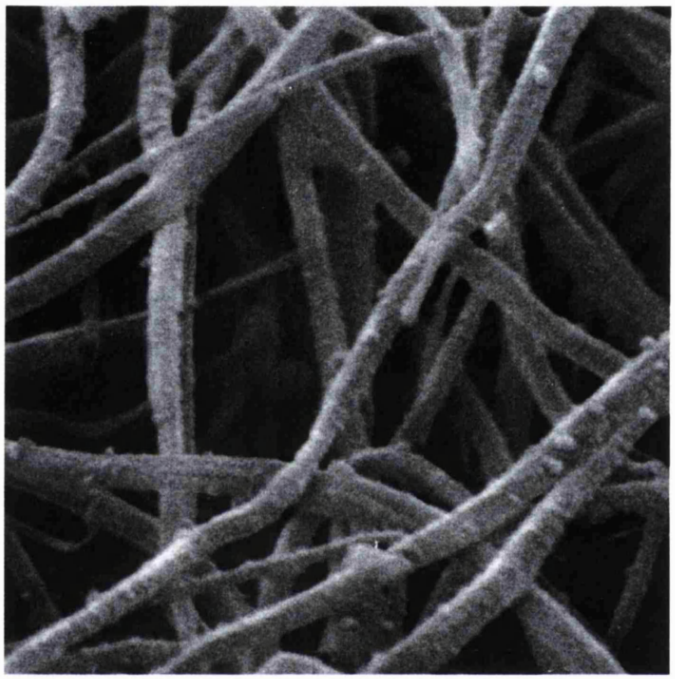


Figure 1.6. Ultrastructure of a fibrin matrix.
Scanning electron micrograph (x10,000 mag) of a fibrin matrix demonstrating random lattice structure and fibril organisation.

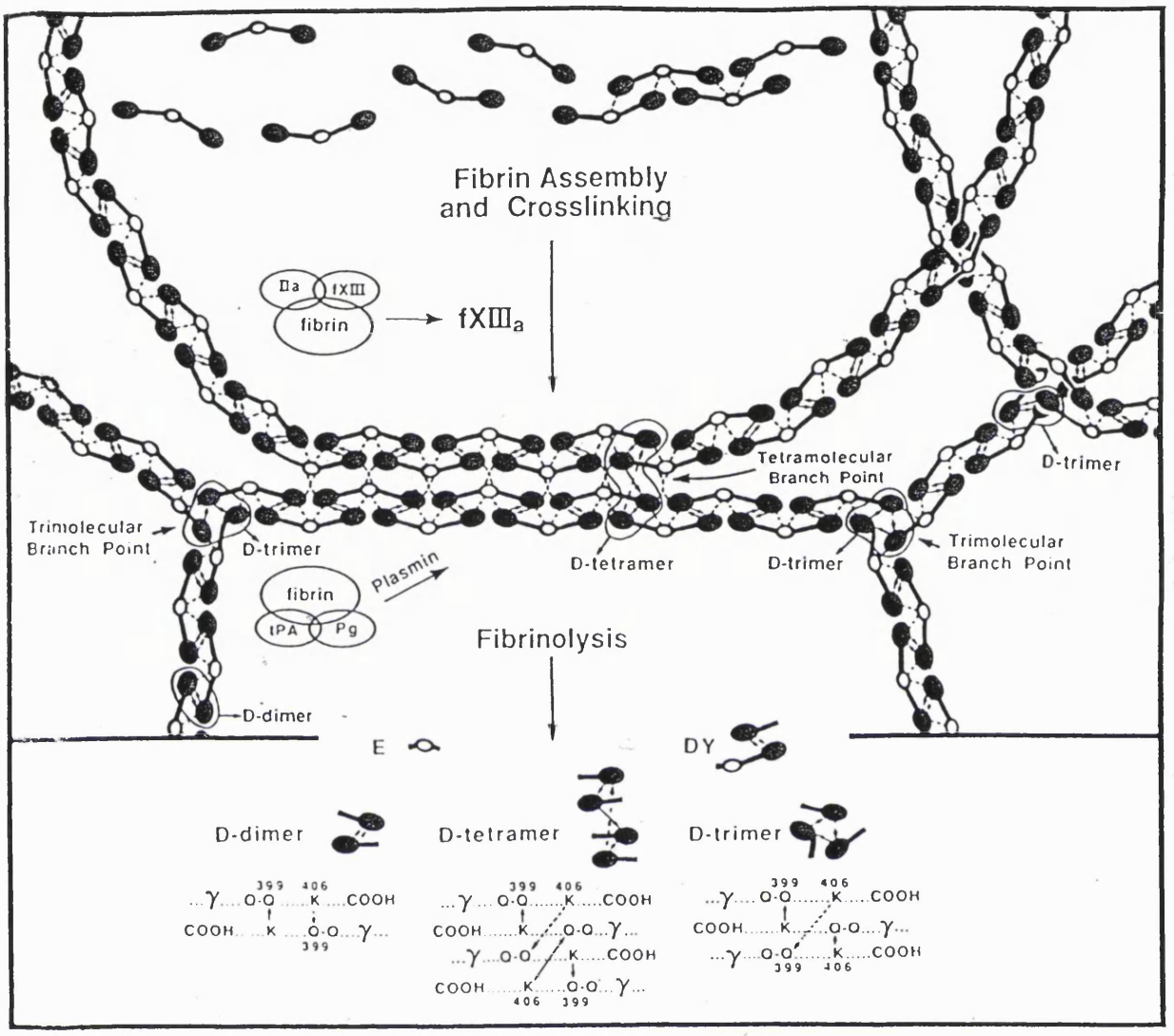


Figure 1.7. Schematic model of fibrin assembly, cross-linking and fibrinolysis
After enzymatic conversion of fibrinogen to fibrin, fibrin monomers assemble in a staggered overlapping manner by noncovalent interactions between D and E domains. These fibrils undergo spontaneous noncovalent lateral associations to form thicker fibres. When cross-linked fibrin undergoes fibrinolysis, the peptides joining the D and E domains are cleaved, leading to the generation of fragment E, and fragment D-containing products (D-dimers, D-trimers and D-tetramers) (taken from Mossesson, 1990).

mitogenic growth factors, which become incorporated in the fibrin matrix (Gray et al., 1990). The presence of growth factors and cytokines initiates the migration of inflammatory cells and fibroblasts into the fibrin clot, promoting attachment and proliferation, and results in the gradual remodelling of the fibrin matrix into granulation tissue (Dvorak, 1986). Furthermore, proteases or their zymogens, including elastase, tryptase, MMPs, cathepsins, plasminogen, their activators and their inhibitors also become incorporated in the clot, promoting fibrin matrix remodelling (Paye et al., 1989).

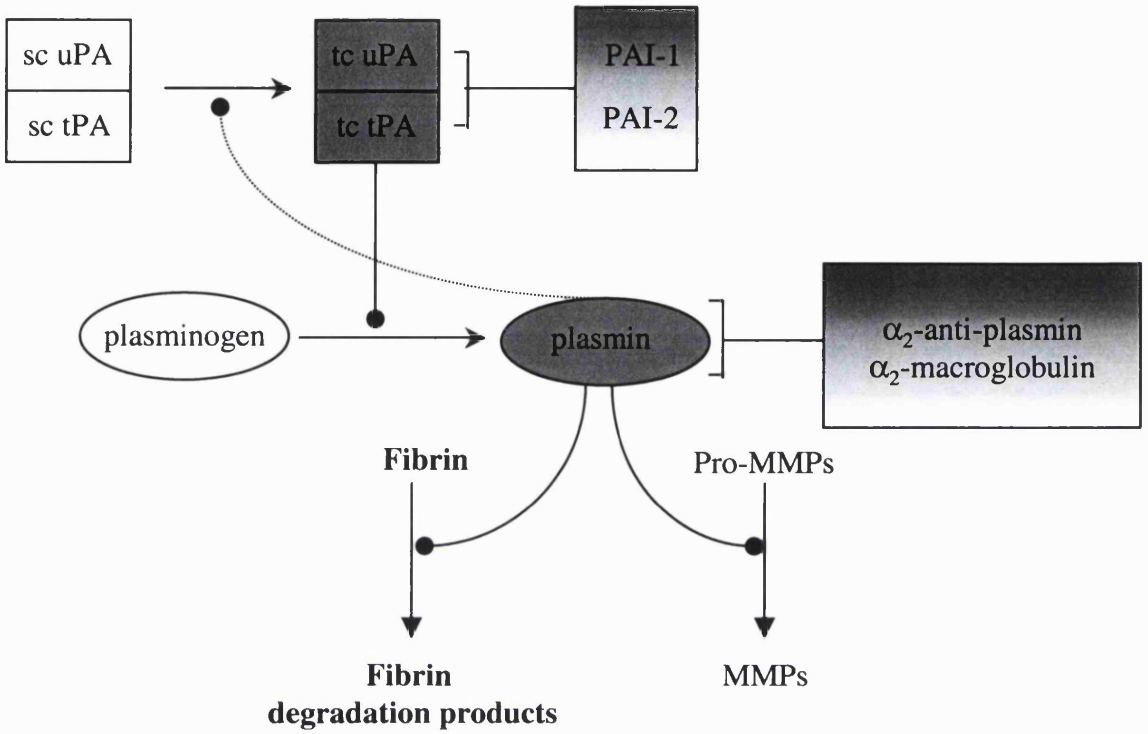
Manipulation of the extracellular matrix through covalent cross-linking of matrix molecules, such as fibrin and fibronectin, by factor XIII, results in a significant modulation of biosynthetic activity of cells, particularly fibroblasts (Paye et al, 1989). Kasai and colleagues (1983) reported that fibroblasts rapidly attached and spread on fibrin cross-linked by FXIIIa and proliferation was stimulated. Paye and colleagues (1989) observed increased biosynthetic activity of collagen in cross-linked fibrin gels compared with fibrin gels in the absence of FXIIIa. Moreover, the extractability of the collagen synthesised in the fibrin gels was significantly reduced in the presence of factor XIIIa, suggesting that the collagen was cross-linked to itself and other components of the fibrin matrix by the transglutaminase activity of FXIII.

1.13 Fibrinolysis

The fibrinolytic system represents a highly regulated enzymatic cascade for extracellular matrix proteolysis, especially in the remodelling of fibrin. Plasminogen is converted into the active serine protease, plasmin, by plasminogen activators and is the main protease involved in fibrin degradation (Figure 1.8). Plasminogen activators comprise tissue-type and urokinase-type plasminogen activator (tPA and uPA respectively) and their activity is regulated predominantly by protease inhibitors, plasminogen activator inhibitors-1 and 2 (PAI-1 and PAI-2).

1.13.1 Plasmin(ogen)

Plasminogen, a single-chain glycoprotein with a molecular weight of 92kDa, is present in the plasma at a concentration of 1.5-2μM (Lijnen and Collen,1995), and is synthesised by hepatocytes in the liver. The plasminogen molecule consists of 971 amino acid residues, coded for by a 52.5kb gene on the long arm of chromosome 6, and consists of 19 exons.



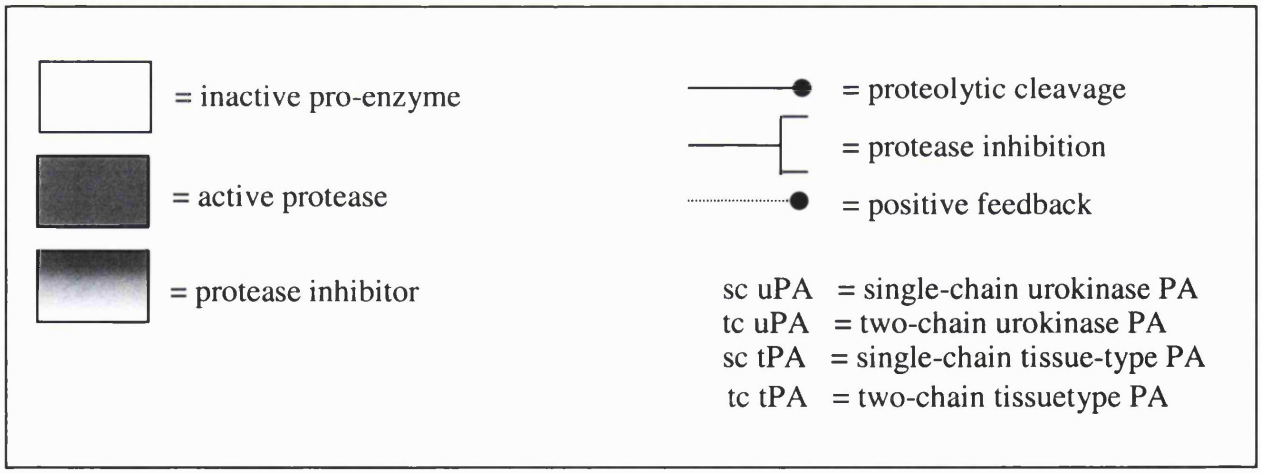


Figure 1.8. The fibrinolytic pathway.
The zymogen plasminogen is converted to the active serine proteinase plasmin, which degrades fibrin into soluble fibrin degradation products, by tissue-type (tPA) or urokinase-type (uPA) plasminogen activator. Inhibition may occur at the level of the plasminogen activator by plasminogen activator inhibitor-1 (PAI-1) or plasminogen activator-2 (PAI-2) or at the level of plasmin, mainly by α_2-antiplasmin.

Plasminogen is converted to plasmin by cleavage of the Arg561-Val562 peptide bond, by plasminogen activators. Coagulation factors XI and XII as well as serum kallikrein can also activate plasminogen, although with low efficiency. The plasmin molecule is a two-chain trypsin-like serine proteinase composed of a heavy chain containing 5 kringle domains (NH_2- terminal) and a light chain (COOH- terminal) with an active site composed of His 603, Asp 646 and Ser 741 (Sottrup-Jensen et al., 1975). Plasmin degrades fibrin into soluble degradation fragments, including X, Y, D and E (Mossesson, 1990). The peptides joining the D and E fragments are cleaved leading to generation of fragment E, fragment E with D-fragments (DY), and fragment D-containing cross-linked multimeric D domains that reflect the type of γ-chain cross-linking that has occurred (e.g., D dimer, D trimer, D tetramer) (Mossesson, 1990).

Plasmin is also capable of degrading other extracellular matrix proteins such as fibronectin and vitronectin (Andreasen et al.,1997) and plays a key role in the activation of matrix metalloproteinases (MMPs), essential for remodelling the extracellular matrix. Plasmin has a specific affinity for proMMP-1 (Rao et al., 1999, Brassart 2001), proMMP-3 and proMMP-9 (Hahn-Dantona et al., 1999). In addition, plasmin has been shown to have affinity for cleavage sequences of latent growth factors, including transforming growth factor-β1 (Munger et al.,1997, Pedrozo et al., 1999).

A 'plasminogen receptor' has been identified on all peripheral blood cells including fibroblasts, neutrophils, monocytes, B and T lymphocytes, epithelial cells, endothelial cells and platelets (Miles and Plow, 1987). Endothelial cells are a particularly rich source of plasminogen receptors (Hajjar et al., 1986). The receptor is believed to have many isoforms that recognise different plasminogen molecules, and can distinguish between plasminogen and activated plasmin. Since the predominant function of the plasminogen receptor is to regulate the activation of plasmin, expression of these receptors on cells of the endothelium and on cells incorporated in the fibrin matrix contribute significantly to fibrinolysis (Redlitz and Plow, 1995).

The most important of the several circulating plasmin inhibitors that play a role in regulating fibrinolysis is α_2-antiplasmin, a rapid and effective inhibitor of plasmin that is also able to inhibit the enzymatic activity of tPA. In the presence of factor XIIIa, α_2-

antiplasmin becomes cross-linked to the Aα chain of fibrinogen or fibrin (Mossesson, 1990). The close association between α_2-antiplasmin and fibrin is important for modulating the initial phases of fibrinolysis (Aoki, 1979). Circulating α_2-antiplasmin is present at a concentration of 1μM, about half the concentration of plasminogen. The half-life of free plasmin is estimated to be 0.1 seconds due to the effectiveness and availability of α_2-antiplasmin. The physiological importance of this reaction for normal haemostatic function is emphasised by the fact that congenital α_2-antiplasmin deficiency results in a severe haemorrhagic disorder characterised by reduced resistance of intravascular thrombi to fibrinolysis (Mossesson, 1990).

A disruption in the balance between profibrinolysis and fibrinolysis inhibition may result in haemorrhage or thrombosis respectively. Under normal physiological conditions, the fibrinolytic system is controlled by specific interactions with various inhibitors, and by the regulation of synthesis and release of plasminogen activators.

1.13.2 Tissue–type plasminogen activator (tPA)

The main role of tissue-type plasminogen activator, or tPA, is in the dissolution of fibrin via the activation of plasmin from plasminogen. The gene for tPA is located on chromosome 8 and consists of 14 exons. Tissue-type PA consists of 527 amino acid residues with a molecular mass of 59kDa, and is produced as a single chain predominantly by vascular endothelial cells, although it can be synthesised by fibroblasts, mesothelial cells and macrophages (Rijken, 1995). The molecule possesses three glycosylation sites and the attached carbohydrates increase the molecular mass of the molecule to 70kDa. The single-chained molecule circulates at a concentration of approximately 100pM and physical characterisation of tPA has shown that the molecule is relatively compact, with the individual kringle domains folded into the molecule, so that the overall appearance is globular (Margossian et al., 1993). The half-life of tPA is approximately 5 minutes due to rapid hepatic clearance and inactivation by its specific inhibitors (Rijken, 1995).

During fibrin clot lysis, the native single-chain tPA is converted to two-chain tPA by plasmin (Rijken, 1995). Tissue-type PA is an atypical serine protease because the single chain form is an active zymogen and in the absence of fibrin is three to six times less

active than two chain tPA (Wallen et al., 1982). Therefore, plasmin generated by single-chain tPA acts in a positive feedback loop, further activating single-chain tPA into two-chain tPA, thereby increasing the yield of plasmin at the fibrin surface (Andreasen et al.,1991). Binding of tPA to fibrin stimulates plasminogen cleavage which is dependant on three separate stages, the release of fibrinopeptide A from the fibrinogen Aα chain by thrombin, the rate of fibrin polymerisation, and the initial rate of plasmin degradation. These events contribute to the binding of tPA and plasmin to the fibrin surface in a sequential and ordered manner, yielding a 'ternary complex'. This ternary complex regulates plasminogen cleavage by tPA and promotes efficient and localised plasminogen activation at the fibrin surface (Lijnen and Collen, 1995). Two domains of tPA are involved in the interaction with fibrin (Rijken, 1995). The first is kringle 2 domain, which harbours a lysine binding site. The second domain involved in fibrin binding is the finger of tPA, which is not affected by lysine or lysine analogues. Cooperation of the two binding sites, in a manner which depends on the structure of the remaining fibrin matrix, results in an optimal interaction between fibrin and plasminogen (Horrevoets et al., 1994).

Localisation of tPA on the cell surface occurs via a 40kDa membrane protein, annexin II (Hajjar et al.,1994). The receptor also binds plasminogen but with a considerably lower affinity. It is present on vascular endothelial cells, fibroblasts, monocytes and smooth muscle cells (Redlitz and Plow, 1995). Receptors that localise tPA to the cell surface promote pericellular plasminogen activation and therefore have profibrinolytic consequences.

The main roles of tPA have been demonstrated by examining knock-out mice deficient in the protease. These mice lysed pulmonary plasma clots at a significantly reduced rate compared to wild-type controls, and injection of endotoxin in the footpad caused venous thrombosis in 55% of tPA-deficient mice compared to 15% of wild-type controls, demonstrating the increased thrombotic phenotype of the tPA -/- mouse (Carmeliet et al., 1994).

Brakman and colleagues (1966) described the presence of a tPA inhibitor in a group of patients with an impaired fibrinolytic system. This inhibitor was later named plasminogen

activator inhibitor-1 (PAI-1), and is a member of the serpin family of inhibitors. PAI-1 reacts very rapidly with single-chain and two-chain tPA, and with two-chain uPA. The rapid inhibition of both tPA and uPA by PAI-1 involves a high affinity interaction which does not depend on the direct binding of the active site on the protease (Lijnen and Collen, 1995). Subsequently, a second plasminogen activator inhibitor (PAI-2) was discovered, which had an affinity for single and two chain-tPA, but not uPA (Kawano et al., 1968).

1.13.3 Urokinase plasminogen activator (uPA)

Urokinase plasminogen activator, or uPA, is a serine protease with a molecular weight of 50kDa and acts in a fibrin-independent cell-bound manner (Lijnen and Collen, 1995). The gene for uPA is located on chromosome 10 and has a length of 6.4kb. Urokinase PA consists of a growth factor-like domain, which is homologous to the growth factor-like domain of tPA, a kringle domain, and after a long connecting peptide, a protease domain, which is 42% homologous to the catalytic domain of tPA (Andreasen et al., 1997). Native uPA is a single-chain zymogen molecule, which can be activated by cleavage of the Lys-Ile peptide bonds by plasmin, plasma kallikrien, factor XIIa, T cell-associated serine proteinase, cathepsin B, cathepsin L, nerve growth factor-γ and human mast cell tryptase (Andreasen et al., 1997). Cleavage results in the formation of the two-chain form, which has an activity at least several hundred-fold higher than that of the single-chain form (Lijnen and Collen, 1995). The concentration of uPA in the circulating blood is around 20pM, with a large proportion of the uPA complexed with PAI-1, while a smaller fraction is in the more inactive pro-uPA form (Andreasen et al., 1994). Urokinase-PA has restricted substrate specificity, with plasminogen being its main substrate. However, recent studies have revealed that uPA may also cleave hepatocyte growth factor / scatter factor and macrophage stimulating protein, which have high degree of sequence homology with plasmin, but are devoid of protease activity (Andreasen et al., 1997).

Whilst the primary role of tPA is to generate plasmin for thrombolysis, the main role of uPA is to generate plasmin in events involving pericellular extracellular matrix degradation (Andreasen et al., 1997) such as cell migration, tissue remodelling, angiogenesis, inflammation and wound healing (Ploug et al., 1991). The process of localised pericellular matrix degradation is achieved by binding of uPA to the cell

surface, via a high affinity interaction with the receptor, urokinase plasminogen activator receptor, uPAR (CD87). Urokinase-PA has been shown to be present on virtually all cell types including fibroblasts, monocytes, lymphocytes, mesothelial cells, endothelial cells, epithelial cells and in particularly high concentrations in tumour cells (Lijnen and Collen, 1995). Binding of uPA to uPAR dramatically accelerates the rate of plasminogen activation six-fold (Ellis et al., 1989), due to co-localisation of uPA and plasminogen at the cell surface. uPAR can bind pro-uPA and uPA produced by either the uPAR-expressing cells themselves or by other cells (Ossowski et al., 1991). Various authors have described uPAR-bound uPA localisation at focal adhesion sites, where integrins cluster and cytoplasmic domains interact with actin filaments, regulating cell shape and structure (Burridge et al., 1988; Clark and Brugge, 1995). It is thought that through the interactions with integrins, such as β1 and β2 subunits, the uPAR receptor may trigger cell-signalling responses that may result in alteration of cell adhesion and migration (Wei et al., 1996). Moreover, there is evidence to suggest that uPAR interacts with ligands other than uPA, such as the extracellular matrix molecule vitronectin (Andreasen et al., 1997). However, recent work has shown that uPA supplies sufficient fibrinolytic potential to clear fibrin from most tissues and supports cell migration and wound healing without the benefit of uPAR or tPA (Bugge et al., 1996; Carmeliet et al., 1998).

Mice deficient in uPA suffer from rectal prolapse and chronic non-healing ulcers, although neither the gene deficiency nor these side effects affect fertility or survival time (Carmeliet et al., 1994). Analysis of tissues isolated from uPA knock-out mice demonstrated occasional fibrin deposits in the liver and intestines, with extensive fibrin deposits in the chronic non-healing ulcerations. The reasons for these deposits remain unclear, although Carmeliet and colleagues (1994) proposed that inflammatory cells deficient in uPA are unable to degrade fibrin and result in delayed wound healing. Furthermore, these mice had significantly decreased clot lysis activity following endotoxin injection, but pulmonary plasma clot lysis remained unaffected, suggesting a normal thrombotic tendency compared with tPA knock-out animals (Carmeliet et al., 1994).

1.14 Interactions of fibrin matrix with fibroblasts

The extracellular matrix (ECM) is not a passive structure but is dynamic, and functions to instruct cellular behaviour (Streuli, 2000). ECM-cell interactions elicit a variety of cellular responses, such as adhesion, spreading, changes in the cytoskeletal organisation and differentiation, formation of focal contacts, stimulation of proliferation and migration, and induction of gene expression. ECM proteins interact directly with the cell surface receptors, known as 'integrins', to initiate signal transduction pathways and to modulate mechanisms triggered by differentiation and growth factors. Modulation of the ECM by remodelling its structure and activity during tissue repair has profound effects on its function and the behaviour of cells residing on or within it.

1.14.1 Cell adhesion and migration

Fibroblasts migrate from healthy tissue to the sites of fibrin deposition to promote granulation tissue formation and remodelling. The fibrin(ogen) molecule contains potential sites that are recognised by specific fibroblast-bound integrins including $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha 4\beta 1$ (Gailit and Clark, 1996). Human fibrin(ogen) contains three potential integrin binding sites, two RGD sequences within the $A\alpha$ chain and a non-RGD sequence in the γ chain, but can also interact with cells through non-integrin receptors. For example fibrin mediates the adhesion and transendothelial migration of leukocytes by acting as a molecular bridge between these cell types through ICAM-1-dependant pathways (Languino et al., 1995).

Knox and colleagues (1987) proposed that the rate-limiting step in the migration of fibroblasts into a fibrin clot was the level of plasminogen activators on the cell surface. These authors suggested that cells do not migrate through the fibrin lattice but rather via localised areas of clot lysis. Analysis of fibrin gels demonstrated areas of decreased fibrin density around fibroblasts as a result of local plasmin activation. Adhesion, spreading and migration of fibroblasts over the surface of a fibrin lattice did not require plasmin activity, however the migration of fibroblasts through a fibrin lattice was entirely plasmin dependent (Knox et al., 1987).

The migration of fibroblasts into the fibrin matrix requires stimulatory signals that initiate the movement from healthy tissue into the injury site. Cytokines and growth factors

released in the fibrin matrix may act as chemoattractants to stimulate fibroblast migration. Furthermore, fibrinopeptides A and B, released by thrombin cleavage of fibrinogen Aα and Bβ chains respectively, are known to be chemoattractants for fibroblasts (Gray et al., 1990). Degradation of the fibrin matrix results in the formation of D monomers, dimers, trimers and tetramers, and these peptides have also been shown to have chemoattractant activity for fibroblasts and macrophages (Senior et al., 1986, Skogen et al., 1988).

1.14.2 Proliferation

Fibrin(ogen) and its cleavage products are mitogens for a variety of cell types, including fibroblasts, endothelial cells, smooth muscle cells and lymphocytes (Sporn et al., 1995). *In vitro*, the proliferation of fibroblast and endothelial cells on fibrin is enhanced by fibrinopeptide B cleavage and exposure of the amino terminus of the fibrinogen Bβ chain (Sporn et al., 1995). This suggests that specific structural features of the provisional fibrin matrix formed at sites of injury may modulate the proliferative response of these cells. Fibrin(ogen) is mitogenic for both lymphoid cell lines and human haemopoietic progenitors, an effect mediated by two possible non-integrin binding sites, mitogenic fibrinogen receptor (MFR) and ICAM-1 (Heron et al., 1998). Studies by Tuan et al. (1996) suggested that dermal fibroblasts cultured in three-dimensional fibrin gels proliferate less rapidly than cells grown on monolayer cultures. However, Gillery et al., (1989) showed no significant proliferation by dermal fibroblasts in fibrin lattices compared with monolayer controls. In addition, cleavage products of fibrin(ogen), fibrinopeptides A and B, also regulate cell proliferation. These peptides are mitogens for fibroblasts *in vitro* and therefore may provide an early stimulus for proliferation during the initial coagulation stage of tissue repair (Gray et al., 1990).

1.14.3 Collagen synthesis

Dvorak (1986) stated that collagen was deposited in intimate association with pre-existing fibrin in tumour tissue. *In vitro,* several studies have shown that the rate of collagen synthesis increased when fibroblasts were cultured in a fibrin matrix compared with cells grown on monolayer culture or in a collagen matrix (Gillery et al., 1989; Coustry et al., 1990; Clark et al., 1995; Lorimier et al., 1996; Tuan et al., 1996).

Furthermore, studies suggest that type I collagen is the predominant matrix molecule synthesised by dermal fibroblasts in response to fibrin gels (Tuan et al.,1996).

Fibrin(ogen) degradation products have also been shown to affect collagen production (Pardes et al., 1995). Physiological concentrations (1μM) of fibrinopeptide A induced a significant up-regulation in the expression of the procollagen type I gene by dermal fibroblasts, demonstrating the ability of fibrinogen cleavage products to enhance collagen synthesis. However, it is not known if fibrin(ogen) degradation products have an effect on collagen synthesis by fibroblasts *in vivo*.

In vivo, collagen co-localises with fibrin-rich lesions (Knighton et al., 1982; Dvorak, 1986) suggesting that fibrin mediates collagen synthesis by fibroblasts. Moreover, other studies suggest that the persistence of fibrin may be associated with a further increase in collagen accumulation. For example, inhibition of fibrinolysis leads to excess fibrin deposition, collagen accumulation and accelerated pulmonary fibrosis following bleomycin-induced lung injury (Eitzman et al., 1996; Swaisgood et al., 2000). In addition, keloid fibroblasts, known to deposit excess collagen, show decreased fibrinolytic activity suggesting that fibrin is associated with the fibrotic phenotype displayed by these overactive scars (Tuan et al., 1996).

1.15 Fibrin deposition and lipodermatosclerosis

Extravascular fibrin deposition has been demonstrated histologically in the skin of patients with lipodermatosclerosis in numerous studies (Browse and Burnand, 1982; Claudy et al., 1991; Falanga et al., 1991; Van de Scheur and Falanga, 1997). Chronic leakage of fibrinogen from blood vessels occurs due to repeated episodes of venous hypertension in the dermal microvasculature (Leu et al., 1991). In addition, increased vascular endothelial growth factor (VEGF) concentrations in the skin of patients with venous disease may result in vascular dilatation and leads to leakage into the interstitium (Peschen et al., 1998; Shoab et al., 1998). Peschen and others (1998) proposed abnormal VEGF expression has local deleterious effects, including hyperproliferation of the vascular endothelium, increased interendothelial pore size and increased permeability. Such effects may explain the increased extravasation of plasma fibrinogen into the interstitium, resulting in the formation of the pericapillary fibrin cuffs. Moreover,

Coleridge-Smith and colleagues (1988) suggested that activated white blood cells sequestered in the dermal microvasculature produce proteases and free radicals that may be potentially damaging to the vascular endothelium, and may perpetuate leakage of plasma fibrinogen from blood vessels.

1.15.1 Reduced fibrinolysis in lipodermatosclerosis

Fibrinolysis contributes significantly to the extent of net fibrin accumulation. There is substantial evidence linking patients with lipodermatosclerosis to fibrinolytic abnormalities. Tissue-type PA has conclusively been shown to be reduced in the plasma (Margolis et al., 1996) and tissue from distal leg skin (Rogers et al.,1999) of patients with LDS. Peschen and colleagues (2000) revealed that the enzymatic activity and localisation of tPA and uPA were significantly altered during the progression of chronic venous insufficiency based on the CEAP classification. They noted a gradual decline in tPA levels and activity accompanied by an increase in the distribution of uPA in LDS tissues. Moreover, increased PAI-1 levels have been noted in fibrin cuffs (Brakman et al.,1992) and were also significantly elevated in the plasma of patients with LDS compared with controls (Margolis et al., 1996). This finding may explain the pronounced reduction in the fibrinolytic activity in patients with chronic venous insufficiency.

Several risk factors relating to the development of deep vein thrombosis have been identified in patients with chronic venous insufficiency. An increasing number of genetic defects have been identified involving factors in the coagulation cascade, including mutations in protein C, protein S, anti-thrombin and fibrinogen genes, which together, are thought to account for 10% of patients with venous thrombosis (Falanga et al., 1990). Hereditary deficiencies of proteins C and S are well-recognised causes of thrombosis (Koeleman et al., 1994). Decreased levels of these proteins in patients with venous disease and LDS may also result in the persistent, unregulated accumulation of fibrin in the skin.

1.16 Summary

The accumulation of fibrin is dependent on the status of the coagulation and fibrinolytic pathways, with a deficient fibrinolytic system predisposing tissue to excessive accumulation of fibrin. Patients with venous dysfunction and LDS have significantly

reduced fibrinolysis and chronic fibrinogen leakage, resulting in fibrin accumulation in the skin. In many fibrotic disorders, excess fibrin deposition is associated with collagen accumulation. Fibrosis in LDS may therefore be explained by the chronic and persistent deposition of fibrin, leading to excessive production of collagen. The following section will describe in detail the synthesis of collagen and its turnover in normal skin, and how this may become unregulated in fibrotic conditions such as LDS.

Section 3: Collagen synthesis and fibrosis

During tissue repair, fibrin matrix is remodelled by various cell types and replaced by a collagen-rich matrix. Collagen deposition by fibroblasts is tightly regulated and involves both co-ordinated synthesis and degradation. In fibrotic disease, an imbalance of these processes occurs and results in the excessive accumulation of matrix proteins, compromising tissue architecture and function. One of the main features of LDS is fibrosis of the dermal and subcutaneous layers of the skin. This section will review the process of collagen synthesis, deposition and degradation, and will summarise potential profibrotic mediators that may play a role in the pathogenesis of fibrotic skin diseases, such as LDS.

1.17 Collagen and the extracellular matrix

Collagen is the most abundant extracellular matrix (ECM) protein and constitutes approximately 25% of the fibrous protein of the body (Murray et al., 1996). The collagens form a complex network of fibres and gels that pervades every organ of the body. Common to all members of the collagen family is a triple-helical domain composed of three polypeptide chains, termed α-chains, with a homologous amino acid sequence. Currently about 33 distinct collagen α-chains have been identified, and these interact to form at least 19 distinct collagen isotypes, designated I-IX. Each isotype possesses different biochemical and physical properties, adopting distinct molecular organisations and performing a range of different biological functions (reviewed by Chambers and Laurent, 1997). Isotypes may exist as homotrimers composed of three identical α-chains (e.g., collagen type III) or as heterodimers consisting of two (e.g., collagen type I) or three different α-chains (e.g., collagen VI).

1.17.1 Structure of the procollagen molecule

The α-chains contain a high proportion of glycine which occurs in a regularly repeating triplet Gly-X-Y, where approximately every third X is proline and every third Y is hydroxyproline (Miller, 1985). The α-chains consist of approximately 1000 amino acids and are 300nm in length. Each polypeptide α-chain forms a left-handed polyproline helix and assembles with two other α-chains to form a stable right-handed triple helix,

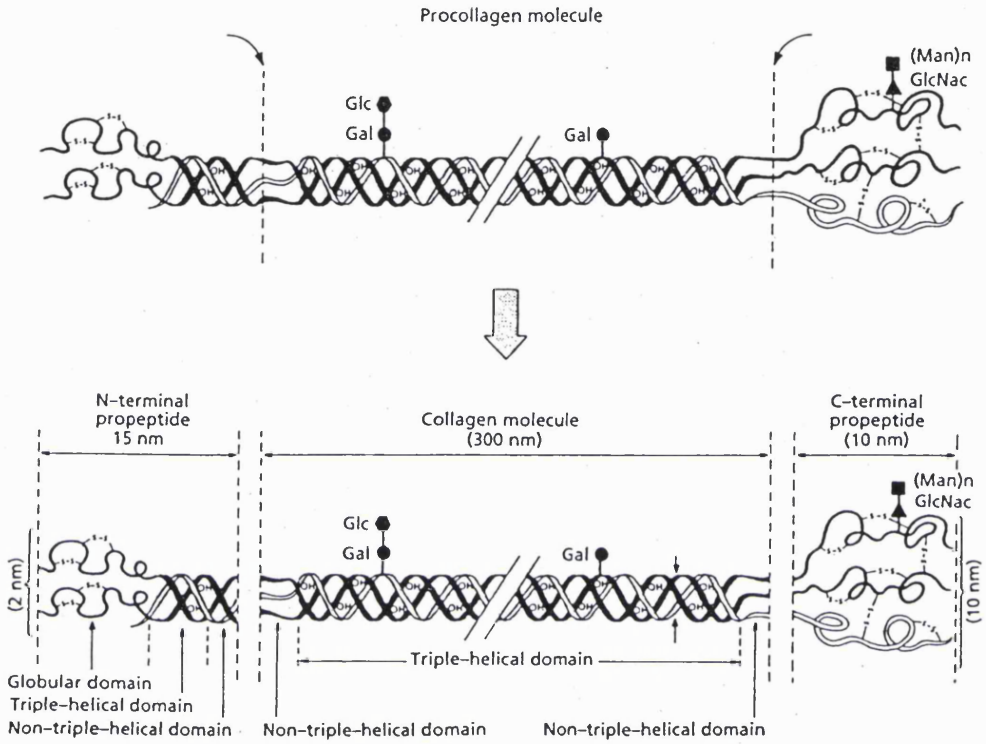


Figure 1.9. Schematic diagram of the structure of the procollagen type I molecule. The molecule is composed of two α1(I) and one α2(1) chain. The central portion of the procollagen molecule is the triple-helical domain which, along with short telopeptides, is deposited in the extracellular matrix as collagen after the excision of the N- and C-terminal propeptides.

stabilised by inter-α-chain hydrogen bonding (Figure 1.9). In this conformation, glycine residues are positioned in the centre of the triple helix and peptide bonds are buried within the interior of the molecule, rendering the triple-helical region highly resistant to proteolysis.

1.17.2 Hierarchical classification of collagen types

The collagen family is divided into two classes, fibrillar and non-fibrillar. The fibrillar sub-family consists of collagen types I, II, III, V and XI, and their primary role is to confer tensile strength to connective tissue. The α-chains of the fibrillar collagens consist of a single triple helical domain forming greater than 95% of the molecule. Following secretion into the extracellular space, interstitial collagen molecules assemble into ordered fibrils through covalent intermolecular bonds between homologous regions within triple-helical domains. The fibrils exhibit a characteristic arrangement and banding patterns, referred to as a 'D-stagger', and are usually composed of copolymers of collagens types I and III, and between collagens I and V in interstitial tissues (reviewed by Chambers and Laurent, 1997).

The non-fibrillar collagens are divided into three groups, consisting of basement membrane collagens (types IV and VII), short-chain collagens (types XIII and X) and fibril-associated collagens with interrupted triple helices (FACIT collagens: types IX, XII and XIV). The most abundant of the non-fibril forming collagens is type IV collagen, which is the major structural scaffold of all basement membranes, where it forms a three-dimensional network in close association with laminin, heparan sulphate and type VII collagen. The main triple helix of the non-fibrillar collagens is interrupted by short non-triple-helical sequences, which give further stability to the molecules, through end-to-end associations of triple-helical regions (Yurchenco and Schittny, 1990).

1.18 Biosynthesis and post-translational modification of collagen

The fibroblast is the predominant cell type responsible for the production of types I and III collagen, although endothelial, epithelial and smooth muscle cells have all shown to be capable of producing these collagens (Bienowski, 1991).

1.18.1 Intracellular processes

Each procollagen α-chain is encoded by separate genes which are widely dispersed throughout the genome. They are characterised by high concentrations of guanine (G) and cytosine (C) residues, due to high levels of proline and hydroxyproline (coding sequences of GGN and CCN respectively) in the Gly-X-Y sequence (deChrombrugghe et al., 1985).

Procollagen DNA is transcribed to pre-mRNA in the nucleus, which is spliced to form functional mRNA before translation in the rough endoplasmic reticulum (RER). The translated product, termed 'preprocollagen α-chain', contains an N-terminal hydrophobic signal peptide, which is thought to facilitate the movement to the RER (Chambers and Laurent, 1997). This signal peptide is proteolytically cleaved shortly after peptide synthesis, by procollagen-N-proteinase. The resulting procollagen α-chain undergoes a series of post-translational modifications, including hydroxylation of specific proline and lysine residues in the Y position of the recurring Gly-X-Y triplet, forming 4-hydroxyproline and hydroxlysine respectively. This hydroxylation process is catalysed by prolyl 4-hydroxylase and lysyl hydroxylase, respectively, and involves the co-factors ferrous iron and ascorbic acid, with co-substrates oxygen and α-ketoglutarate. Hydroxylation of proline residues is essential for the stability of the triple helix. Underhydroxylated procollagen is unable to form stable triple helices and is susceptible to rapid degradation intracellularly (Steinmann et al., 1981). Certain hydroxylated residues are subsequently glycosylated post-translationally (reviewed by Kivirikko and Myllyla, 1984). The extent of glycosylation varies widely among different collagen types in different tissues, and also with age. The function of this glycosylation remains unclear, but it is not thought to be essential for secretion. A final modification involves the transfer of mannose-rich oligosaccharides on to asparigine-linked carbohydrate units in the C-propeptides of fibrillar collagens and some domain in non-fibrillar collagens (Olsen et al., 1977).

In fibrillar collagens, juxtaposition of cysteine residues in three adjacent procollagen α-chains results in the formation of inter- and intra-disulphide bonds between opposing

lysine and hydroxlysine residues. Triple-helix formation then proceeds in a 'zipper'-like fashion from the C-terminus to the N-terminal domains. The procollagen molecules are subsequently transported from the rough endoplasmic reticulum to the golgi body, where they are packaged into secretory vesicles.

1.18.2 Extracellular processes

During or immediately following secretion, the N- and C-propeptides of fibrillar collagens are enzymatically cleaved by procollagen N-proteinase and procollagen C-proteinase respectively. Cleavage of propeptides leads to spontaneous assembly into fibrils at the plasma membrane, which are initially held together by electrostatic forces and lack tensile strength until they are cross-linked by a series of covalent bonds. Crosslink formation is due to the action of lysyl oxidase, which oxidises susceptible lysine and hydroxylysine residues to aldehydes, leading to the formation of covalent intra- and intermolecular links (reviewed by Chambers and Laurent, 1997).

1.19 Regulation of collagen synthesis and deposition

Metabolism of ECM molecules such as collagen involves both co-ordinated synthesis and degradation of matrix components. Collagen deposition is regulated at several levels including modulation of procollagen synthesis, intracellular and extracellular degradation, and the number of resident collagen-synthesising cells in the tissue. An imbalance in these processes results in the accumulation of matrix proteins, and fibrosis ensues. Thus, fibrosis may be due to an increase in procollagen gene expression and/or synthesis, decreased degradation and/or an increase in the number of collagen-producing cells, through proliferation or migration at the site of injury (Trojanowska et al., 1998). These events commonly occur in many organs other than skin, including kidneys, liver and heart, and are likely to involve similar mechanisms in each (Gressner and Bachem, 1994).

1.19.1 Regulation of procollagen gene expression and synthesis

The rate of procollagen synthesis is determined primarily by steady-state levels of procollagen mRNA which in turn are determined by a dynamic equilibrium between the rate of procollagen gene transcription, mRNA processing and mRNA degradation. A host

of chemical mediators (including cytokines, growth factors, hormones and vitamins), physical factors (e.g. mechanical stress, hypoxia) and ECM interactions modulate procollagen production (reviewed by Chambers and Laurent, 1997) (Table 1.2). Of these, TGF-β_1 is one of the most potent stimulators of collagen synthesis and has been implicated as the key mediator in the pathogenesis of fibrotic disease (Massague, 1990; Trojanowska et al., 1998).

TGF-β_1 is synthesised in a latent form consisting of a latency-associated protein (LAP) and a latent TGF-β binding protein (LTBP). Latent TGF-β_1 is incorporated in the extracellular matrix and is activated following cleavage by certain proteases, such as plasmin and MMPs (Massague, 1990). It is produced by a number of different cells, including haematopoietic, mesenchymal, platelets and epithelial cells and increases collagen deposition, through up-regulation of procollagen gene transcription and increased procollagen mRNA stability (Massague, 1990). Furthermore, TGF-β_1 reduces the proportion of newly synthesised collagen that is degraded and promotes the down-regulation of collagen-degrading proteases and an up-regulation in their inhibitors (McAnulty et al., 1991). Abnormal expression of TGF-β_1 has been associated with fibrotic skin diseases such as scleroderma (Kulozik et al., 1990; Border and Ruoslahti, 1992). Indeed, repeated subcutaneous injections of TGF-β_1 in the skin of a mouse resulted in sclerotic skin changes, with significantly increased levels of collagen deposited, in particular types I and III (Shinozaki et al., 1997).

In addition to the effects of cytokines and growth factors on procollagen production by fibroblasts, the ECM directly affects cell behaviour and collagen synthesis (reviewed by Streuli, 1999). Indeed, several studies have demonstrated an association between fibrin deposition and collagen synthesis. *In vitro,* studies have shown that the rate of collagen synthesis increased when fibroblasts were cultured in a fibrin matrix compared with cells grown in monolayer cultures or in a collagen matrix (Gillery et al., 1989; Coustry et al., 1990; Clark et al., 1995; Lorimier et al., 1996; Tuan et al., 1996). Similar effects on collagen production have been demonstrated on other ECM molecules such as fibronectin (Ostberg et al., 1995).

Agent	Procollagen synthesis	Cell type
Angiotensin II	Increased	Human lung fibroblasts
Ascorbate	Increased	Human skin fibroblasts
CTGF	Increased	Human skin fibroblasts
C-propeptides	Increased	Human lung fibroblasts
N-propeptides	Decreased	Human lung fibroblasts
EGF	Decreased	Human lung fibroblasts
Endothelin-1	Increased	Human lung fibroblasts
Fibrinopeptide B	Decreased	Human skin fibroblasts
Fibrinopeptide A	Increased	Human skin fibroblasts
Glucocorticoids	Decreased	Human smooth muscle
Histamine	Increased	Human lung fibroblasts
Insulin	Increased	Human lung fibroblasts
IGF-1	Increased	Human lung fibroblasts
IL-1	Decreased	Human skin fibroblasts
IL-4	Increased	Human skin fibroblasts
IL-6	Increased	Human skin fibroblasts
IFN-γ	Decreased	Human skin fibroblasts
Mechanical load	Increased	Rat cardiac fibroblasts/ Human lung fibroblasts
PDGF	Increased	Rat cardiac fibroblasts
PGE_2	Decreased	Human lung fibroblasts
TGF-β_1	Increased	Human skin fibroblasts/ Human lung fibroblasts
Thrombin	Increased	Human lung fibroblasts
TNF-α	Decreased	Human skin fibroblasts
tPA	Increased	Human skin fibroblasts

Table 1.2. Mediators of collagen synthesis *in vitro*.
Agents are shown with respect to cell lines on which their activity has been well documented.

1.19.2 Regulation of collagen degradation

Degradation of collagen occurs at both intracellular and extracellular levels. The intracellular pathway of procollagen degradation occurs soon after synthesis and is likely to occur in the lysosomes, since inhibition of the lysosomal proteases cathepsins B, D and L, prevents intracellular procollagen degradation (Berg et al., 1980). The rate of extracellular degradation of collagen is slower than intracellular breakdown, since the triple helical structure of the fibrillar collagens is highly resistant to proteolytic cleavage, especially when fibrils are heavily cross-linked (Schneir et al.,1984). Proteases capable of cleaving collagen extracellularly, comprise of the matrix metalloproteases (MMPs) including interstitial collagenases, gelatinases and the stromelysins. The matrix metalloproteinase (MMP) gene family encodes twenty-three metal-dependant endopeptidases (designated MMP1 to MMP-23) with activity against most ECM molecules (Nagase and Woessner, 1999). MMPs are secreted as zymogens and become activated only following cleavage of their amino pro-domains (Nagase and Woessner, 1999). MMPs are derived from mesenchymal cells and hemopoeitic cells, including macrophages and keratinocytes. All MMPs have a putative Zn^{2+} binding site and have regions of homology throughout the family that may have importance in the activation of latent proforms to active enzymes (Nagase and Woessner, 1999). MMPs all require Ca^{2+} for stability and exhibit preferred cleavage specificity for the N-terminal side of hydrophobic residues. In order that MMP proteolysis does not lead to widespread destruction of the extracellular matrix, activation is closely orchestrated on the cell surface (Streuli, 1999).

Secreted pro-MMPs are activated *in vitro* by proteinases and non-proteolytic agents such as SH-reactive agents (mercaptoethanol or dithiothreitol), mercurial compounds, reactive oxygen and denaturants. In all cases activation requires the disruption of the cysteine-Zn^{2+} (cysteine switch) interaction, and the removal of the propeptide proceeds (Nagase and Woessner 1999). *In vivo*, most pro-MMPs are likely to be activated by tissue and plasma proteinases and opportunistic bacterial proteinases. Using fibrinolytic protease deficient mice, Carmeliet and colleagues (1997) suggested that the fibrinolytic pathway was a pathophysiologically significant activator of MMPs. Plasmin has been shown to

activate pro-MMP-1 and pro-MMP-3 (Nagase et al., 1991). MMP-3 is a potent activator of pro-MMP-9 which in turn degrades collagenous proteins (Ramos-Desimone et al., 1999). Thus collagen degradation is in part regulated by the activity of the fibrinolytic proteases, tPA, uPA and plasmin.

Interstitial collagenase, MMP-1, and neutrophil collagenase, MMP-8, uniquely cleave the interstitial collagens at a 'Gly-Ile' or 'Gly-Leu' bond within the native helical structure, three-quarters the distance from the N-terminal end (Nagase and Woessner, 1999). The primary sequence of this collagenase-sensitive site is an important factor in determining the susceptibility to further proteolysis. Wu and colleagues (1990) showed that most substitutions around the scissile bond are unfavourable and yield uncleavable or poorly susceptible collagen molecules. Cleaved portions of the helix rapidly lose their triple-helical conformation and the denatured products are susceptible to the action of a number of less specific proteases, such as the gelatinases (MMP-2 and MMP-9), elastase and cathepsin G (reviewed by Nagase and Woessner, 1999).

MMP activity is regulated by a group of protease inhibitors, including the tissue inhibitors of matrix metalloproteases (TIMPs 1-4). The major form, TIMP-1 is synthesised and secreted by mesenchymal cells and macrophages, and forms a high affinity, irreversible complex with the active forms of the MMPs. Protease activity is further inhibited by the circulating antiproteinase, α_2- macroglobulin, which inactivates all serine proteases. Other circulating MMP inhibitors include the serpins, α_1-antitrypsin and α_2-antiplasmin (Nagase and Woessner, 1999). The expression and synthesis of protease inhibitors may play an equally important role in the degradation of collagen as the proteases themselves. Decreased concentrations of active MMPs, or indeed of a specific MMP isotype (such as the interstitial collagenases), coupled with increases in inhibitor concentrations, may have profound negative effects on degradation and remodelling of the extracellular matrix. Moreover, MMPs play a major role in the release and activation of latent growth factors in the ECM (Nagase and Woessner, 1999). Diminished growth factors levels or activities may have further adverse consequences in processes such as tissue repair, where these factors are critical in influencing cell

behaviour, particularly in the presence of specific matrix molecules, such as the fibrillar collagens.

1.20 Fibroblast-collagen interactions

Cell-extracellular matrix interactions can directly regulate cell behaviour, either through receptor mediated signalling or by modulating their cellular responses to growth factors in the ECM. Studies have shown that integrins $\alpha1\beta1$ and $\alpha2\beta1$ are the major receptors responsible for collagen binding and regulate remodelling by human dermal fibroblasts grown in three-dimensional collagen gels (Langholz et al., 1995). Integrin $\alpha1\beta1$ induces the down-regulation of procollagen gene expression by fibroblasts, whereas $\alpha2\beta1$ integrin mediates the up-regulation of gene expression and synthesis of collagenase (MMP-1), a fibrillar collagen degrading protease (Langholz et al., 1995, Xu et al., 1997). One consequence of increased expression of MMPs in a collagen-rich environment, is that remodelling reveals cryptic sites previously unrecognised by cell surface receptors (reviewed by Streuli, 1999). Proteolysis of collagen type I exposes integrin binding sites that further induce cells to synthesise proteases, to assemble protein complexes involved in cell adhesion, or to induce down-regulation of collagen gene expression (Messent et al.,1998). Furthermore, studies have shown that interactions between fibroblasts and collagen matrix result in reduced proliferation (Mauch et al.,1988).

1.21 Collagen deposition and lipodermatosclerosis

LDS skin is characterised by excessive accumulation of collagen (Leu et al., 1991; Kirsner et al., 1993; Brinckmann et al., 1996; 1999) resulting in complete disruption of the interface between the dermal and subcutaneous fat layers. Leu and colleagues (1991) reported that the collagen fibres in sclerotic areas of LDS skin were densely packed and linearly orientated, similar to scar tissue. In addition, Brinckmann et al., (1996) demonstrated a marked increase in the amount of trivalent cross-links in the dense collagen matrix of LDS, of which levels were comparable to those of skeletal tissue. Subsequent studies by the same group revealed increased hydroxylation of lysyl residues in the post-translational modification of procollagen in LDS skin (Brinckmann et al., 1999). This event resulted in altered fibrillar architecture, with parallel collagen bundles 1nm larger in LDS skin compared with normal control skin. The factors that may cause

the increased accumulation of collagen and cross-linking in LDS-affected skin are not known.

1.22 Summary

Collagen deposition is a fundamental event in tissue repair and provides structural support to maintain tissue integrity following injury. Collagen metabolism by fibroblasts is tightly regulated and involves both co-ordinated synthesis and degradation. In LDS, an imbalance of these processes occurs and results in collagen accumulation in the dermal and subcutaneous layers, compromising tissue architecture and pre-disposing the skin to ulceration. Although the macrovascular changes that occur in LDS are known, the cellular and molecular mechanisms by which venous hypertension mediates collagen accumulation are not clear. However, it is well documented that patients with LDS have significantly reduced fibrinolysis and chronic fibrinogen leakage, resulting in fibrin accumulation in the skin. In many fibrotic disorders excess fibrin deposition is associated with collagen accumulation. Therefore, the effects of chronic and persistent fibrin deposition may perpetuate fibrosis in LDS. This thesis will examine the role of fibrin in collagen deposition by dermal fibroblasts, and aims to elucidate cellular and molecular mechanisms that may regulate this event in fibrotic disease development.

1.23 HYPOTHESIS AND AIMS

Excessive extracellular matrix accumulation, in particular collagen, in the dermal and subcutaneous layers is a prominent feature of skin affected by LDS. The degree of fibrosis in LDS skin is directly related to the formation of chronic venous ulcers and impairs their subsequent healing. However, the cellular and molecular mechanisms and factors that mediate excessive collagen deposition remain unknown. Chronic fibrin deposition in the skin and an impaired fibrinolytic system are also features associated with LDS. During tissue repair, fibrin deposition provides a provisional matrix promoting inward migration of tissue repair cells, and is gradually replaced by granulation tissue, rich in collagen. However, persistent fibrin deposition in organs is a feature of many fibrotic diseases, including pulmonary fibrosis, atherosclerosis, glomerulonephritis and scleroderma. Thus, the fibrin matrix may play a significant pathological role in the development of these fibrotic conditions as well as in LDS.

This thesis will employ novel strategies to examine the overall hypothesis that **in LDS, reduced fibrinolysis and persistent fibrin deposition are associated with excessive collagen accumulation by dermal fibroblasts, leading to the development of fibrosis.** New approaches will be used to investigate the role of fibrin and its persistence in regulating collagen deposition both *in vitro* and *in vivo*, and to elucidate cellular and molecular mechanisms regulating this fundamental event of tissue repair. The specific aims of this thesis are:

1) To characterise fibrotic skin changes associated with LDS, including procollagen gene expression and cell proliferation, using histological, *in situ* hybridisation and immunohistochemical techniques.
2) To investigate the effect of fibrin matrix in the production of collagen by fibroblasts using *in vitro* and *in vivo* model systems.
3) To assess the effect of persistent fibrin matrix due to decreased fibrinolysis on the further accumulation of collagen in these *in vitro* and *in vivo* systems.
4) To determine the effect of reduced fibrinolysis and persistent fibrin matrix on procollagen gene expression by dermal fibroblasts, and the role of plasmin-mediated MMP activation on collagen accumulation.

In addition, more specific hypotheses and aims will be introduced in each individual chapter.

Aim 1 – To characterise fibrotic skin changes associated with lipodermatosclerosis

The development of fibrotic skin changes in patients with venous disease will be investigated in skin biopsies taken from 3 groups of patients divided according to the CEAP classification system. These individuals include normal controls (CEAP Class 0), patients with chronic venous insufficiency but no visible skin changes (CEAP Class 3) and patients with LDS (CEAP Class 4). Extracellular matrix distribution and cellularity will be examined histologically by Masson's trichrome and haematoxylin and eosin techniques respectively. The mechanisms that regulate fibrotic changes will be assessed using *in situ* hybridisation for procollagen type I gene expression and immunocytochemical detection of proliferating cell nuclear antigen (PCNA) for cell proliferation. Inflammatory cells will be analysed by histological and immunohistochemical techniques as possible sources of profibrotic mediators. In addition, the distribution of fibrin will be assessed together with collagen accumulation.

Aim 2 – To investigate the effect of fibrin matrix on the production of collagen by dermal fibroblasts using *in vitro* and *in vivo* model systems

Fibrin deposition in the dermis is a prominent feature of LDS. Collagen deposition has been reported to be intimately associated with pre-existing fibrin matrix (Dvorak, 1986). Thus, collagen production by human dermal fibroblasts in fibrin matrix will be examined both *in vitro* and *in vivo* using novel approaches. *In vitro,* non-retracting fibrin lattices will be used to compare the collagen production by dermal fibroblasts in a three-dimensional fibrin matrix to that produced by fibroblasts on fibrin monolayers or tissue culture plastic. Procollagen production will be analysed by HPLC measurement of hydroxyproline. The effect of fibrin matrix deposition on collagen production will be examined *in vivo*, by a novel, standardised murine skin fibrosis model, a sensitive and accessible way of examining the mechanisms regulating fibrin remodelling and subsequent collagen deposition over time in a physiological system. Collagen deposition will be measured by HPLC analysis of hydroxyproline and by histological analysis of fibrin-injected tissue.

Aim 3 – To assess the effect of persistent fibrin matrix due to decreased fibrinolysis on the further accumulation of collagen in *in vitro* and *in vivo* systems

Fibrin deposition in LDS and other fibrotic conditions is both chronic and persistent, with reduced levels of fibrinolytic proteases and increased levels of their inhibitors. Therefore, the effect of reduced fibrinolysis and persistent fibrin deposition on the accumulation of collagen by dermal fibroblasts will be examined in *in vitro* and *in vivo* models developed for this study. Models will be modified to encompass the effects of reduced fibrinolysis. Procollagen production by human dermal fibroblasts in fibrin gels will be assessed, in the presence or absence of the plasmin inhibitor, α_2-antiplasmin, by HPLC analysis of hydroxyproline. The advent of genetically modified animals permits the use of novel experimental approaches to address this hypothesis and so fibrinolytically-deficient mice will be used. Collagen production by dermal fibroblasts isolated from plasminogen activator-deficient animals will be assessed in fibrin gels and reduced fibrinolysis will be confirmed using a novel, fluorescently-labelled fibrin degradation assay. Furthermore, the effects of persistent fibrin deposition and reduced fibrinolysis will be examined *in vivo* in fibrinolytic-deficient mice.

Aim 4 – To determine the effects of reduced fibrinolysis and persistent fibrin matrix on procollagen gene expression by human dermal fibroblasts, and the role of plasmin-mediated MMP-1 activation on collagen accumulation in fibrin gels

The mechanisms by which reduced fibrinolysis and persistent fibrin deposition may mediate collagen production by dermal fibroblasts remain to be elucidated. Direct fibroblast-fibrin interactions may result in the up-regulation of procollagen gene expression and thus procollagen type I mRNA levels will be measured in fibroblasts grown in fibrin gels by Northern analysis. In addition, evidence suggests that the fibrinolytic system plays a major role in the activation of collagen-degrading proteases, in particular MMP-1. The effects of reduced fibrinolysis on MMP-1 activation by dermal fibroblasts in persistent fibrin gels will be investigated using a specific MMP-1 activity assay. The subsequent effect of reduced active MMP-1 levels on collagen accumulation will be examined in this system by HPLC analysis of hydroxyproline.

In summary, this thesis aims to understand how fibrotic skin changes develop as a result of reduced fibrinolysis and persistent fibrin deposition, and may provide improved strategies for the development of effective therapies for LDS and other fibrotic disorders in which chronic fibrin deposition is a feature.

Chapter 2
MATERIALS AND METHODS

2.1 Materials

All chemicals were of analytical grade or above and obtained from Sigma-Aldrich Company Ltd (Poole, UK) unless otherwise indicated. All water used for the preparation of buffers was distilled and deionised using a Millipore Water Purification System (Millipore R010 followed by Milli-Q Plus; Millipore Ltd, Watford, UK). Solvents used for the preparation of high pressure liquid chromatography (HPLC) buffers and solutions were of HPLC grade and obtained from BDH-Merck. Ltd (Lutterworth, UK). Sterile tissue culture dishes, polypropylene centrifuge tubes and pipettes were obtained from Corning Costar Ltd (High Wycombe, UK) unless otherwise specified. Other disposable sterile plasticware was purchased from Sterilin Ltd (Ashford, UK). Sterile tissue culture medium (DMEM), sterile tissue culture grade amino acids, trypsin/EDTA and antibiotics were obtained from Life Technologies Ltd (Paisley, UK). Foetal calf serum (heat inactivated) was purchased from Imperial Laboratories (Andover, UK).

Human fibrinopeptides A and B, human fibrinogen, human thrombin, murine fibrinogen and murine thrombin were all purchased from Sigma-Aldrich Company Ltd (Poole, UK). Porcine transforming growth factor-β type I was purchased from R&D Systems Europe Ltd (Abingdon, UK) and was diluted to 1ng/μl in 10μl aliquots. All enzymes and growth factors were prepared in DMEM culture medium, aliquoted and stored at -70°C.

2.2 Collection of human material

Adult human skin samples were collected to set up normal primary fibroblast cell cultures and for histological and *in situ* hybridisation studies.

2.2.1 Patient selection

Informed consent and ethical approval from the Royal Free and University College London Medical School Ethics committee was obtained before studies were commenced. Thirty-six patients were recruited and classified into three groups according to the

International Society for Cardiovascular Surgery / Society for Vascular Surgery CEAP classification (Porter and Moneta, 1995) and their clinical details are summarised in **Appendix A Tables 1-3**. Patients in Class 0 (n=12) were undergoing coronary artery by-pass surgery and demonstrated no signs of venous disease as demonstrated by colour ultrasonography and air plethysmography analyses. Patients in Class 3 (n=12) were undergoing varicose vein stripping and demonstrated varicose veins, reticular veins and oedema with no detectable skin changes on physical examination. Class 4 patients (n=12) presented with skin changes ascribed to lipodermatosclerosis, including skin thickening and hyperpigmentation. The average ages of class 0, 3 and 4 groups were 61 ± 12, 44 ± 3 and 41 ± 4 years respectively. Sex was evenly distributed in Classes 3 and 4, although there were more males than females in Class 0 controls.

2.2.2 Biopsy collection

One 4mm full thickness excision of skin was removed from the medial malleolus of the distal leg by punch biopsy (Steifel, Bucks, UK) under general anaesthetic. Biopsy sites were not sutured. Skin biopsies from patients in Classes 3 and 4 were taken by Mr Coleridge-Smith (Department of Vascular Surgery, Middlesex Hospital, University College Hospital, London) and patients in Class 0 were biopsied by Mr Pudsley (Department of Cardiovascular Surgery, Middlesex Hospital, University College Hospital, London).

2.2.3 Tissue Processing

Each biopsy was immediately fixed in 4% paraformaldehyde buffered in PBS, pH7.4 for 24 hours, and then placed in 70% ethanol for 24 hours at 4°C. The tissue was processed for paraffin wax embedding by dehydration with ascending concentrations of alcohol, and cleared in 100% xylene. Subsequent to embedding in wax, 5μm sections were prepared using a microtome (Shandon, UK). Sections were collected on poly-L-lysine-coated slides (BDH, Lutterworth, UK) air-dried for 10 minutes then baked at 60°C for 1 hour. All slides were subsequently stored at room temperature prior to histological, immunocytochemical or *in situ* hybridisation analysis.

2.3 Histological analysis

Sequential sections were stained using routine histological staining methods (Bancroft and Stephens, 1996) and the changes in tissue architecture, cellularity and extracellular matrix distribution was assessed between the skin samples.

2.3.1 Haemotoxylin and eosin staining

Haemotoxylin and eosin is the most suitable stain to demonstrate the general histological architecture of a tissue. Its particular value is its ability, with proper differentiation, to distinguish between the nucleus (purple) and the cytoplasm of different cell types, and between the different types of connective tissue fibres and matrices, by staining them different shades of red and pink.

Sections were dewaxed in 2 changes of xylene and rehydrated through graded alcohols (2x 100%, 1x 90%, 1x 70% and 1x 50%) to tap water. Slides were immersed in Mayer's haemotoxylin (BDH, Lutterworth, UK) for 10 seconds and then washed in running tap water. The haemotoxylin was differentiated in 1% acid alcohol (1% HCl, 70% ethanol) and then washed in running tap water for 5 minutes. Sections were counter-stained with 1 % eosin (Sigma, UK) for 30 seconds, washed in running tap water and dehydrated through graded alcohols to xylene and coverslipped with DPX mountant (BDH, Lutterworth, UK).

2.3.2 Massons trichrome staining

Many trichrome stains are available for the differential demonstration of the connective tissues. The term 'trichrome stain' is a general name for a number of techniques which selectively demonstrate collagen fibres, muscle fibres and erythrocytes. Three dyes are used, one of which is usually a nuclear stain. Massons trichrome stained collagen blue, fibrin red and nuclei blue/black. The technique is best suited to distinguish collagen from other components of the tissue.

Sections were dewaxed and rehydrated through graded alcohols to tap water. Tissue sections were stained for 10 seconds in Celestine blue stain (celestine blue 1.25g, ferric

ammonium sulphate 12.5g, glycerin 35ml (all BDH, Lutterworth, UK), water 250ml) and washed in running tap water, before counter-staining for 10 seconds with Mayer's haemotoxylin (BDH, Lutterworth, UK). Sections were then washed in running tap water before being immersed in 1% ponceau fuschin (Sigma, Poole, UK) in 1% acetic acid. The slides were washed in distilled water and collagen decolourised by immersion in 1% phosphomolybdic acid (Sigma, Poole, UK) for 2 minutes. Finally, the sections were stained with 0.5% soluble blue (methyl blue, Sigma, Poole, UK) in 2.5% acetic acid. Sections were washed in distilled water and dehydrated through graded alcohols to xylene, mounted in DPX mountant (BDH, Lutterworth, UK) and coverslipped. Nuclei stained blue-black, cytoplasm and erythrocytes stained red and collagen stained blue.

2.3.3 Martius scarlet blue trichrome staining

The main features of the martius scarlet blue technique are the use of small molecule yellow dye, together with phosphotungstic acid in alcoholic solution, to selectively stain red blood cells and early fibrin deposits. On treatment with a medium-sized molecule red dye, mature fibrin and muscle are stained red. Phosphotungstic acid blocks the staining of collagen and final treatment with a large molecule blue dye demonstrates collagen deposition.

Sections were dewaxed and rehydrated through graded alcohols to water. Sections were stained with celestine blue staining solution (celestine blue 1.25g, ferric ammonium sulphate 12.5g, glycerin 35ml, water 250ml) and then Mayer's haemotoxylin (BDH, Lutterworth, UK) for 10 seconds each before being thoroughly washed in running tap water. Slides were washed in 95% alcohol and stained in martius yellow (Sigma, Poole, UK) for 2 minutes. Sections were washed in distilled water, stained in brilliant crystal scarlet solution (Sigma, Poole, UK) for 10 minutes before treatment with phosphotungstic acid for 5 minutes (BDH, Lutterworth, UK). Tissue sections were counter-stained in soluble blue solution (BDH, Lutterworth, UK) for 10 minutes, rinsed in 1% acetic acid and dehydrated through graded alcohols to xylene.

2.4 Immunohistochemical techniques

Immunohistochemical techniques were used to identify various inflammatory cell types, such as macrophages, mast cells, neutrophils and lymphocytes, proliferating cells, vascular endothelial cells and fibrinogen and its degradation products in skin samples. Positive staining of cells was semi-quantitated by counting cell number in a defined area (see section 2.7 Scoring).

Sections were dewaxed and rehydrated through graded alcohols to tap water. If required, tissue sections were initially treated with pronase (Sigma, Poole, UK) or citric acid buffer (10mM anhydrous citric acid, Sigma, Poole, UK) with microwaving, promoting antigen retrieval. Sections were treated with 0.3% H_2O_2 (Sigma, Poole, UK) in PBS (pH7.4) for 20 minutes to block endogenous peroxidase activity, in a humidified chamber at room temperature. The slides were washed three times in PBS for 5 minutes and then incubated for 20 minutes with the appropriate blocking serum (6% in PBS) (Dako, Cambridge, UK), or serum derived from the host animal of the secondary antibody (e.g. a secondary antibody raised in a goat would require goat serum blocking). Well-characterised primary antibodies (Table 2.1) were diluted in PBS as appropriate and incubated on the sections for 1 hour at room temperature in a humidified chamber. Initial dilution studies with the primary antibodies were conducted to establish optimal antibody concentrations prior to each study.

Table 2.1. Primary antibodies used for immunocytochemistry studies
* denotes either protease or microwave/citric acid treatment

Marker	Ab raised against	Ab raised in	Dilution	Source	Secondary Ab raised in
Proliferating cell nuclear antigen (PCNA) *	Dividing cells	Mouse IgG	1:75	Dako	Goat
Human CD68 *	Macrophages	Mouse IgG	1:100	Dako	Goat
Human CD15s	Neutrophils	Mouse IgM	1:50	Serotec	Goat
Mast cell Tryptase *	Mast cells	Mouse IgG	1:100	Serotec	Goat
Human CD3	T lymphocytes	Mouse IgG	1:50	Serotec	Goat
Vimentin	Mesenchymal	Mouse IgG	1:100	Dako	Goat
Von willebrand factor	Vascular endothelium	Rabbit IgG	1:300	Dako	Goat
Fibrinogen	Fibrin(ogen) Peptides	Mouse IgG	1:500	Dako	Goat

The sections were washed in PBS three times and incubated with biotinylated secondary antibody (Dako, Cambridge, UK) for 1 hour at room temperature in a humidified chamber, diluted to 1 in 200 in PBS. Sections were washed in PBS three times and incubated with streptavidin-conjugated horseradish peroxidase (Dako, Cambridge, UK) for 30 minutes at 1 in 100 dilution, in PBS. The slides were washed for a further 15 minutes in PBS and peroxidase activity was detected using the chromagen 3-3'diaminobenzidine substrate (FAST DAB) according to the manufacturer's instructions (Sigma, Poole, UK) for up to 10 minutes. After washing in tap water, sections were counterstained with either Mayer's haemotoxylin or 1% eosin for 30 seconds. The slides were washed in running tap water and dehydrated through graded alcohols before mounting the tissue sections with DPX mountant (BDH, Lutterworth, UK).

Negative controls for each antibody consisted of a section incubated with PBS or immunoglobulins of the primary antibody host animal, instead of the primary antibody. The tissue was treated with secondary antibody and streptavidin-linked peroxidase as with the test sections. No positive signal was observed in the negative control sections, indicating there was no non-specific staining.

2.5 *In situ* hybridisation techniques

In situ hybridisation techniques allow specific nucleic acid sequences to be detected in morphologically preserved tissue sections. *In situ* hybridisation techniques were modified from those of Shahzeidi and others (1994), and were used to examine the distribution of cells expressing procollagen type I (COL1A1) mRNA in tissue sections.

2.5.1 Plasmid preparation and gene cloning

The procollagen type I α-chain DNA was inserted into a plasmid for transformation into E.coli bacteria (see 'Bacterial transformation' **Appendix B1.2**) before purification of the DNA (see 'Small-scale preparations of DNA' **Appendix B1.3**) and synthesis of the riboprobes. Briefly, the 1.5kb human procollagen type I (COL1A1) (Access no. Hf677, ATCC, USA) cDNA fragment was cloned into the expression vector pGEM7zf+ (Promega, Southampton, UK) following restriction with the restriction enzyme ECOR1

(Promega, Southampton, UK). The vector contained an ampicillin resistance gene and dual opposed Sp6 and T7 promoters flanking the multiple cloning site, allowing RNA to be transcribed from either end of the insert (Figure 2.1).

2.5.2 Linearisation of plasmid

Purified pGEM7zf+ plasmids containing the COL1A1 insert were diluted in diethylpyrocarbonate-treated (DEPC, Sigma, Poole, UK) water to a concentration of 1μg/μl, following analysis by spectrophotometry (see 'Spectrophotometric evaluation of nucleic acids' **Appendix B1.4**). The construct was linearised with restriction enzymes HindIII and XbaI (Promega, Southampton, UK) at 12 units/μl supplied with appropriate enzyme buffers, according to manufacturer's instructions (Figure 2.1). Approximately 1 unit of enzyme activity was used per 1μg of uncut DNA. In a final volume of 40μl, 20μg of plasmid DNA was linearised, and reaction mixtures prepared in sterile microfuge tubes on ice. Reaction mixtures were incubated at 37°C for 2 hours and then a 2μl aliquot was removed and examined by gel electrophoresis on a 1% agarose gel (see 'Agarose gel electrophoresis of nucleic acids' **Appendix B1.5**), to confirm linearisation. Linearised plasmids were purified by phenol:chloroform (see 'Phenol-chloroform purification of DNA' **Appendix B1.6**), precipitated and resuspended in DEPC-treated water to a concentration of 1μg/μl. The samples were stored at -40°C prior to *in vitro* transcription.

2.5.3 Preparation of digoxigenin-labelled riboprobe by *in vitro* transcription

Nucleic acid labelling with digoxigenin (DIG) was performed using protocols developed by Boehringer Mannheim, UK. Digoxigenin-labelled riboprobes were synthesised by transcription *in vitro* according the manufacturer's protocol (Roche Diagnostics, UK). Briefly, T7 and SP6 RNA polymerases were used to generate sense and anti-sense probes in the presence of digoxigenin-labelled UTP. *In vitro* transcription mixtures containing 4μl (1μg) of linearised cDNA template, 2μl of 10 x transcription buffer (400mM Tris-HCl pH8, 60mM $MgCl_2$, 100mM dithiothreitol, 20mM spermidine, 100mM NaCl, 1 U/μl RNase inhibitor), 10μl of DEPC-treated water, 2μl of 10 x DIG RNA labelling mix (10mM ATP, 10mM CTP, 10mM GTP, 6.5mM UTP, 3.5mM DIG-UTP, pH7.5) and 2μl of SP6 or T7 RNA polymerase, were prepared on ice.

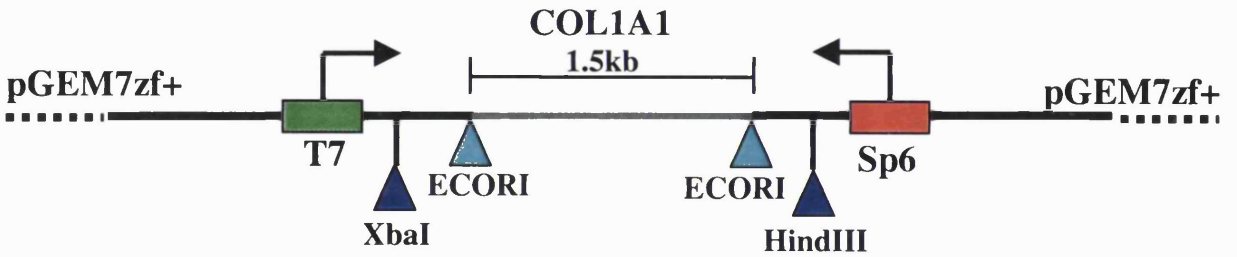


Figure 2.1. Schematic map of procollagen type I gene sequence (COL1A1) cloning sites into vector pGEMzf7+. The COL1A1 insert was cloned following restriction of the plasmid and insert with ECORI restriction enzyme. *In vitro* transcription of the clone resulted in the formation of anti-sense and sense RNA strands from the T7 and Sp6 promoter sites respectively.

Samples were incubated at 37°C for 2 hours and a 2µl aliquot examined by agarose electrophoresis of RNA (see 'RNA electrophoresis' **Appendix B1.7**) to verify the size of the transcripts. Samples were compared with two dilutions of RNA ladder (Promega, Southampton, UK), for semi-quantitative evaluation of riboprobe yield (1µg cDNA yields 10µg RNA). To terminate the reaction, 2µl of EDTA (Sigma, Poole, UK) was added. The reaction mixture was diluted with DEPC-treated water to give a final ribroprobe concentration of 25ng/µl and stored at -40°C. The specificity of the riboprobe for procollagen type I α-chain mRNA was tested by Northern analysis (see 'Northern analysis of procollagen type I riboprobe specificity' **Appendix B1.8**).

2.5.4 Slide Preparation

Superfrost ® microscope slides (BDH, Lutterworth, UK) were washed in double-distilled deionised water and immersed in acetone for 2 minutes. The slides were subsequently treated with freshly prepared 2% 3-aminopropyltriethoxysilane (BDH, Lutterworth, UK) in acetone for 5 minutes, rinsed in distilled water and air-dried.

2.5.5 Prehybridisation

Slides were immersed twice in fresh xylene for 5 minutes to dewax samples and sections were rehydrated by immersion in ethanol series, 100% ethanol for 2 minutes, 90%, 70% and 25% ethanol. Slides were briefly rinsed in RNase-free DEPC-treated water and immersed in RNase-free PBS for 10 minutes. Sections were subsequently treated with 100mM glycine (BDH, Lutterworth, UK) in PBS for 10 minutes, followed by further washing in PBS.

Sections were treated individually with 20µg/ml proteinase K (Sigma, Poole, UK) in PBS at 37°C for 45 minutes, in a humidified chamber. Sections were refixed in 4% paraformaldehyde for 5 minutes at 4°C and then acetylated with 0.25% acetic anhydride in 0.1M triethanolamine (Sigma, Poole, UK) for 10 minutes. They were then treated with pre-hybridisation buffer containing 40% deionised formamide, 10% dextran sulphate, 1x Denhardt's solution (0.02% Ficoll, 0.02%ployvinylpyrrolidone, 10mg/ml RNase-free

bovine serum albumin), 4 x SSC, 10mM dithiothrietol, 1mg/ml yeast tRNA and 1mg/ml denatured and sheared salmon sperm DNA (Gibco, Paisley, UK) at 37°C for 10 minutes in a humidified chamber, to equilibrate the tissue.

2.5.6 Hybridisation

In order to denature the secondary structure of the RNA riboprobes, 8µl of anti-sense and sense probes (25ng/µl respectively) were heated to 65°C for 10 minutes in a water-bath. Simultaneously, 10µl of salmon sperm DNA was denatured in a water-bath at 100°C for 5 minutes. Both the salmon sperm DNA and riboprobe mix were added to 250µl of hybridisation buffer (containing 40% deionised formamide, 10% dextran sulphate, 1x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10mg/ml RNase-free bovine serum albumin), 4 x SSC, 10mM dithiothrietol, 1mg/ml yeast tRNA and 1mg/ml denatured and sheared salmon sperm DNA) preheated to 65°C. Excess prehybridisation solution was removed from the incubating slides and 10µl of hybridisation solution containing the anti-sense riboprobe or the sense riboprobe was added, so that the sense samples provided negative controls for each anti-sense stained tissue section. The aim of the 'sense' negative control was to demonstrate the absence non-specific binding of the RNA. Finally, the slides were cover-slipped using RNAse-free forceps and sections were incubated overnight at 37°C in a humidified chamber.

2.5.7 Post-hybridisation

Coverslips were removed by immersion in 2x SSC solution (Saline sodium citrate) solution. All slides were washed in 2xSSC, 1xSSC and 0.1xSSC at 37°C, for 30 minutes each before equilibrating the sections for 10 minutes in detection buffer (100mM Tris-HCl, 150mM NaCl, pH7.6). Sections were treated with anti-digoxigenin antibody (Roche Diagnostics, UK) conjugated to alkaline phosphatase, diluted to 1:200 detection buffer (100mM Tris-HCl and 150mM NaCl), for 2 hours at room temperature. Slides were washed for 10 minutes in two changes of Tris-HCl/ NaCl buffer, before treatment with NBT/BCIP substrate solution (Dako, Cambridge, UK) for 2 minutes. The sections were counter-stained with 1% eosin for 10 seconds, washed in tap water, then mounted in aqueous 'Immu-mount' (Shandon, Basingstoke UK) and coverslipped.

2.6 Microscopy and photography

The sections were examined using an Axioscop 2 Zeiss Microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) and photographed with a Zeiss DX (Carl Zeiss Ltd, Welwyn Garden City, UK) camera using Kodak Extachrome 64T tungsten film.

2.7 Scoring system

Positively-stained cells were scored following *in situ* hybridisation and immunohistochemistry procedures. Briefly, the dermis in the section was divided into papillary, reticular and deep dermal layers and two adjacent 500μm^2 fields were counted in each dermal layer and the subcutaneous fat layer, by the investigators (A. deGiorgio-Miller, S. Herrick and R. McAnulty) under x 200 magnification using an eye-piece graticule. The average number of positive cells per 500μm^2 was calculated for each layer, and the total number of cells in the three dermal layers and subcutaneous fat was recorded for all patients in each of the classification groups.

2.8 Statistical analysis

All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired students t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

2.9 Determination of collagen deposition in response to fibrin *in vitro*

Collagen production by dermal fibroblasts in three-dimensional fibrin lattices and in monolayer cultures was assessed *in vitro*. For this purpose, human dermal fibroblasts were isolated from skin biopsies taken from the legs of control patients.

2.9.1 Isolation and culture of human dermal fibroblasts

Normal human dermal fibroblasts were routinely cultured from skin biopsies taken from around the medial malleolus of the distal leg from patients undergoing coronary bypass surgery. Under sterile conditions, the tissue was chopped finely with sterile scalpel blades (Swann-Morton, Sheffield, UK) and rinsed several times in explant media containing

20% foetal calf serum, 3% fungizone, 2% glutamine and 1% penicillin/streptomycin (Life Technologies, Paisley, UK) in Dulbecco's Modified Eagle Media (DMEM, Life Technologies, Paisley, UK) to remove traces of blood. Tissue explants were then placed into $10cm^2$ petri dishes (Sterilin, UK), previously scored to generate grooves for cell adhesion, and was covered with 2mls of DMEM supplemented with penicillin (200μg/ml), streptomycin (200μg/ml), L-glutamine (4mM), 1% fungizone (Sigma, Poole, UK) and 20% FCS. Explants were incubated in a humidified atmosphere of 10% CO_2 in air at 37°C for 16 hours before further explant media was added to the cultures. The media was routinely changed every 4 days until a confluent culture was established (approximately 3 weeks). At confluence, cells were trypsinised from the dish using 0.25% trypsin/EDTA (Life Technologies, Paisley, UK) solution and replated in a $75cm^2$ culture flask, and incubated in a humidified atmosphere of 10% CO_2 in air at 37°C in standard DMEM media (10% FCS, 100μg/ml penicillin and 100μg/ml streptomycin). The cells were maintained under standard tissue culture conditions (See "Routine tissue culture techniques" **Appendix C1.1**) Fibroblasts used in all experiments were between passage 6 and 10.

2.9.2 Fibroblast characterisation

As primary cultures of human dermal fibroblasts were used throughout this thesis, characterisation was performed by immunocytochemistry using antibodies against cytoskeletal and cell surface proteins. At confluence, cells were trypsinised as described above and plated into 8-well chamber slides at a density of 10,000 cells per well and incubated until 70-80% confluency was reached. Cultures were washed twice with PBS, fixed in cold (-20°C) methanol for 30 minutes (cytoskeletal proteins) or 4% paraformaldehyde in PBS (surface proteins) at room temperature for 3 minutes. Cells were washed three times with PBS and stored at 4°C in PBS prior to staining. Non-specific binding was blocked with 0.6% serum (prepared from the species in which the secondary antibody was raised). The cells were then incubated with individual primary antibodies diluted in PBS/2% BSA (Sigma, Poole, UK) for 45 minutes at room temperature: anti-vimentin (1:40, clone V9), anti-α-smooth muscle actin (SMA) (1:1000, clone 1A4), anti-desmin (1:100, clone DE-R-11), anti-myosin (1:1000, clone hSM-V) and anti-cytokeratin (1:100, clone LP34). Cells were subsequently incubated with an

appropriate secondary antibody (1:200 in PBS) conjugated to the fluorochrome fluoroisothiocyanate (FITC) for 60 minutes at room temperature. Cells were washed three times in PBS for 5 minutes and mounted. Cells were visualised under an inverted microscope (Axioscop, Zeiss, Germany). Positive control cells, Madin-Darby Canine Kidney (MDCK) epithelial cells were set up to confirm staining.

A representative example of the staining pattern exhibited by the primary cell lines is shown (Figure 2.2). Primary cell lines in culture stained positive for vimentin, and negative for myosin, desmin and cytokeratin. In general, the two 'human skin fibroblast' (HSF) cell lines (HSF-1 and HSF-5), stained negative for α-smooth muscle actin, although positive cells were occasionally observed. MDCK cells stained positive for vimentin and cytokeratin only, and negative for all other markers. On the basis of these results, it was concluded that cultures of dermal fibroblasts had been isolated.

2.10 Fibroblast/fibrin gel preparation

Confluent cultures of human dermal fibroblasts (passage 6-10) were trypsinised and resuspended at a concentration of 500,000 cells per ml in 12ml serum-free DMEM, supplemented with 50μg/ml ascorbate and 0.2mM proline (Sigma, Poole, UK). The cell suspension was passed through a pipette several times to ensure a single cell suspension. Human fibrinogen (Sigma, Poole, UK) was added to the cell suspension at a final concentration of 3mg/ml (9μM), and the solution was mixed. Human thrombin (10μl) (Sigma, Poole, UK) stock solution was added to a final concentration of 2nM. The suspension was mixed thoroughly and 1ml was aliquoted into each well of a 12-well plate (Falcon, New Jersey, USA) (500,000 cells/well). The fibrin gels set after incubation for 6 hours at 37°C in 10% CO_2 in air, and then 1ml of the test solution was added to the gel (6 wells per test for collagen analysis; 4 wells per test for cell counts) (Figure 2.3). Test solutions were either serum-free DMEM alone (with ascorbate and proline), or serum-free DMEM (with ascorbate and proline) containing bovine transforming growth factor-β1 (R&D Systems, UK) at a final concentration on 1ng/ml. Cells were incubated for 24, 48 and 72 hours at 37°C in 10% CO_2 in air. After each 24 hour incubation period, 10μl of stock ascorbate (Sigma, Poole, UK) solution was added to a final concentration of 50μg/ml to restore reduced ascorbate levels. An identical 4-well plate was set up for each

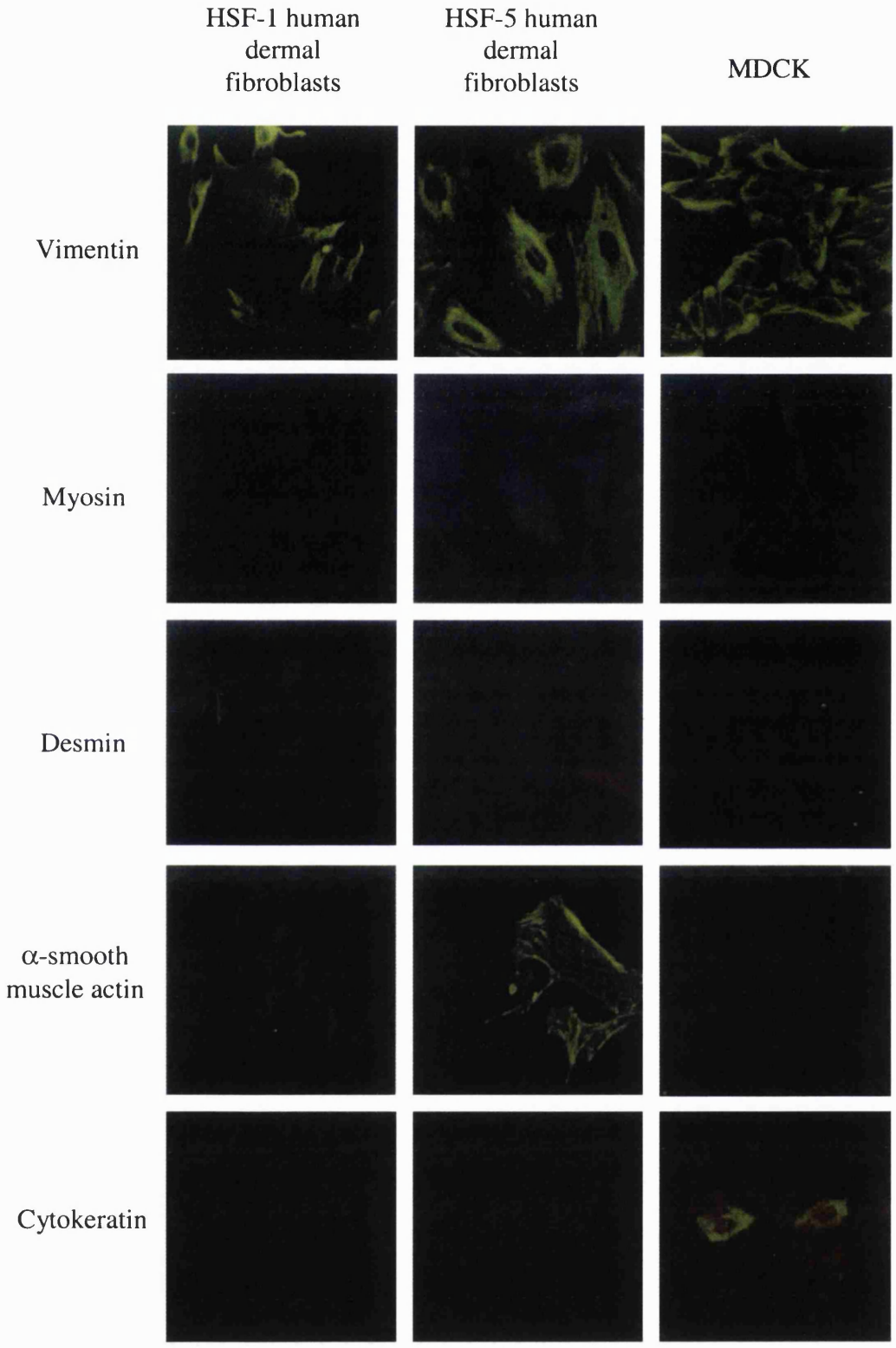


Figure 2.2. Characterisation of adult human dermal fibroblast cell lines (HSF-1 and HSF-5) by immunocytochemistry. Fibroblasts were stained for various cytoskeletal and surface proteins to determine fibroblast phenotype. Madin-darby canine kidney cells (MDCK) were used as a control cell line.

experiment to assess basal collagen synthesis before application of the test solutions. The experiment was stopped by freezing and storing the fibrin gels at -20°C, prior to collagen production analysis or performing cell counts.

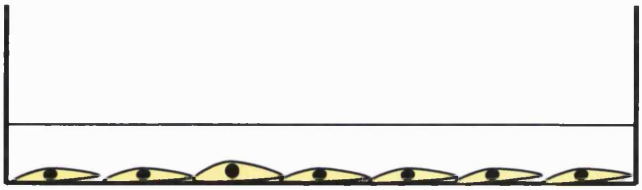
2.11 Fibrin and plastic monolayer preparation

Collagen production by dermal fibroblasts in three-dimensional fibrin gels was compared with that of fibroblasts seeded as a monolayer on fibrin-coated plastic, or plastic alone (Figure 2.3). Human fibrinogen (9μM) was dissolved in serum-free DMEM and human thrombin was added to a final concentration of 2nM. The mixture was mixed gently before adding and immediately removing 1ml of the solution to each well of a 12-well plate (Falcon, New Jersey, USA). The fibrinogen and thrombin solution was left to dry for 30 minutes. Fibroblast cell suspension (500,000 cells/ml) in 1ml test solution, with and without TGF-β1 (1ng/ml), was added to each fibrin-coated or uncoated plastic well (6 wells per condition for collagen analysis and 4 wells for cell counts). Cells were incubated for 24, 48 and 72 hours at 37°C in 10% CO_2 in air. After each 24-hour time period, the ascorbate levels were replaced (final concentration of 50μg/ml).

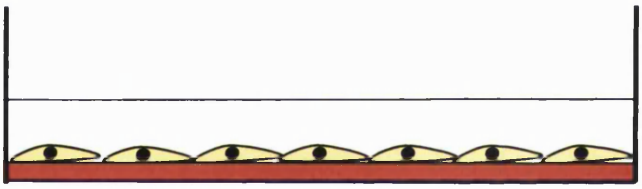
In addition, the effect of human fibrinopeptides A and B (Sigma, Poole, UK) (final concentration of 10-5M-10-7M), fibrinogen alone (final concentration of 9μM) (Sigma, Poole, UK) or thrombin alone (final concentration of 2nM) (Sigma, Poole, UK) on procollagen production was examined in dermal fibroblasts grown on tissue culture plastic. Briefly, 1ml of serum-free DMEM containing the various components independently (with proline and ascorbate) was added to confluent dermal fibroblasts on tissue culture plastic (500,000 cells/well) and was incubated at 37°C in 10% CO_2 in air for 48 hours (6 wells per condition for collagen analysis and 4 wells for cell counts). Each experiment was repeated atleast twice with two different adult human dermal fibroblast cell lines (HSF1 and HSF-5).

2.12 Cell counts

For cell count analysis, supernatant was removed from the fibrin gel, fibrin monolayer or tissue culture plastic cultures and each well was washed twice with 1ml PBS. To each well, 1ml of trypsin/EDTA solution was added and the plates were incubated for 20



Dermal fibroblasts ($5x10^5$ cells) grown on tissue culture plastic



Dermal fibroblasts ($5x10^5$ cells) grown on a fibrin monolayer

Dermal fibroblasts ($5x10^5$ cells) grown in three-dimensional fibrin gels

Figure 2.3. *In vitro* culture systems used for the analysis of collagen production by dermal fibroblasts grown in three-dimensional fibrin gels, on fibrin monolayers and on tissue culture plastic. Procollagen production was assessed by measurement of hydroxyproline by HPLC.

minutes at 37°C. The cell suspension was passed through a 19-guage syringe several times to ensure a single suspension of cells. The number of cells per well was calculated using a hemocytometer (BDH, Lutterworth, UK) and 4 cell counts were obtained for each condition.

2.13 HPLC analysis of collagen deposition

Collagen production by human dermal fibroblasts in three-dimensional fibrin cultures, on fibrin monolayers or on tissue culture plastic, was assessed by the measurement of hydroxyproline by high performance liquid chromatography (HPLC).

2.13.1 Sample filtering

Fibrin gels (2mls) and monolayer cultures (1ml) were thawed, scraped with a cell scraper (Orange Scientific, UK) and cell layer and media were transferred into a labelled tube. Each well was washed with 1ml of PBS and was transferred to the corresponding tube. Protein in the cell suspension was precipitated by the addition of ice-cold absolute ethanol to a final concentration of 67% and tubes were stored at 4°C overnight. Proteins were separated from free amino acids and small peptides (< 3 amino acids) by filtration through 0.45μm pore filters (Millipore, Watford, UK) using a vacuum unit (Millipore, Watford, UK). The protein left on the filter was washed twice with 1ml 67% ethanol. The filter was transferred to a pyrex hydrolysis tube and proteins hydrolysed in 6M HCl at 110°C for 16 hours. The hydrolysates were then mixed with a small spatula-load of charcoal (to decolourise the solution), and filtered through a 0.65μm pore filter to remove the charcoal (Millipore, Watford, UK). From this hydrolysate, 100μl was transferred into a 1.5ml microfuge tube and liquid evaporated under vacuum on a sample concentrator (Speed-vac, Savant, UK) prior to analysis.

Procollagen deposition by cultured fibroblasts was assessed by measuring hydroxyproline (OHpro) in both the cell layer and media by HPLC, using a method previously described by Campa et al. (1990). Hydroxyproline (OHpro) represents approximately 12% of the primary sequence of procollagen (Laurent et al., 1981) and is essential for the formation of the collagen triple helix. The amino acid is not present to a significant level in any other protein with the exception of elastin (approx. 2% OHpro), C1q component of

complement (4.3% OHPro), acetylcholinesterase (5% OHpro) and surfactant apolipoproteins A and D. These proteins have not been demonstrated to be produced in significant amounts by isolated fibroblasts *in vitro* and therefore measuring OHpro content in the fibroblast cultures is a specific determinant of procollagen synthesis.

2.13.2 Derivitisation of samples

Hydroxyproline (OHpro) was isolated and measured by reverse phase HPLC of 7-chloro-4nitrobenzo-2-oxa 1,3-diazole (NBD-Cl)-derivitised hydrolysates (Campa et al., 1990). Briefly, each dried sample was re-dissolved in 100µl HPLC-grade distilled water, buffered with 100µl 0.4M potassium tetraborate (pH 9.5) and reacted with 100µl NBD-Cl in methanol, incubated in the dark at 37°C for 20 minutes. To each sample, 50µl of 1.5M HCl was added to stop the reaction and 150µl 167mM sodium acetate (FSA, Loughborough, UK) in acetonitrile (26% w/v) was then added to the samples to make them compatible with the initial running buffer. Samples were filtered (type GV, pore size 0.22µm, Millipore, Wayford, UK) and a 100µl aliquot loaded onto the HPLC column and eluted with an acetonitrile gradient.

2.13.3 Instrumentation and chromatographic conditions

Derivitised samples were separated on a Beckman System Gold HPLC (Beckman, High Wycombe, UK) with a reverse phase cartridge column (LiChroCART LiChropher 250 x 4mm, 5µm particle size, 100RP-18) protected by a directly coupled pre-column (LiChrosorb, 4 x 4 mm, 5µm particle size, 100RP-18). The columns were continuously maintained at 40°C in a heated column oven. Buffers were degassed and the HPLC column equilibrated in running buffer A (160ml acetonitrile, 1840ml water, 13.6g sodium acetate, pH6.7 with orthophosphoric acid) for 40 minutes. The first two samples run each day were OHpro standard solutions (Sigma, Poole, UK) and were used in the quantitation of the unknown samples.

NBD-Cl derivatives in the test samples and standards were eluted with an acetonitrile gradient, by increasing the concentration of buffer B (750ml acetonitrile, 250ml water) over time. Post-column detection was achieved by monitoring absorbance at 495nm using

a flow-through variable wavelength monitor and the signal was processed using an on-line chromatography computing integrator (Beckman Systems Gold Integrator) for quantitative analysis. OHpro elutes from the column between 5 and 6 minutes after injection. The remaining amino acid derivatives in the sample were eluted as the proportion of the organic buffer (acetonitrile) was increased. The total running and column regeneration time was 25 minutes for each sample.

2.13.4 Calculation of procollagen levels

The OHpro content in each sample was determined by comparing the peak area of the sample obtained on a chromatogram to those generated from standard solutions derivitised and separated under identical conditions. All values obtained for EtOH-insoluble fractions were corrected for the amount of OHpro in the protein-bound, OHpro measured in the combined cell layer and media at the onset of the incubation period by subtracting the value of EtOH-insoluble fraction of the t = 0 hours sample. The t = 0hours sample represented the level of procollagen synthesised before the application of the test solutions.

Calculation for the measurement of procollagen synthesis:

$$\frac{\text{Total } \mu\text{l of acid dried}}{\text{Volume of aliquot dried}} \times \frac{500\mu\text{l reaction mix}}{100\mu\text{l on column}} \times \text{HPLC value} \times \frac{1}{1000} = \text{nmols OHpro}$$

To correct for cell number: $\frac{\text{nmoles of OHpro}}{\text{cell number}} \times 10^5 = \text{nmoles OHpro} / 10^5 \text{ cells}$

These values represent procollagen synthesised over the incubation period. Parallel identical cultures were included to obtain cell counts in order to calculate procollagen synthesis per cell. All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired students t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

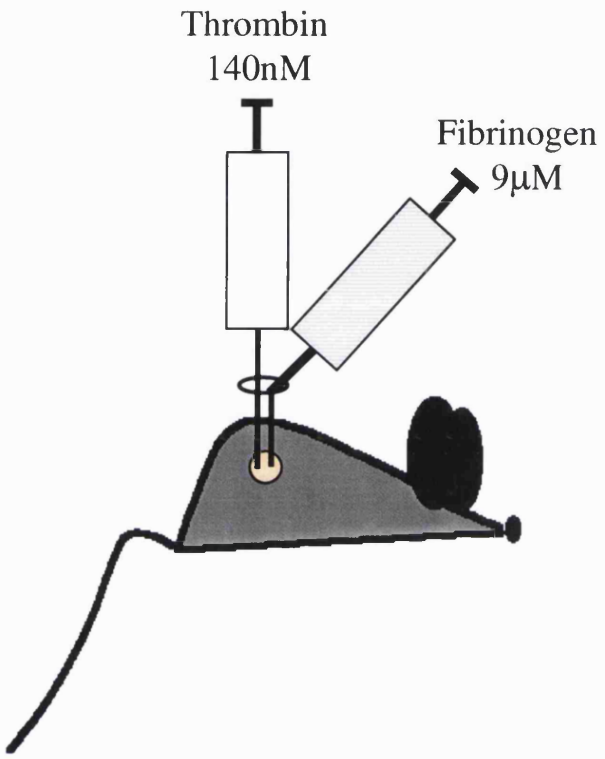
2.14 Subcutaneous injection of fibrin in Balb-c mice

Male Balb-c mice of 8-10 weeks of age (approximately 20g) were purchased from Harlan Laboratories, UK. Animals (n=12) were anaesthetised with 4% halothane in air and the back of each animal was shaved. Injection sites, 3cm from the base of the skull and 1cm either side of the spine, were tattooed with permanent ink. On one side, 25µl of murine fibrinogen [9µM] (Sigma, Poole, UK) in sterile PBS (with 264µg/ml $CaCl_2$), and 25µl of murine thrombin [140nM] (Sigma, Poole, UK) (with 264µg/ml $CaCl_2$), both at physiological concentrations, were injected simultaneously through two separate syringe needles (25-gauge) into adjacent sites (Figure 2.4). On the opposite side, 50µl of sterile PBS was injected, 25µl through two adjacent needles. The injections were repeated at the same injection sites twice a week for three weeks. Animals were housed individually for the duration of the experiment and were weighed before each injection to ensure no weight loss and ill health.

One week after the final set of injections, the animals were killed and the skin from the backs of the animals removed using sterile instruments. A full-thickness 8mm punch biopsy (Stiefel Laboratories, Bucks, UK) was taken at each injection site, including the peritoneum, and samples were snap-frozen in liquid nitrogen prior to collagen analysis by HPLC analysis (n=6 for each treatment). A second set of animals underwent the same treatments and biopsies were examined histologically. Full-thickness 8mm punch biopsies were taken at days 1, 3, 5, 7 and 14 after the final injection (n=4 for each time point). Fibrin-injected, PBS-injected and normal skin biopsies were fixed in 4% paraformaldehyde in PBS (pH7.6) and processed for wax histology as described in section 2.2.3.

2.15 Analysis of collagen deposition in skin samples by HPLC

Frozen 8mm punch biopsies were transferred to a pyrex tube containing 1ml of 6M hydrochloric acid. Skin samples were hydrolysed at 110°C for 16 hours, followed by addition of charcoal and filtering through a 0.65µm filter (Millipore, UK). From the stock hydrolysate solution, 200µl of the hydrolysed sample was evaporated and derivitised (see section 2.13.2) for HPLC analysis.



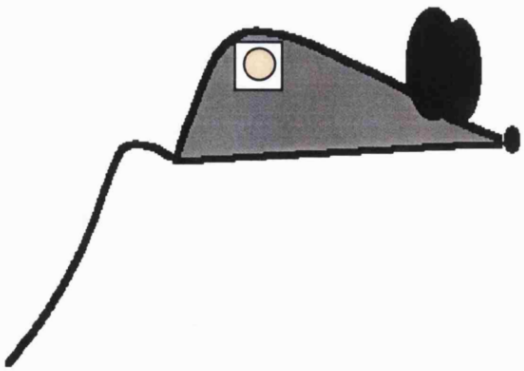
- **Animal anaesthetised and sites were shaved**

- **Sites tattooed 3cm from the base of the skull, 1cm either side of the spine.**

- **2 subcutaneous injections at each site per week for 3 weeks.**

On right side:
25μl murine fibrinogen [9μM]
25μl murine thrombin [140nM]

On left Side:
25 μl sterile PBS
25 μl sterile PBS



Analysis

8mm punch biopsy from fibrinogen-treated, PBS-treated and untreated skin sites 1 week after final injection for HPLC analysis (n=14), and at 1, 3, 5, 7 and 14 days post-injection for wax histology (n=6).

Figure 2.4. Protocol for subcutaneous injections of fibrinogen and thrombin in male BALB-c mice and subsequent analysis of biopsy samples.

The collagen values per 8mm biopsy were calculated as follows:

$$\text{HPLC value} \times \text{derivitised ratio} \times \text{hydrolysed ratio} \times \frac{131}{10^9} \times 8.1967 = \textbf{mg collagen per biopsy}$$

All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired students t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

2.16 Histological analysis of fibrin and collagen deposition following subcutaneous injections of fibrin

Skin samples were embedded in paraffin wax and twelve 5μm sections were collected at 200μm intervals using a microtome (Shandon, UK). Sections were collected on poly-L-lysine slides (BDH, Lutterworth,UK), and stained with Haemotoxylin and Eosin, and Martius Scarlet Blue trichrome. Measurements of skin layer depths (μm) were conducted on the dermis (from the base of the epidermis to the top of the panniculus carnosus muscle layer) and fascia (bottom of the panniculus carnosus to the top of the peritoneum), by image analysis using the KS300 imaging system (Carl Zeiss Ltd, Welwyn Garden City, UK) on a pre-calibrated screen. Collagen and fibrin densities in the fascial layer of skin sections (μm^2) were measured by 'full-colour thresholding' analysis, where collagen was measured by the area covered by 'blue' stain, and fibrin was measured by the area covered by 'red' stain, using the KS300 imaging system. Five measurements were taken per section, from five sections each 200μm apart, from each blinded skin sample (25 readings per sample, n=4) by two independent scorers (A. de Giorgio-Miller and Mr S. Bottoms). All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired student's t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

2.17 Subcutaneous injection of fibrin in plasminogen activator deficient mice

To examine the role of persistent fibrin deposition and reduced fibrinolysis on the deposition of collagen in the skin, plasminogen activator deficient mice were used for *in*

vivo and *in vitro* studies. Breeding pairs of plasminogen activator deficient mice were a gift from Professor Peter Carmeliet, University of Leuven, Belgium and were genetically modified to be deficient for either tPA or uPA (for review Carmeliet et al., 1994). The genotype of the mice was confirmed by PCR analysis (see **Appendix D**). Repeated subcutaneous injections of fibrinogen and thrombin were administered to tPA deficient (n=14), uPA deficient (n=14) and wild-type mice (n=14) as described previously (Section 2.14). One week after the final injection animals were killed and full thickness 8mm punch biopsies were taken from injection sites and from normal skin. Samples were processed for collagen content by HPLC analysis (n=10 for each condition) or histological analysis (n=4 for each condition) as described for Balb-c mice.

2.18 Isolation and culture of mouse dermal fibroblasts

Primary mouse dermal fibroblasts were isolated from the skin of wild-type and plasminogen activator-deficient animals. An area of shaved skin approximately 25cm^2 was dissected from four animals of each group with sterile instruments and collected in 20ml of DMEM media containing trypsin (0.25%), 300μg/ml penicillin and streptomycin and 300μg/ml fungizone (Gibco, Paisley, Scotland). Skin samples were incubated for three hours at 37°C with regular agitation to allow the epidermis to be peeled from the dermis using sterile instruments. The remaining dermis was cut into small pieces and transferred into a sterile tube containing 20ml of bacterial collagenase (1mg/ml) (Gibco, Paisley, Scotland) in DMEM media. Following vigorous shaking, the mix was incubated for 2 hours at 37°C in a shaking water bath. The resulting cell suspension was filtered through a 100μm pore mesh (Falcon, UK) and the flow-through was collected and diluted 1:1 with FCS, to quench collagenase activity before centrifugation at 1000rpm for 5 minutes. The cell pellet was resuspended in 20% FCS in DMEM media containing penicillin (100μg/ml) and streptomycin (100μg/ml). Cells were seeded in 75cm^2 tissue culture flasks and media was routinely changed every 4 days until the cells reached confluence. Primary cultures were trypsinised (passage 1), split 1:3 and continued to be incubated in 10% FCS/DMEM in a humidified atmosphere of 10% CO_2 in air at 37°C.

2.19 FITC-fibrin gel degradation

Fibrinolysis by dermal fibroblasts in fibrin gels in the presence or absence of α_2-antiplasmin, or by dermal fibroblasts isolated from the skin of plasminogen activator-deficient mice was assessed using a novel assay of fibrinolysis. Fibrinogen (Sigma, Poole, UK) was conjugated to fluorescein isothiocyanate (FITC) according to the manufacturer's instructions (Sigma, Poole, UK). Briefly, 5mg of human fibrinogen was dissolved in 1ml of phenol red-free DMEM media (Life Technologies, Paisley, UK) (containing penicillin and streptomycin at 300μg/ml) and 250μl of FITC dissolved in 0.1M carbonate-bicarbonate buffer was added drop-wise. The reaction vial was protected from the sunlight by aluminium foil and was incubated for 2 hours at room temperature with constant agitation. The Sephadex G-25M filtration column was equilibrated following addition of 30ml of sterile PBS. The FITC-fibrinogen solution was applied to the top of the column and the eluent was collected in 1ml fractions following the addition of 10ml of PBS. Fractions were retained in the dark and the absorbance was measured by spectrophotometer at 280nm to calculate the concentration of labeled fibrinogen (typically approximately 2mg/ml). Fibrin-FITC conjugate eluted was pooled and stored at 4°C for up to one week.

Fibrinogen-FITC conjugated solution was diluted in the required volume of phenol red-free DMEM medium, and unlabelled fibrinogen was added to restore the concentration of 3mg/ml (9μM). Human dermal fibroblasts or plasminogen activator deficient fibroblasts were added to the fibrinogen-FITC solution (500,000 cells per ml) and the cell suspension was passed through a pipette several times to ensure a single cell suspension. The plasmin inhibitor, α_2-antiplasmin (Sigma, Poole, UK) (5μl), was added to the fibrinogen-FITC-cell suspension in phenol-red-free DMEM media to a final concentration of 1μM. Characterisation of the assay, by adding increasing concentrations of α_2-antiplasmin (0.25μM - 2μM), showed that 1μM was sufficient to significantly reduced fibrinolysis by dermal fibroblasts. Fibrin gels (1ml) were clotted following the addition of 2nM thrombin as described previously (Section 2.10), and the fibrin gels set after incubation for 6 hours at 37°C in 10% CO_2 in air. To each fibrin gel, 1ml of serum-

free phenol-red-free DMEM media (containing penicillin and streptomycin) was added to the gels and incubated for 24, 48 or 72 hours.

At each time-point, 1ml of the media was removed, the fibrin gel was centrifuged for 1 minute at 2000 rpm in a microfuge tube, and the remaining solution was combined with the media. The media containing FITC-conjugated fibrin degradation products was mixed thoroughly and 200µl was added to an opaque Packard Optiplate 96-well plate (Packard Bioscience, Pangbourne, UK). Fluorescence from the FITC-fibrin degradation products was measured using a Cyto-fluor multi-well plate reader (Series, 4000, Perceptive Biosystems, Massachussetts, USA) at a wavelength of 495nm (in arbitrary units). In addition to fibroblast-populated fibrin gels, fibrin-FITC gels without cells were prepared and overlaid with serum-free phenol-red-free media (n=6 wells) or media containing 2µM plasmin (n=6 wells) (Sigma, Poole, UK). Media from gels without cells provided a measure of the natural degradation of the fibrin gel with time, and the addition of plasmin to gels demonstrated the total level of FITC in the gel at a given time-point. All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired students t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

2.20 Measurement of procollagen production by dermal fibroblasts in fibrin gels in the presence of α_2-antiplasmin

To assess the role of reduced fibrinolysis and persistent fibrin deposition on the production of procollagen by dermal fibroblasts in three-dimensional fibrin gels, the fibrinolytic inhibitor α_2-antiplasmin (Sigma, Poole, UK), was added to give a final physiological concentration of 1µM. Fibrin gels were prepared as described previously (Section 2.10), with the exception that α_2-antiplasmin, dissolved in DMEM media, was added to the dermal fibroblast/fibrinogen solution prior to the addition of thrombin. Gels were allowed to set for 6 hours at 37°C in 10% CO_2 in air prior to the addition of 1ml overlaying serum-free DMEM media, and were incubated for 24, 48 or 72 hours in the presence of ascorbate and proline as described previously (Section 2.10). Procollagen production by dermal fibroblasts in this system was assessed by HPLC measurement of

hydroxyproline (Section 2.13). An additional 4 wells were prepared for each condition for cell counts (Section 2.12). All data are presented as mean ± standard errors of the mean (SEM) for n=6 wells for each condition. Statistical analysis was performed using an unpaired students t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

2.21 Northern analysis of procollagen gene expression by dermal fibroblasts in fibrin gels in the presence of α_2-antiplasmin

Procollagen gene expression by dermal fibroblasts in fibrin gels in the presence of α_2-antiplasmin was assessed by Northern analysis. Fibroblast-populated fibrin gels (500,000 cells per well) were prepared with the fibrinolytic inhibitor α_2-antiplasmin (1μM) as described previously (Section 2.20) and procollagen gene expression by the HSF-1 fibroblasts was examined after 24, 48 and 72 hours. In addition, fibroblasts were grown on tissue culture plastic with and without α_2-antiplasmin, their procollagen gene expression was examined, and used as controls for cells in fibrin gels.

2.21.1 RNA isolation and Northern blot

The reagents used for RNA isolation were of molecular biology grade (from Sigma, Poole, UK unless otherwise stated). All water used was distilled and deionised by Mill-Q Plus. As a further precaution, water was also treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) overnight.

After 24, 48 or 72 hours the media was discarded from each fibrin gel and 1ml of TRIzol reagent was added according to the manufacturer's instructions (Gibco, Paisley, UK). RNA was isolated from total cell lysate in three separate stages. Initially, samples were shaken with 0.2ml chloroform (Sigma, Poole, UK), centrifuged for 15 minutes at 10,000rpm at 4°C and the supernatant transferred to a new microfuge tube. This fraction was mixed with 0.5ml of isopropanol (Sigma, Poole, UK) and centrifuged for a further 10 minutes at 10,000rpm at 4°C. The RNA pellet was washed with 75% ethanol, re-centrifuged for 5 minutes at 10,000rpm and air-dried. RNA was resuspended in 20μl of

DEPC-treated water and quantitated by spectrophotometer at a wavelength of 260nm/280nm.

The RNA samples were denatured by heating to 65°C in a water-bath for 15 minutes and then loaded onto a 1% formaldehyde/agarose gel and run at a constant voltage of 80V in 1 x 3-(N-morpholinopropanosulfonic acid) (MOPS) running buffer. The integrity of the RNA and the uniformity of the loading were confirmed by the presence of the 18S and 28S ribosomal RNA bands identified by ethidium bromide staining using the phosporimager (Fujifilm, Japan). RNA was transferred to a nylon membrane (Amersham Pharmacia Biochem UK Ltd, Little Chalfont, UK) by Northern blot in 20 x SSC solution overnight (Figure 2.5). A glass tray was filled with x20 SSC and an inverted stage was placed on the tray base. A wick made from 3MM Whatman Paper (Whatman, Maidstone, UK) was placed on the inverted stage and both sides were immersed in the x20 SSC. The gel was placed RNA-side down on the wick and air bubbles expelled. A pre-wet membrane was placed over the gel with forceps, and covered with three wet pieces of 3MM Whatman paper, and three dry pieces of Whatman paper on top. Clingfilm was wrapped around the blot apparatus to stop the gel short-circuiting. Towels were placed onto the blot apparatus, and covered with a glass plate and a 500g weight overnight. The transferred RNA was then cross-linked to the nylon membrane using an UV cross-linker (Stratalinker, Stratagene, UK) to maintain the RNA permanently on the membrane.

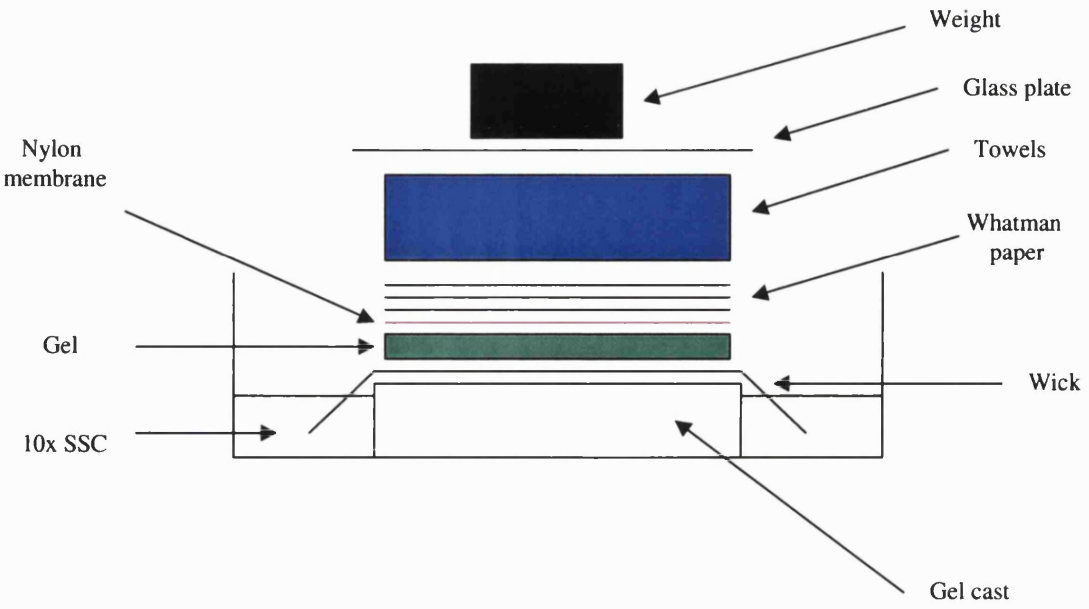


Figure 2.5 Diagram showing the assembly of Northern blot apparatus

2.21.2 Hybridisation and autoradiography

Following transfer, the membrane was pre-hybridised in a solution containing 30ml DEPC-treated water, 12.5ml 20X SSC, 5ml 50X Denhardt's solution (10g/L Ficoll 400, 10g/L polyvinylpyrrolidone, 10g/L BSA Fraction V), 2.5ml 10% sodium dodecyl-sulphate (SDS) and 250μg salmon sperm DNA (denatured at 100°C). The membrane was placed in a hybridisation tube, with RNA facing inwards. The tube was placed in a hybridisation oven (Bachofer, Germany) at 65°C for 90 minutes. During this time, the cDNA probe for procollagen α1(I) was radio-labelled with [^{32}P] d-CTP using a random priming DNA labelling kit (Megaprime DNA labelling kit) as per manufacturer's instructions (Amersham Pharmacia Biochem UK Ltd, Little Chalfont, UK). Briefly, the Hf677 procollagen clone (25ng in 15μl) was heated for 10 minutes at 65°C. Primers (5μl) were added to this solution and were mixed and boiled for 5 minutes at 100°C. To this solution, 10μl of labelling buffer, 13μl of DEPC-treated water, 2μl of Klenow enzyme, and 5μl of [^{32}P] d-CTP was added. The reaction mixture was incubated at 37°C for 1 hour. Finally, 5μl of 0.2M EDTA were added to stop the reaction and the solution was heated at 100°C for a further 5 minutes.

Labelled probe was purified using ProbeQuant G-50 Micro Columns (Amersham, New Jersey, USA) according to manufacturer's instructions. The resin in the column was suspended by vortexing, and then the column was centrifuged at 800g for 1 minute. The reaction mixture containing the labelled probe (50μl) was applied to the resin, and the column was centrifuged in a microfuge tube at 800g for a further 2 minutes. The purified sample in the flow-through was retained for the hybridisation procedure.

Pre-hybridisation solution was discarded and fresh pre-hybridisation solution without salmon sperm DNA was added to the membrane. The purified, labelled probe was added to the tube and the membrane was incubated overnight at 65°C in the hybridisation oven. Following hybridisation, the solution was discarded and the membrane was washed with 2X SSC/0.1% SDS at room temperature for 10 minutes. Membranes were then washed with fresh 0.2%/0.1% SDS for 1 hour with constant agitation. The membrane was

wrapped in cling-film and exposed to a phosphorimager autoradiographic screen (Fujifilm, Japan).

2.21.3 Semi-quantitation of procollagen type I gene expression

The signal generated from labelled, hybridised procollagen type I mRNA was quantitated by Advanced Image Data Analysis (AIDA) software (Raytek Ltd, Milton Keynes, UK). Absorbance values of the signal (from both procollagen bands) representing the bands for α1(I) procollagen mRNA were normalised relative to loading of total RNA in the same sample. This was determined by analysis of the ethidium bromide-stained rRNA band by the AIDA software. Procollagen gene expression is shown as the ratio obtained from procollagen/18s RNA readings (in arbitrary units) and is expressed as mean ± standard error, when at least n=3 wells were used for each condition.

2.22 MMP-1 activation by dermal fibroblasts with reduced fibrinolysis in fibrin gels

The role of reduced fibrinolysis on MMP-1 activation was examined in the same *in vitro* fibrin gel system. MMP-1 activation in the conditioned media of fibroblast-populated fibrin gels was assessed using a specific MMP-1 substrate cleavage assay (Calbiochem, Nottingham, UK). Briefly, hydrolysis of a specific MMP-1 cleavage site in the substrate releases the highly fluorogenic 7-methoxycoumarin-4-yl acetate (MCA) from the 2,4-dinitrophenyl quencher molecule (DPA), resulting in an increase in fluorescence at 398nm (Figure 2.6). The assay system was initially calibrated using increasing concentrations of active MMP-1 standard, purchased from Calbiochem (Nottingham, UK).

Human dermal fibroblasts were prepared in fibrin gels as described in Section 2.20. One millilitre of conditioned media from fibrin gels with and without α_2-antiplasmin for 24, 48 and 72 hours was collected, and 50μl was mixed with 50μl MMP-1 substrate solution (10mg/ml) according to manufacturer's instructions. In addition, 50μl samples were were treated for 1 minute with 50μl aminophenylmercuric acid (APMA) (final concentration of 2mM) (Calbiochem, Nottingham, UK) or 50μl EDTA (20mM final concentration) (Sigma, Poole, UK) at room temperature. The addition of APMA resulted in complete

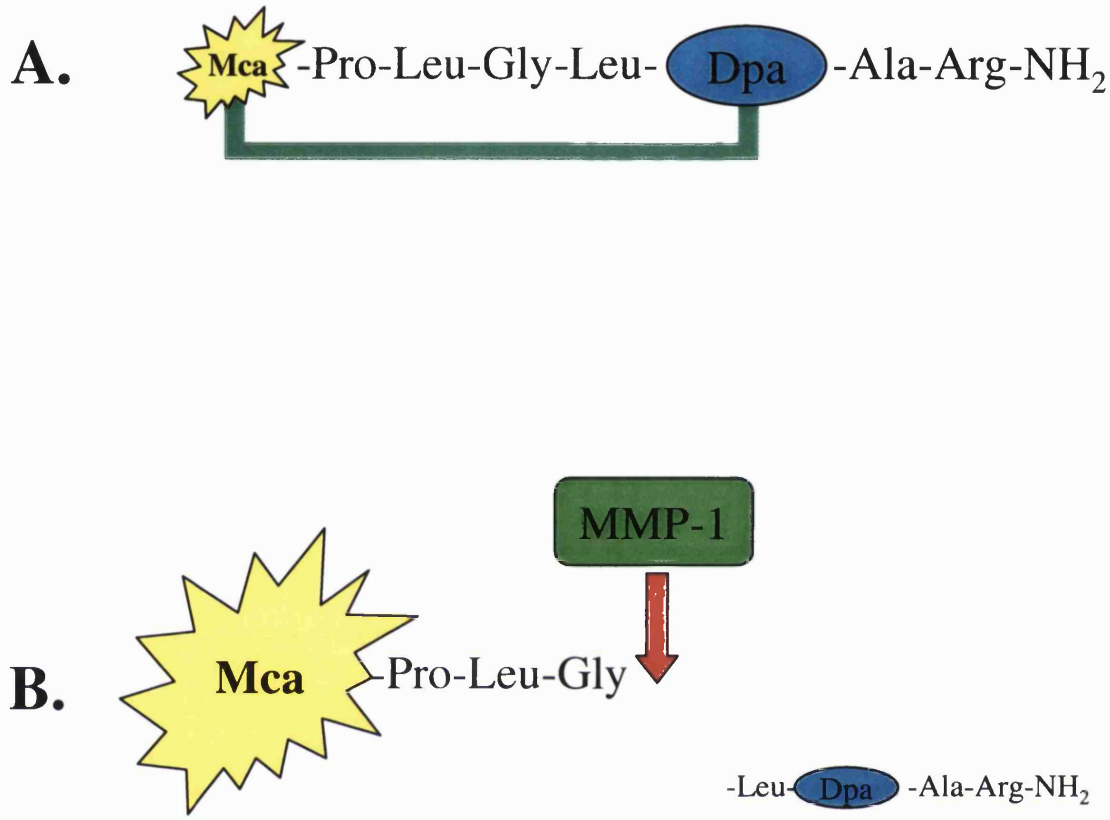


Figure 2.6. Fluorescent MMP-1 substrate assay.
A. Synthetic substrate specific for MMP-1, consisting of 7-methoxycoumarin-4-yl-acetyl group (MCa) linked to 2,4-dinitrophenyl quencher molecule (Dpa), which inhibits Mca fluorescence. **B**. Hydrolysis of Gly-Leu bond by MMP-1 separates the highly fluorogenic Mca group from the quencher molecule resulting in an increase in fluorescence (measured at 398nm) and inactivation of MMP-1.

activation of MMP-1 in the conditioned media, representing total MMP-1 levels. In contrast, the addition of EDTA to the conditioned media resulted in the inhibition of all metal-dependent enzymes, such as the MMPs, demonstrating any activity not due to MMP-1. Standard MMP-1 concentrations were added to each Optiplate 96-well plate (Packard Bioscience, Pangbourne, UK) to ensure calibration of the assay. The increase in fluorescence at 398nm was measured against time by Cytofluor multi-well plate reader series 4000 (Perceptive Biosystems, Massachussetts, USA) and the active and total active MMP-1 concentrations were calculated using the following calculation:

$$\text{MMP-1 concentration (ng/ml)} = \frac{\left(\frac{\delta F \times c \times V}{F \times v}\right)}{A}$$

δF = change in fluorescence following complete peptide cleavage (arbitrary units)
c = concentration of substrate used [2.5μM]
V = volume of hydrolysis reaction (0.1ml)
F = fluorescence of substrate at the concentration used for fluorimeter calibration (12)
v = volume of media added (0.05ml)
A = MMP-1 activity (15U per ng)

Data represents n=6 wells for each condition and are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired student's t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

2.23 The effect of MMP-1 inhibition on collagen accumulation by dermal fibroblasts in fibrin gels

To examine if inhibition of MMP activity in this three-dimensional fibrin gel system affects the accumulation of collagen, a synthetic MMP-1 (MMP-1*i*) (MWt. 491) inhibitor was added to the culture medium. The water soluble peptide preferentially inhibits interstitial collagenase at relatively low concentrations (1nM) but is also capable of inhibiting gelatinases and stromelysins (Odake et al., 1994). The process of fibrillar collagen

degradation is dependent on cleavage by MMP-1 (reviewed by Chambers and Laurent, 1997) and so this inhibitor provided an excellent means to examine the effects of MMP-1 inhibition on the accumulation of collagen in fibrin gels. Briefly, human dermal-fibroblast-populated fibrin gels were prepared with and without α_2-antiplasmin as described previously (Section 2.20). Once the fibrin gels had set, 1ml of serum-free DMEM media containing MMP-1*i* (1μM) (Calbiochem, CN Biosciences, Nottingham, UK) was added to each well. Fibrin gels were incubated for 48 hours at 37°C in 10% CO_2 in air. After this time the gels and media were combined and processed for HPLC analysis of hydroxyproline by methods described previously (Section 2.13). Data are presented as mean ± standard errors of the mean (SEM) for n=6 wells. Statistical analysis was performed using an unpaired student's t-test for single group comparisons. Differences were considered statistically significant at $p<0.05$.

Chapter 3
RESULTS AND DISCUSSION

Fibrotic skin changes associated with lipodermatosclerosis

3.1 Introduction

Many aspects of the pathophysiology of venous disease remain to be clarified, in particular the initial phases of tissue injury leading to fibrotic skin changes in the distal limb that ultimately result in chronic venous ulceration. There are few studies documenting the histological changes associated with LDS development and progression. The aims of this chapter were to characterise the fibrotic skin changes of LDS and to offer novel insights into the cellular and molecular mechanisms of venous disease progression.

Biopsies (n=12) of distal leg skin were taken from patients defined by the CEAP classification of venous disease (Porter and Moneta, 1995) and were divided into 3 groups. Class 0 patients represented control skin with no evidence of chronic venous insufficiency, Class 3 patients had chronic venous insufficiency without skin changes characteristic of LDS, and Class 4 patients had LDS skin changes, including skin thickening and hyperpigmentation. The tissue samples were processed for wax histology and initially stained with H&E to assess cellularity, Massons trichrome for collagen distribution and Martius Scarlet Blue for fibrin deposition. Histological features of the skin were compared between the three patient groups. Skin samples were also analysed for procollagen gene expression by *in situ* hybridization to examine the distribution of procollagen-synthesising fibroblasts within the various skin layers, and cell proliferation and inflammatory cell infiltrate were examined by immunocytochemical techniques.

3.2 Results

3.2.1 Histological appearance of LDS skin compared with control skin

Normal control skin (Class 0)

Normal skin was composed of an epidermis, an underlying connective tissue component, the dermis, and a subcutaneous fat layer (subcutis or panniculus) (Figure 3.1A). The epidermis consisted of stratified, squamous epithelial cells, or keratinocytes, resting on a basement membrane. The underlying dermis was sub-divided into three layers, the papillary, reticular and deep dermis. The papillary layer comprised of loose connective tissue and was invaginated to form 'dermal papillae' between the rete ridges of the epidermis. The reticular layer was composed of loosely arranged collagen bundles, with epithelial structures, such as sweat glands and hair follicles. The deep dermal layer, found between the reticular and subcutaneous fat layers was composed of thicker, more densely arranged collagen bundles. The subcutaneous fat, a layer of adipose tissue that connects the skin to the superficial fascia, was sharply dermarcated from the dermis.

The dermis and subcutaneous fat layer of normal skin samples were well vascularised, with arterioles and venules of the cutaneous microcirculation forming two plexuses. The upper papillary plexus is a horizontal network in the papillary dermis from which the nutritive capillary loops of the dermal papillae arise. The lower horizontal plexus (rete cutaneum), found at the dermal-subcutaneous interface, is formed by perforating vessels from the underlying muscle and fat tissue, giving rise to arterioles and venules that directly connect the lower plexus to the upper plexus, and provide a blood supply to sweat glands and hair follicles. There were few mesenchymal cells scattered within the dermal layers and inflammatory cells were scarce. There was no evidence of erythrocyte extravasation or fibrin deposition by Martius Scarlet Blue staining or immunohistochemistry.

Skin associated with chronic venous insufficiency without LDS (Class 3)

Distal leg skin taken from patients classified as CEAP Class 3 showed a similar histological appearance to control skin (Figure 3.1B) following H&E, Masson Trichrome and Martius Scarlet Blue staining, with no evidence of fibrin deposition or increased

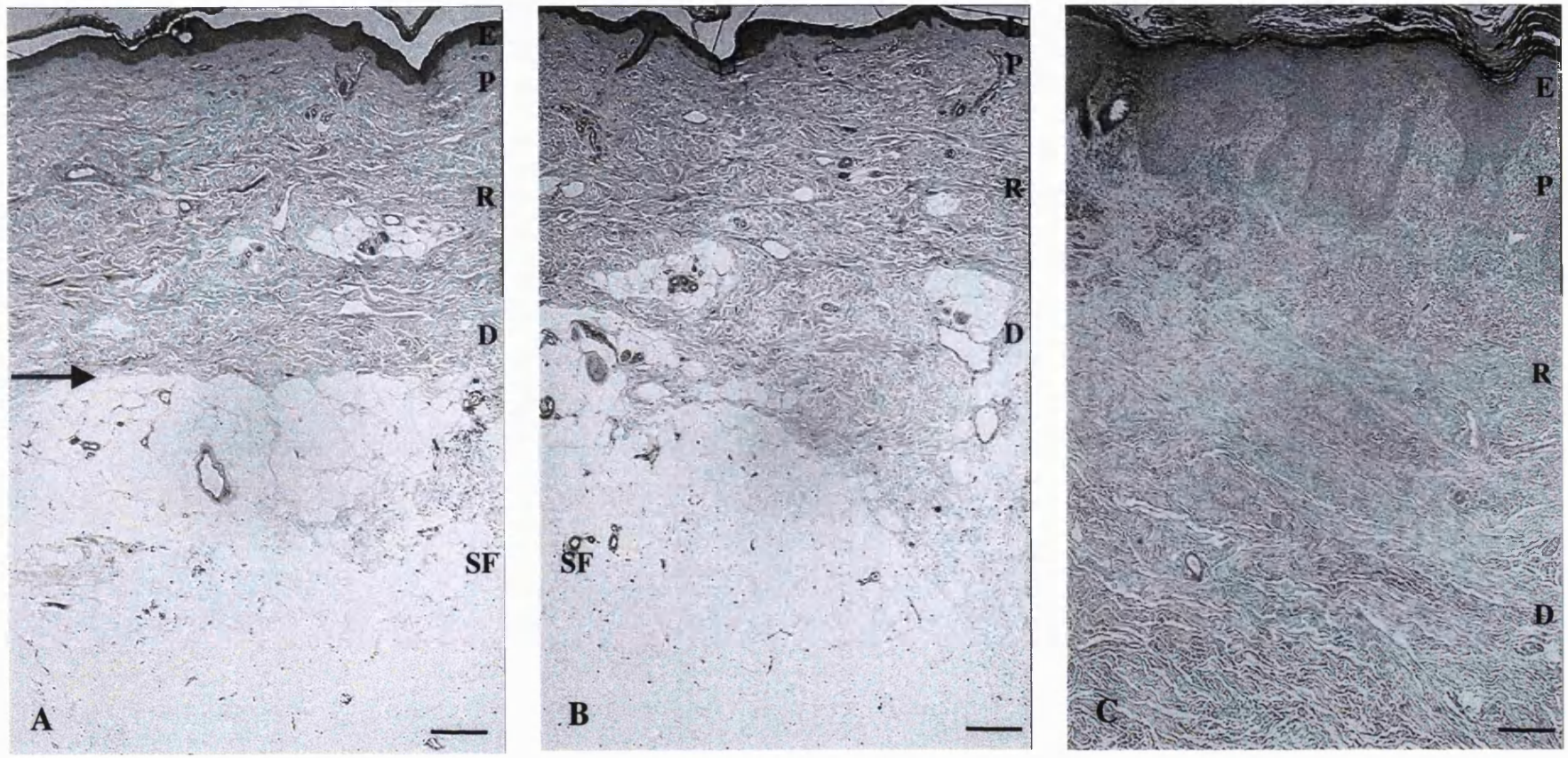


Figure 3.1. Histological appearance of normal skin (A), Class 3 skin (B) and Class 4 LDS skin (C) from the distal leg. The epidermis (E) rests above the dermis, which is divided into papillary (P), reticular (R) and deep layers (D). The dermal-subcutaneous fat (SF) interface is well-defined (arrow). In Class 4 LDS skin, the epidermis was thickened and irregular with a significantly deeper dermis and complete disruption of the dermal-subcutaneous fat interface.Bar represents 100μm.

extracellular matrix deposition. However, although the subcutaneous fat layer appeared normal in the majority of patients, three biopsies showed minor disruption of the dermal-subcutaneous fat layer interface with collagen deposited in a disorientated manner.

LDS skin (Class 4)

The histological appearance of LDS skin was markedly different from normal skin and Class 3 skin (Figure 3.1C). The epidermis of LDS skin showed an abnormal and irregular thickening, or acanthosis, particularly marked in the rete pegs (epidermal papillae) (Figure 3.2A). In some samples, the epidermis showed a thickening of the keratin layer (hyperkeratosis) and spongiosis, the separation of the epithelial cells due to accumulation of fluid, a prominent feature of acute dermatitis. The thickness of the epidermis was measured using image analysis and was significantly thicker in LDS skin (0.268 ± 0.08mm) compared with control skin (0.076 ± 0.008mm) and skin from Class 3 patients (0.075 ± 0.006 mm) ($p<0.01$).

LDS skin had the same distribution of dermal components as control skin but the architecture of the deep dermal and subcutaneous fat layers was significantly different. A prominent feature of all the specimens was a thickened dermis with densely arranged collagen fibres, often aligned in parallel bundles (Figure 3.2B). This was accompanied by a substantial reduction in the subcutaneous fat layer thickness, and in some samples little fat tissue was present. Skin samples from control patients showed a clear subcutaneous-dermal interface, however, this divide was irregular and undefined in LDS skin. Indeed, the increase in the depth of the dermis was confirmed by measuring the distance from the base of the epidermis to the subcutaneous fat layer, or the bottom of the biopsy if no fat layer was present. The biopsies were of a standard depth in all patient groups (3.756 ± 0.213 mm (n=36)). The depth of the dermal layer was significantly increased in LDS skin (2.97 ± 0.21mm) compared with control skin (1.76 ± 0.12mm, $p<0.01$) and Class 3 skin (1.51 ± 0.1mm) (Figure 3.3).

Another feature of LDS skin was an increased number of spindle-shaped cells, fibroblast-like in morphology, throughout the interstitium and clustered around blood vessels in the

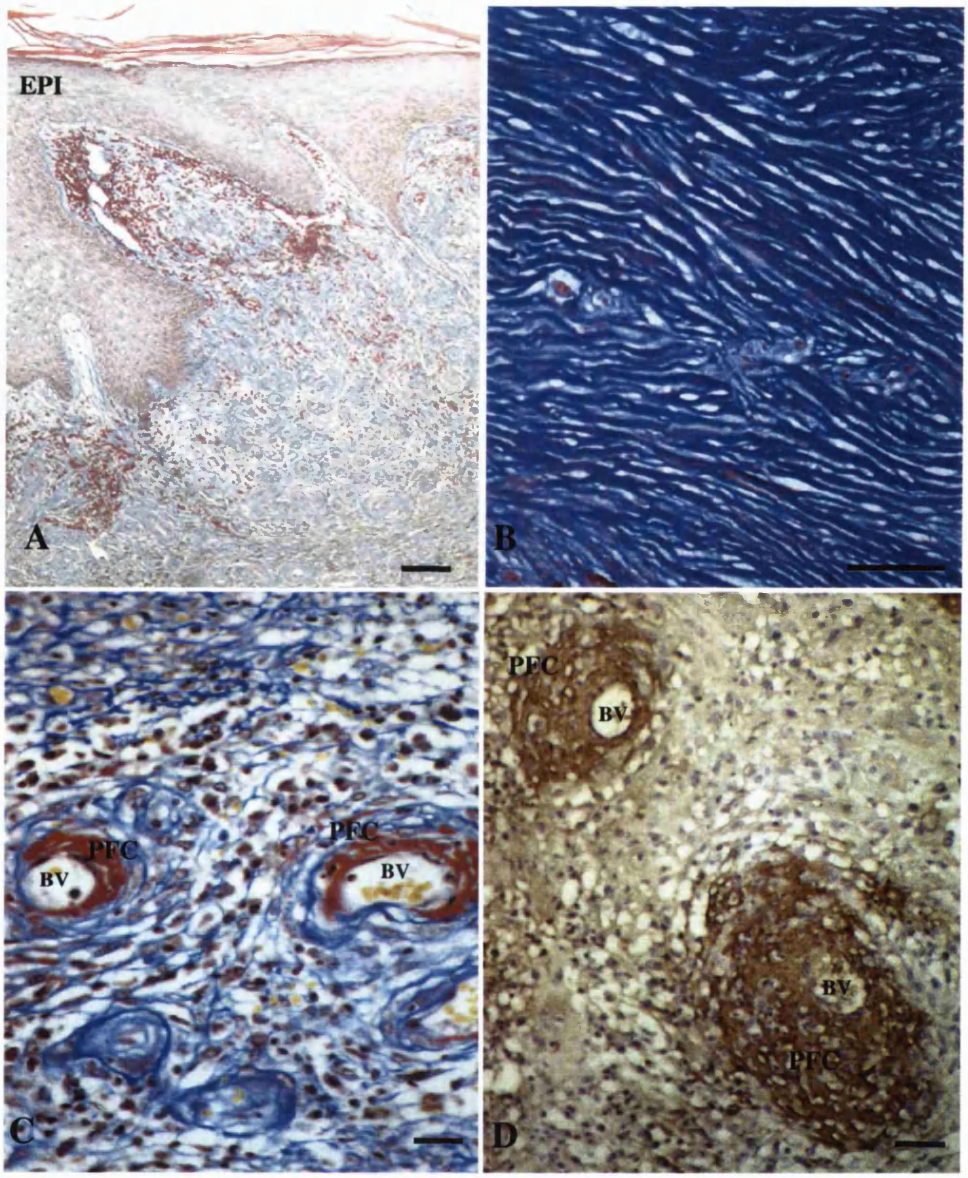


Figure 3.2 Prominent histological features of lipodermatosclerosis
A. Thickened and irregular epidermis (EPI) in LDS skin. **B**. Parallel-arranged collagen bundles (blue) in the dermis of LDS skin. **C**. Pericapillary fibrin cuffs (PFC, red) with collagen bundles (blue), extravasated erythrocytes (yellow) and cellular infiltrate (purple) in dermis of LDS skin. **D.** Diffuse leakage of plasma fibrinogen from blood vessels (BV) and pericapillary fibrin cuff (PFC, brown) formation in dermis of LDS skin, shown by immunohistochemistry for fibrin(ogen). Bar represents 50μm.

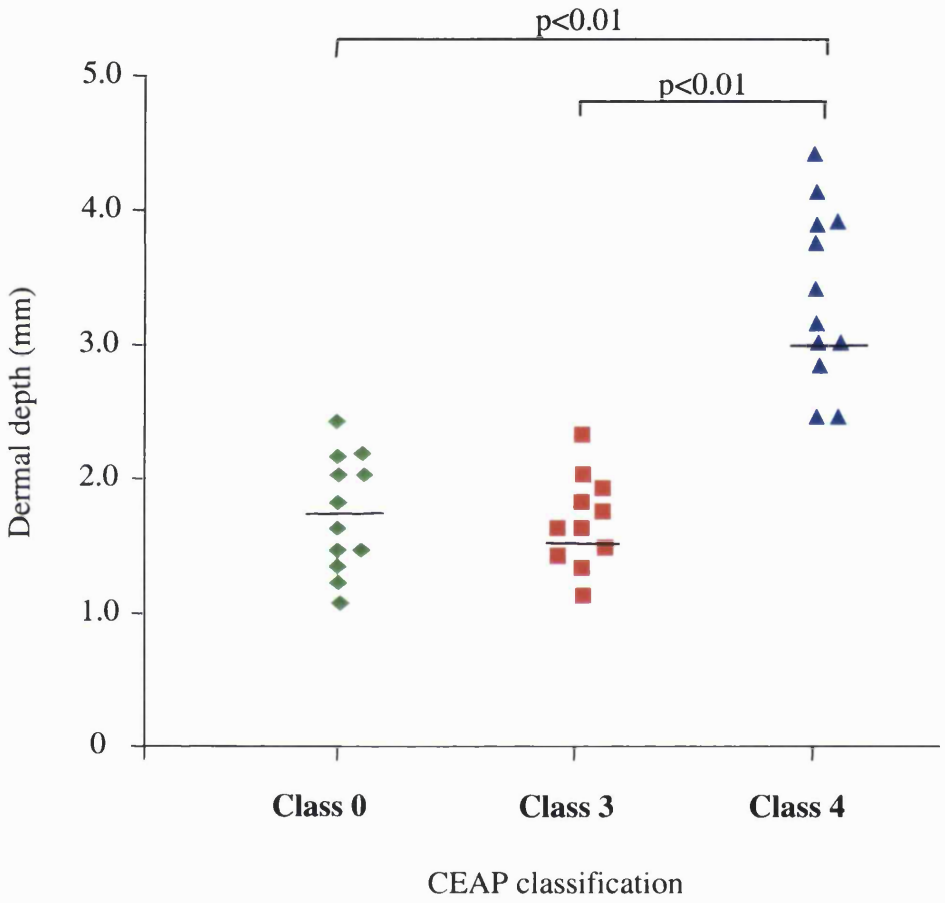


Figure 3.3 Dermal depth measurements of CEAP Class 0, 3 and 4 skin samples. Mean depth of dermis was significantly increased in Class 4 LDS skin samples compared with Class 3 and Class 0 controls (n=12).

reticular and deep dermal layers. Furthermore, there was an accumulation of brown-pigmented haemosiderin in the papillary dermis, a by-product of erythrocyte degradation, which appeared to be both cellular and deposited in the interstitium. Blood vessels in the LDS-affected tissue were generally enlarged, with thickened walls and larger vessel lumens. In the papillary dermis, there were clusters of small vessels that were often surrounded by a dense collagen matrix and numerous extravasated erythrocytes, not observed in normal skin. Immunocytochemical techniques using antibodies against von Willebrand factor, showed an increase in the number of blood vessels in several LDS skin samples, however, this was not significant compared with controls (Figure 3.7A).

Martius scarlet blue trichrome staining demonstrated the presence of pericapillary fibrin cuffs surrounding many of the smaller blood vessels in the papillary and reticular layers of the dermis, but were predominantly observed around the larger vessels of the upper capillary plexus (Figure 3.2C). Immunolocalisation of fibrin(ogen) showed that fibrin cuffs were larger and in greater numbers than originally shown by the histological trichrome stain (Figure 3.2D). In addition, fibrin(ogen) was observed around blood vessels in the papillary, reticular and deep dermal layers, although no positive staining was found around vessels in the subcutaneous fat layer.

3.2.2 Procollagen type I gene expression in LDS skin compared with control skin

To assess the distribution and number of cells producing collagen in LDS skin, digoxigenin-labelled riboprobes were used to stain procollagen mRNA-expressing cells in skin sections, by *in situ* hybridisation. Hybridisation with the anti-sense riboprobe showed specific procollagen type-I gene expression in the skin samples examined (Figure 3.4 A, B and C) and this was confirmed using scleroderma and post-operative adhesion samples as positive controls (data not shown). Negative controls using the 'sense' probe showed an absence of non-specific binding (Figure 3.4D).

Positive cells appeared to be 'spindle-like' in nature, suggesting that fibroblasts were the major cells expressing the procollagen type I gene. Cells appeared to cluster around thickened capillaries but were also abundant between collagen fibres, especially in the

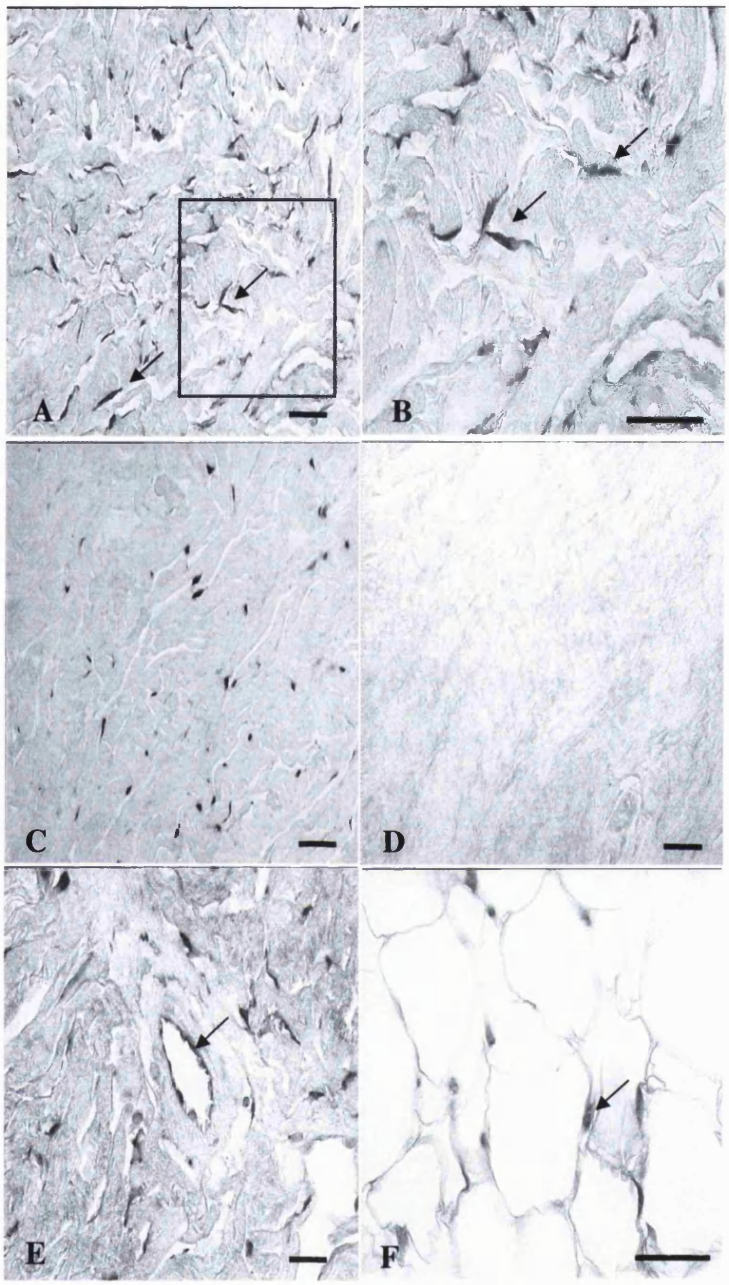


Figure 3.4 ***In situ*** **hybridisation of procollagen α1(I) mRNA in the dermis of LDS skin (A and B, low and high magnification) (black), compared with control skin (C). D**. No non-specific staining of sense riboprobe in dermis of LDS skin. **E**. Procollagen type I gene expression by vascular endothelial cells in LDS skin (arrow). **F**. Procollagen type I gene expression by fibroblast-like cells in the subcutaneous fat septae (arrow). Bar represents 50μm.

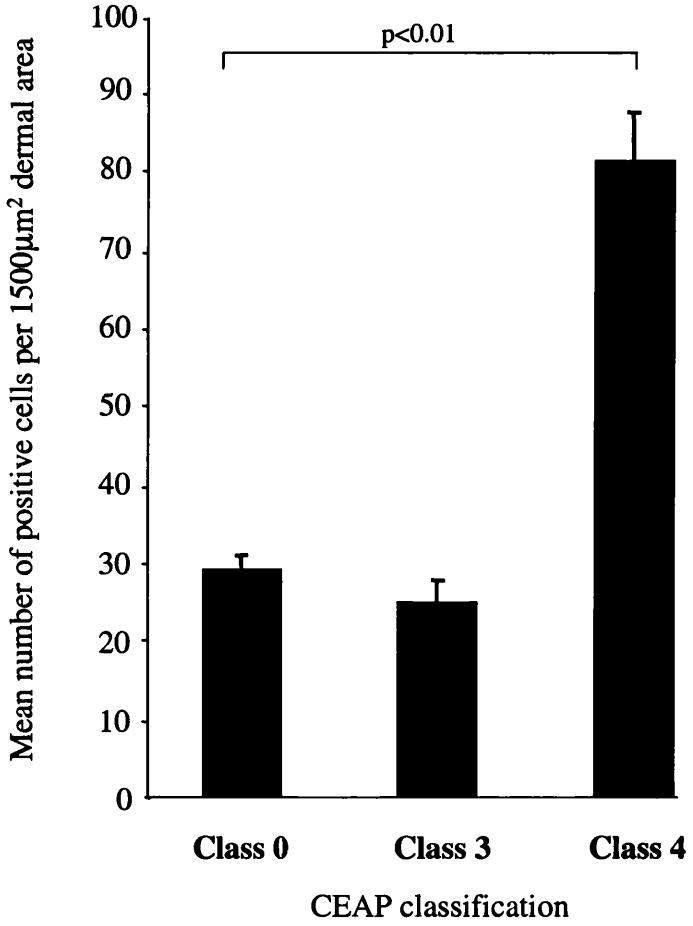


Figure 3.5. Procollagen type I (α(I)) gene expression in LDS
The number of cells expressing procollagen type I was significantly increased in CEAP Class 4 LDS skin compared to CEAP Class 3 and Class 0 controls by *in situ* hybridisation (mean ± standard error, n=12).

lower dermis. Positive staining was often observed in the vascular endothelium of vessels in the deep dermis of LDS skin, not observed in control or Class 3 samples (Figure 3.4E). Fibroblasts of the thickened fat septae appeared to be actively expressing procollagen type I mRNA in LDS samples (Figure 3.4F). Overall, there was a significantly increased number of positive cells in LDS samples compared with controls ($p<0.01$, Figure 3.5). In addition, the distribution of positive cells within each layer varied with a significantly greater number of cells in the deep and reticular dermal regions of LDS compared with both Class 3 and control skin ($p<0.01$, Figure 3.6).

To determine whether there was an increased proportion of cells producing procollagen mRNA or an overall increase in the number of cells per unit area of the dermis, the distribution of all mesenchymal cells in the skin was examined by immunocytochemical detection of vimentin (Figure 3.7B and C). There was a significantly greater number of positive cells in LDS skin (539.55 ± 112.76) compared with Class 3 skin (251.33 ± 30.26) and control skin (292.46 ± 41.6) ($p<0.01$). Overall, there were no differences in the proportion of fibroblasts producing procollagen type I (by *in situ* hybridisation) in the dermis of LDS skin (16.5 ± 2.8%) compared with CVI skin (15.3 ± 1.6%) and normal skin (14.8 ± 1.8%). Although an increase in the proportion of procollagen-producing fibroblasts was observed in the deep dermis of LDS skin (22.3 ± 5.5%) when compared with Class 0 controls (15.3 ± 2.3%), it was not statistically significant.

3.2.3 Cell proliferation in LDS skin compared with control skin

As well as an increase in the production of collagen, fibrosis is also characterised by enhanced cell proliferation and therefore PCNA immunocytochemistry was used to examine cell proliferation in all skin samples. Immunocytochemistry showed proliferation of the basal keratinocytes in all sections and this was used as a positive control (Figure 3.7D). Cell proliferation was more abundant in the papillary dermis, compared to the reticular and deep dermal layers in skin from all patient groups (Figure 3.8A). There was a significant increase in the number of proliferating cells in LDS skin compared to controls, and in skin from Class 3 patients compared to Class 0 controls

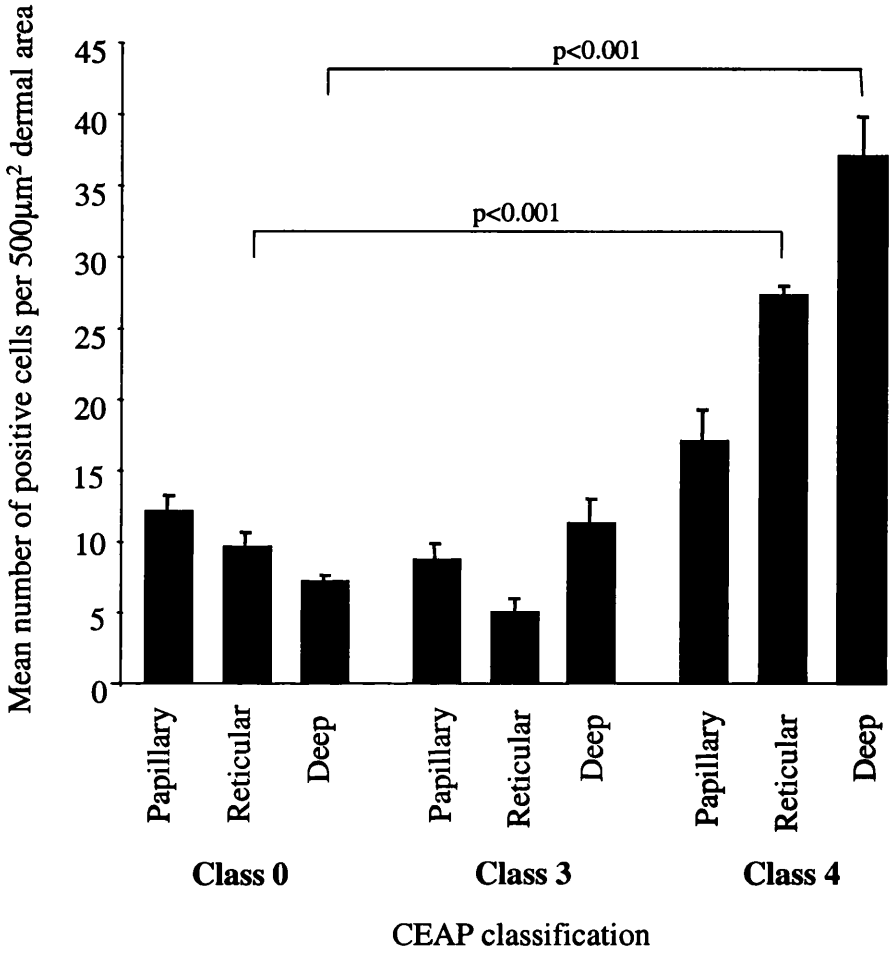


Figure 3.6. Procollagen type I (α(I)) gene expression in LDS
The number of procollagen type I expressing cells was significantly increased in the reticular and deep dermal layers of Class 4 LDS skin compared to CEAP Class 3 and Class 0 controls by *in situ* hybridisation (mean ± standard error, n=12).

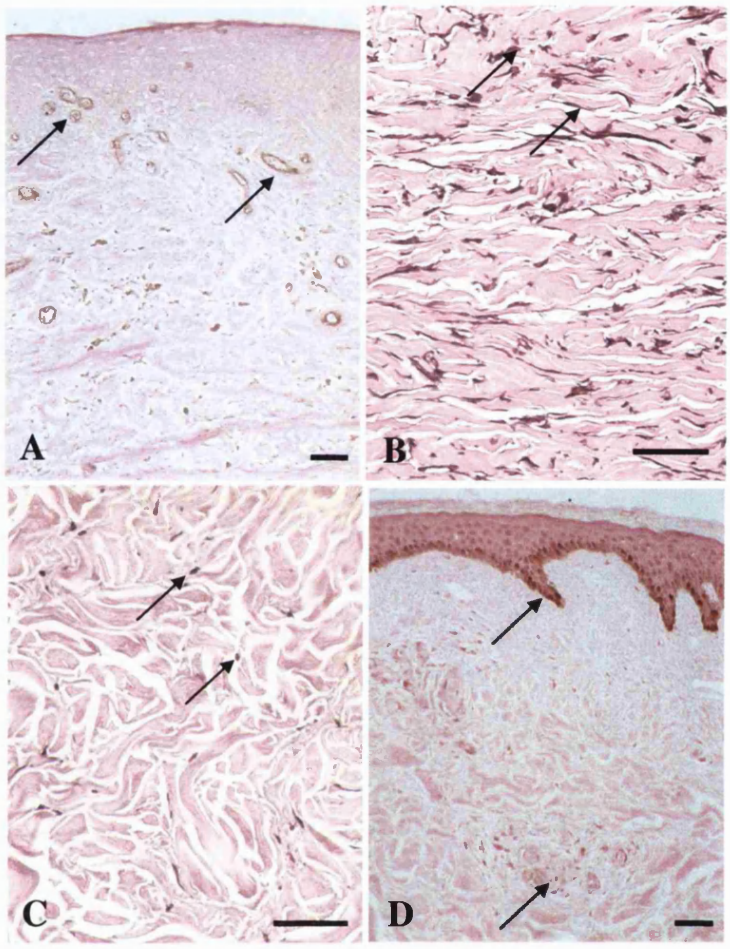
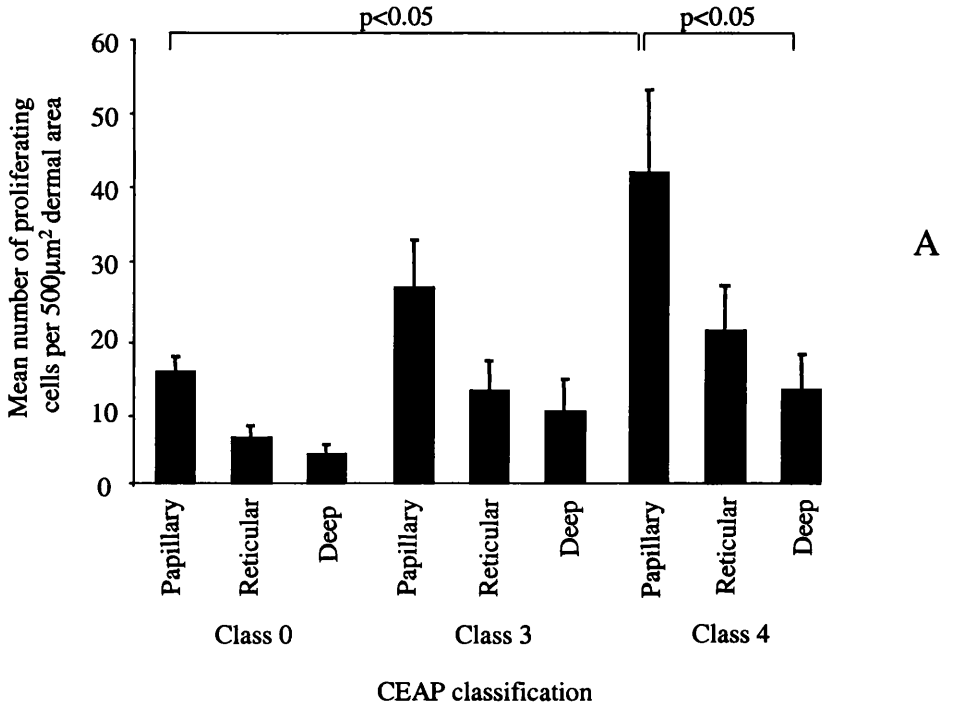


Figure 3.7. Histological appearance of lipodermatosclerosis.
A. Distribution of blood vessels (arrows) in LDS skin compared with control skin, shown by immunohistochemistry for von Willebrand factor. **B**. Increased numbers of fibroblasts (arrows) in the dermis of LDS skin compared with control skin (**C**), shown by immunocytochemistry for vimentin. **D**. Cell proliferation in LDS skin by PCNA immunocytochemistry, demonstrating proliferation of the basal keratinocyte layer (arrow) and positive cells in the dermis (arrow). Bar represents 50μm



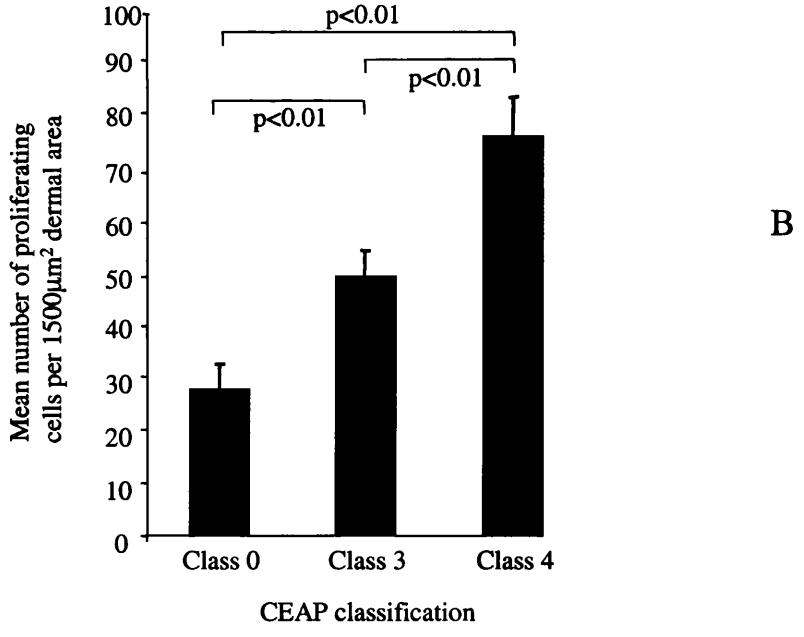


Figure 3.8. Cell proliferation in LDS skin

A. There was a significant increase in the number of proliferating cells in the papillary dermis of LDS skin compared with Class 3 and Class 0 controls, assessed by PCNA immunocytochemistry. **B.** Cell proliferation was enhanced in the skin of patients with Class 4 LDS and Class 3 compared to Class 0 controls (mean ± standard error, n=12).

($p<0.01$, Figure 3.8B). In LDS skin, proliferating cells were observed surrounding small blood vessels in the papillary dermis, although numerous cells were also positively stained in the interstitium. Evidence of cell proliferation in the subcutaneous fat septae was not observed in any of the skin samples.

3.2.4 Inflammatory cell distribution

To determine the source of potential mediators of collagen synthesis by fibroblasts, the distribution of inflammatory cells was examined. Inflammatory cells including macrophages, mast cells, neutrophils and T-lymphocytes were visualised immunocytochemically using well-characterised primary antibodies (Figures 3.9 and 3.10). There was no significant increase in the number of macrophages in LDS skin compared with control skin (Figure 3.11). However, the number of macrophages was significantly greater in the papillary dermis compared with reticular and deep dermal layers in all patient groups. In the control skin, macrophages were generally observed in or around the larger vessels of the papillary dermis whereas in LDS they appeared to be more widespread (Figure 3.9 A and B). Few macrophages were observed in the subcutaneous fat layer in any patient group.

As with macrophages, there was no significant increase in the number of mast cells or neutrophils in the skin of patients with LDS compared to Class 3 alone and controls (Figure 3.9 C and D). However, there were significantly greater numbers of mast cells in the papillary dermis of all three patient groups compared to the reticular and deep dermal layers (Figure 3.11). Mast cells were usually observed in and around the major blood vessels of the upper plexus in the papillary layer of control skin and Class 3 skin, in a similar distribution to the neutrophils. However, mast cells in LDS skin were often seen in the interstitium a distance from the vasculature, close to the basement membrane and basal keratinocyte layer of the epidermis. Neutrophils were usually observed in close proximity to blood vessels, with few cells in the deep and subcutaneous layers of the skin (Figure 3.10A and B). There was no increase in the number of T-lymphocytes in LDS skin compared with controls (data not shown). However, unlike macrophages,

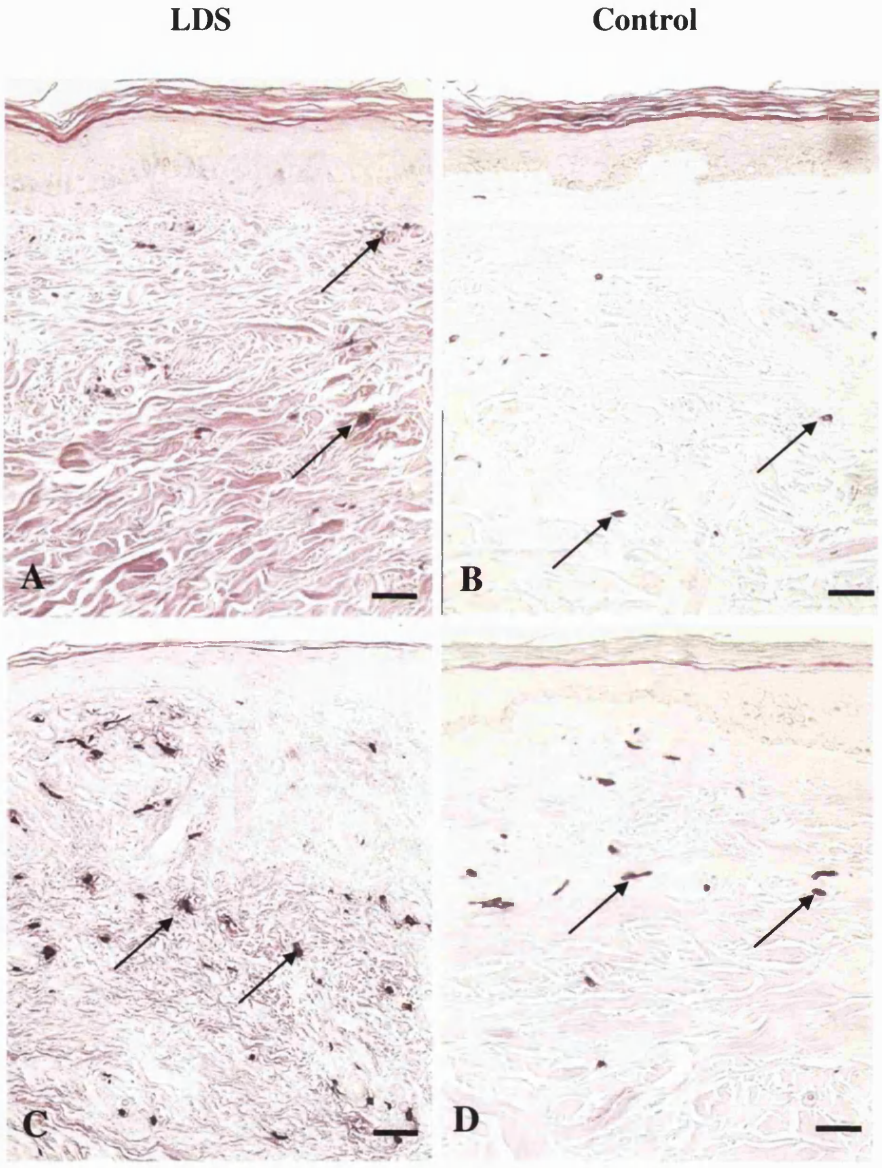


Figure 3.9 Inflammatory cell distribution in LDS skin by immunocytochemistry
A. Macrophages (CD68) (arrows) in LDS skin compared with control skin (**B**). **C**. Mast cell tryptase demonstrating presence of mast cells (arrows) in the dermis of LDS skin, compared with normal skin (**D**). Bar represents 50μm.

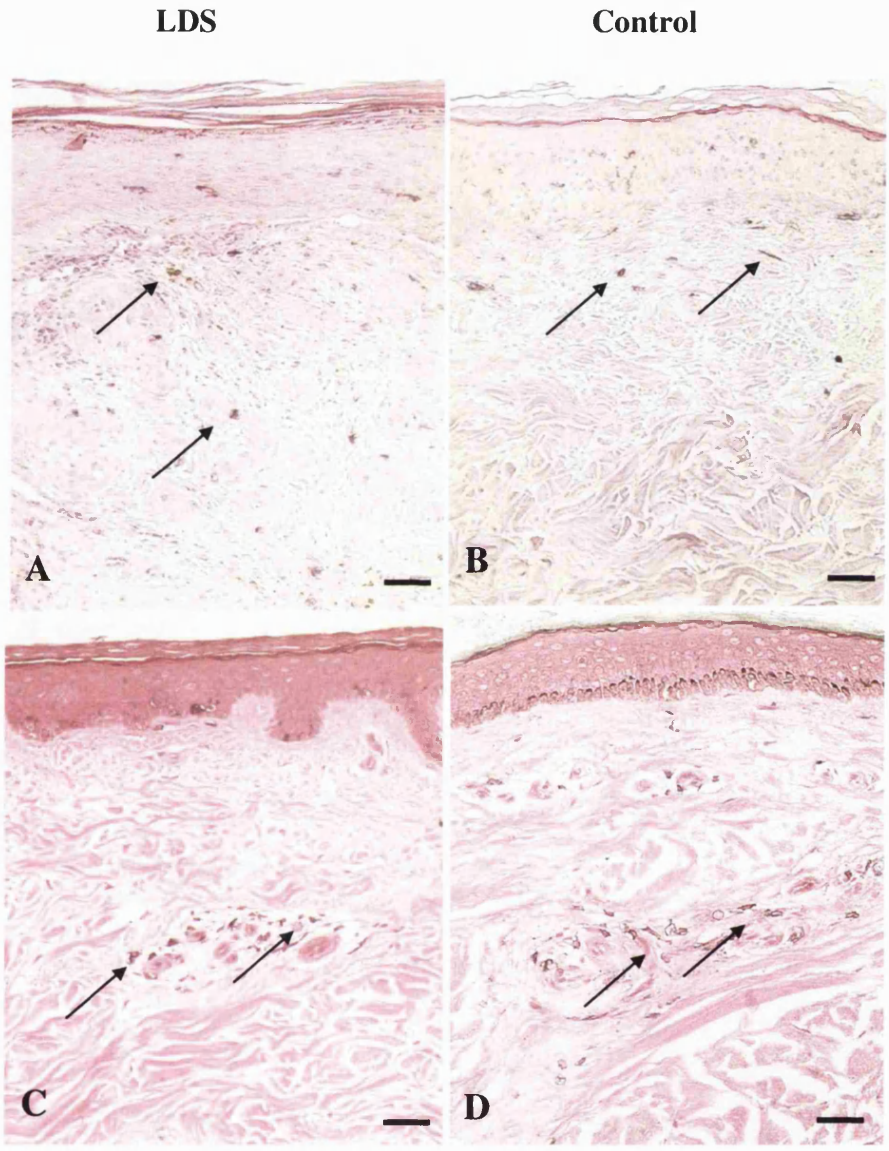


Figure 3.10 Inflammatory cell distribution in LDS skin by immunocytochemistry
A. Neutrophils (CD15s) (arrows) in the dermis of LDS skin compared with control skin (**B**). **C**. T-lymphocytes (CD3) (arrows) in the dermis of LDS skin and normal skin (**D**). Bar represents 50μm.

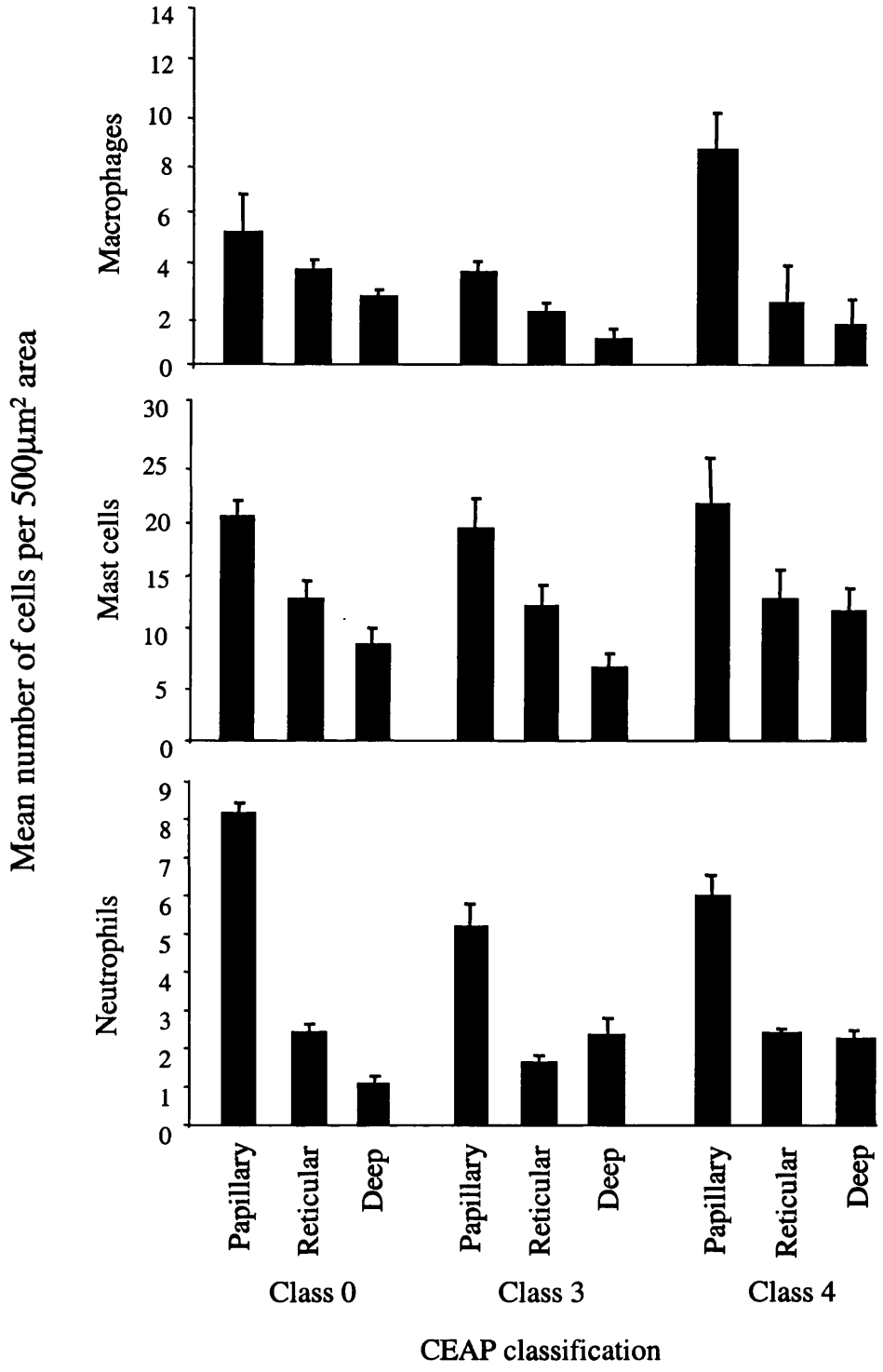


Figure 3.11. Distribution of inflammatory cells in LDS skin
There was no significant increase in the number of macrophages, mast cells or neutrophils in the skin of patients with CEAP Class 4 LDS compared to Class 3 and Class 0 controls (mean ± standard error, n=12).

neutrophils and mast cells, there were no differences in the distribution of T-lymphocytes in the dermal layers (Figure 3.10C and D).

In summary, the most striking histological change in LDS skin was observed in the dermal and subcutaneous fat layers, with complete disruption of the interface resulting in the extension of the dermis into the subcutaneous fat layer. This dermal expansion appeared to be due to increased deposition of extracellular matrix, in particular collagen. This was confirmed by *in situ* hybridisation demonstrating a significantly increased number of cells producing procollagen type I mRNA in LDS skin compared with Class 3 and control skin. However, there was no increase in the proportion of fibroblasts actively producing procollagen type I in LDS skin. Cell proliferation was significantly increased in both Class 4 LDS skin and Class 3 skin, compared with controls, indicating a progression in the pathogenesis of venous disease in that proliferation may precede collagen production. There was no evidence of increased inflammatory cell infiltrate in LDS skin by immunocytochemistry. A prominent feature of LDS skin was the abundant deposition of fibrin in the dermal and subcutaneous fat layers, particularly surrounding blood vessels as pericapillary fibrin cuffs.

3.3 Discussion

Previously, there was a great deal of uncertainty regarding the histological nature of LDS, resulting in problems in defining its development. Many aspects of the pathophysiology of venous disease remain to be clarified, in particular, the consequences of venous insufficiency which lead to fibrotic skin changes of LDS and the development to skin ulceration. To address this issue, a series of biopsies were collected from three patient groups representing progressive stages of venous disease, classified by the CEAP system (Porter and Moneta, 1995). These included patients from Class 0 (no signs of venous disease), Class 3 with chronic venous insufficiency but without skin changes and Class 4 patients with LDS.

Histological assessment of LDS biopsies (Class 4 CEAP) demonstrated extensive fibrotic changes in the dermal and subcutaneous layers characterised by excessive accumulation of collagen, in comparison with control skin biopsies. These findings support those of others demonstrating that LDS skin is composed of excessive quantities of collagen type I by HPLC (Brinckmann et al., 1999) and immunolocalisation (Higley et al., 1995). In addition, the interface between the dermal and subcutaneous fat layer was not defined due to thickening of the dermis and/or remodelling of the fat tissue. These findings imply that a tissue repair process is initiated in LDS since the deposition of fibrous tissue is a feature of repair rather than destruction. Other fibrotic conditions, including scleroderma and keloid scarring are also characterised by extensive deposition of collagen in the dermis and subcutaneous fat tissues (Browse et al., 1977). There is strong evidence to suggest that the underlying mechanism leading to increased collagen deposition in these disorders is enhanced procollagen gene expression by dermal fibroblasts (Peltonen et al., 1990).

To examine if the accumulation of collagen in LDS skin was due to increased collagen synthesis, *in situ* hybridisation was performed using specific procollagen type I riboprobes. Collagen gene expression by fibroblasts was indeed increased in LDS skin compared with Class 3 and Class 0 controls, with significantly increased numbers of procollagen type I-producing cells in the dermal and subcutaneous fat layers. The presence of procollagen-producing cells in the subcutaneous fat septae support the

hypothesis of Browse (1982), suggesting that there was progressive replacement of the subcutaneous fat layer by fibrotic tissue. This is the first evidence to fully support the theory that the subcutaneous fat is progressively replaced by collagen-rich tissue in LDS. The differentiation of fat-storing cells is also known to occur in fibrotic liver disease (Abdel-Aziz et al., 1991). Fat-storing cells differentiate into a more fibroblast-like phenotype, thereby capable of producing excessive amounts of ECM molecules, such as fibrillar collagens type I and III, and fibronectin. It is possible that chronic venous hypertension causes vascular injury to the subcutaneous layer, possibly via mechanical trauma or by leukocyte activation and free radical damage. Injury to the vasculature may result in leakage of plasma factors into the interstitium, in particular fibrinogen and its degradation products, which may mediate the production of ECM proteins by fibroblast-like cells in the subcutaneous fat septae.

Immunocytochemical detection of the mesenchymal marker, vimentin, demonstrated that there was no change in the proportion of fibroblasts producing procollagen in both LDS and in control samples, but that the overall number of fibroblasts in LDS skin was significantly increased compared with control samples. Studies have shown that fibroblasts consist of several heterogeneous populations of cells (Fries et al., 1994) and that fibroblast hyperplasia and excessive matrix deposition observed in fibrotic disorders such as scleroderma and pulmonary fibrosis, may be the consequence of a convergence of a fibroblast subset, positively selected during an inflammatory response (Jeleska et al., 1996). Data from the present study suggests that such a fibroblast population in the deep dermis of patients with LDS does not exist, but collagen accumulation in LDS may be the result of an increased number of fibroblasts in the tissue, either due to migration from local tissues or enhanced proliferation of resident fibroblasts.

Immunolocalisation of proliferating cells showed that there was significantly increased proliferation in LDS tissues compared with Class 3 and control skin, mainly in the papillary dermis. In addition, Class 3 skin had significantly increased numbers of proliferating cells compared with control skin. One of the aims of this study was to correlate the clinical features of venous disease progression with histological

characteristics and to assess whether such a progression of events exists. As only LDS skin (Class 4) showed increased numbers of procollagen-producing cells, whereas Class 4 and Class 3 showed increased proliferation compared with controls, data suggests that cell proliferation precedes collagen gene expression and deposition in the skin of patients with chronic venous disease. This progression in cellular events in venous disease has not been demonstrated before. However, the mechanisms and factors that cause some patients to progress from Class 3 to Class 4 skin changes remain unknown. Examination of the patient clinical data showed that there was no correlation between severity and duration of venous disease, or indeed the age of the patients in Classes 3 and 4, indicating that other factors in combination with the effects of venous hypertension play a role in mediating the development of fibrotic skin changes.

Many groups have suggested that 'white blood cell trapping' in the microcirculation of the skin may be the underlying cause of LDS and subsequent ulceration in patients with chronic venous disease (Coleridge-Smith et al., 1988; Thomas et al., 1988; Scott et al., 1991; Rosner et al., 1995; Saharay et al., 1997). Studies have provided histological evidence for an increased inflammatory cell infiltration, in particular macrophages and T-lymphocytes, in the skin of patients with LDS (Thomas et al., 1988; Scott et al., 1991; Rosner et al., 1995; Saharay et al., 1997). However, in contrast to these studies, our results suggest that there was no increase in the number of macrophages, neutrophils, mast cells or T-lymphocytes in the skin of patients with LDS. This data agrees with the findings of Pappas and coworkers (1997), which had also divided patients into classes according to CEAP classification of venous disease (Porter et al., 1995). The discrepancies between these observations and previous reports may be due to the fact that prior reports were restricted to qualitative descriptions of ulcer-affected LDS skin, with insignificant numbers of patients and non-uniform biopsy sites and sizes. In failing to discriminate between ulcer-affected LDS skin and LDS skin not complicated by ulceration, the resultant inflammatory profiles may have been biased by the presence of low grade infection and inflammation associated with chronic wounds.

An alternative explanation for the absence of inflammation observed in the LDS tissues in the current study, may be due to the time of sampling. The patient age, duration and severity of chronic venous insufficiency gave no indication of the rate of progression of LDS. Therefore, it is a possibility that the inflammatory phase causing the LDS skin changes may have resolved. In addition, it may be that the inflammatory phase preceding LDS skin changes was still to occur in the Class 3 patients, and thus chronic inflammatory infiltrates were not observed in either patient group. However, this proposal seems unlikely given the number of patients used in each group. Similar findings by Kirsner et al. (1993) reported that the final stage in the histological progression of the disease included pronounced sclerosis of the subcutaneous layer with diminished or absent inflammatory infiltrate. It is possible that the patients used in our study were at the final stage of LDS progression, as the histological observations of the current study were similar to those described by Kirsner as the second 'chronic phase'. All of the patients in Class 3 CVI and Class 4 LDS groups in the present study were undergoing varicose vein surgery, and therefore a defined set of patients with similar venous disease pathologies were used, all having incompetent superficial veins. No other study has used samples from such a defined set of patients, and therefore patient groups of other studies may include individuals with alternative venous disease pathologies, including calf muscle pump dysfunction or incompetent deep or perforating veins. On the basis of this evidence, it becomes increasingly more important to sub-classify patients with LDS, perhaps by histological skin changes, and to correlate those skin changes with other factors such as degree and duration of venous insufficiency. Perhaps in this way, the progression and pathogenesis of the disease may be further elucidated.

Although fibrosis is usually associated with increased inflammatory cell infiltrates and elevated growth factor levels (Mutsaers et al., 1997), the absence of inflammation in LDS skin suggests a substantial reduction in the levels of inflammatory mediators of collagen synthesis, such as transforming growth factor-β. The indications are that the up-regulation of procollagen gene expression in LDS skin may involve an alternative mechanism. A prominent feature of LDS skin was excessive fibrin deposition in the dermis. Dvorak (1986) noted that collagen is deposited in intimate association with pre-

existing fibrin and therefore enhanced procollagen gene expression observed in patients with LDS may be due to the effects of fibrin(ogen) and/or its degradation products. *In vitro* evidence suggests that fibrin matrix provides a provisional scaffold which is associated with increased collagen synthesis (Coustry et al., 1990; Gillery et al., 1989) and cell proliferation (Tuan et al., 1996). Moreover, persistent fibrin deposition has been associated with many fibrotic disorders, including scleroderma (Ames et al., 1997) pulmonary fibrosis, acute respiratory distress syndrome, atherosclerosis and tumorigenesis (Brown et al., 1989).

Fibrinogen has been shown to exist in numerous structural forms, many of which have been shown to differ in functional properties and levels (Henschen-Edman, 1995). Polymorphisms in the fibrinogen gene may result in an increased thrombotic potential, either through altered fibrinogen levels or changes in the molecular structure. This may make individuals more susceptible to deep vein thromboses in the superficial venous system, which have been demonstrated in 94% of patients with LDS (Gross et al., 1993). Degradation products of fibrin(ogen), such as fibrinopeptides A and B, have been shown to be potent mitogens and chemoattractants for fibroblasts and may play a role in regulating cell proliferation and migration in LDS (Gray et al., 1990, Gray et al., 1995). Coagulation cascade proteases such as thrombin and factor Xa have been shown to be potent inducers of proliferation (Chen et al.,1975 and Blanc-Brude, PhD thesis, 1999). Thrombin has been shown to be a potent inducer of procollagen synthesis and proliferation by fibroblasts (Chambers et al., 1998). The presence of pericapillary fibrin cuffs and excessive fibrin deposition in the interstitium demonstrates that thrombin is present in the interstitium of LDS skin, possibly residing within the fibrin matrix. Furthermore, patients with LDS have been shown to have partial or total deficiencies of coagulation cascade inhibitors protein C and protein S (Falanga et al., 1990) and these deficiencies may make patients more susceptible to deep vein thrombosis and the resultant venous hypertension, via superficial vein incompetence.

In summary, this study has shown that both procollagen type I gene expression and proliferation were increased in the skin of patients with LDS compared with Class 3

CEAP and control skin. In Class 3 samples, proliferation appeared to occur prior to procollagen type I gene expression, with increased numbers of proliferating cells compared with control skin. Procollagen type I-expressing cells were predominantly found in the deep dermal layers of LDS with some activity observed in the subcutaneous fat septae, suggesting that perhaps the dermis and the subcutaneous fat tissue undergo fibrosis simultaneously. There was no difference in the number of macrophages, neutrophils or lymphocytes in Class 3 or Class 4 skin compared with control skin suggesting that inflammation appears to have no role in the pathogenesis of LDS indicating an alternative mechanism of collagen accumulation. In accordance with previous reports, this study has shown that persistent fibrin deposition is a characteristic feature of LDS. Although the role of fibrin as a barrier to the diffusion of oxygen and nutrients remains in doubt, this thesis proposes that fibrin matrix is associated with the production of collagen by dermal fibroblasts. The next chapter will address this hypothesis, examining the production of collagen by dermal fibroblasts in fibrin matrix both *in vitro* and *in vivo*. Furthermore, persistent fibrin deposition through reduced fibrinolysis, results in further accumulation of collagen associated with LDS and other fibrotic diseases where persistent fibrin deposition is a key feature. The effects of persistent fibrin deposition and reduced fibrinolysis on collagen production will be examined in subsequent chapters.

Chapter 4
RESULTS AND DISCUSSION

The role of fibrin in the regulation of collagen production

4.1 Introduction

Excessive collagen accumulation in the dermis is the hallmark feature of LDS. The previous chapter showed an increased number of procollagen-producing fibroblasts in the skin of patients with LDS. Inflammatory cytokines and growth factors are proposed to be the main mediators of increased collagen synthesis in fibrotic disorders, however, only a mild inflammatory infiltrate was observed in LDS skin. Fibrin deposition throughout the dermis was also a prominent feature of LDS skin. It was proposed that collagen deposition is intimately associated with pre-existing fibrin deposits (Dvorak, 1986). Further evidence suggests that fibrin matrix is also associated with fibrotic conditions. Fibrin(ogen) and its degradation products have been shown to regulate several cellular functions, including fibroblast chemotaxis, proliferation and to stimulate angiogenesis. The aim of this chapter was to examine the hypothesis that collagen production by dermal fibroblasts is regulated by fibrin matrix, using both *in vitro* and *in vivo* model systems.

4.2 Results

In vitro results

The aim of these studies was to assess the effect of a three-dimensional fibrin matrix on collagen production by human dermal fibroblasts, compared with two-dimensional cultures involving fibrin monolayers or uncoated tissue culture plastic. Fibrin(ogen) matrices were prepared at physiological concentrations (9μM) but with decreased levels of thrombin (2nM) to polymerise the fibrin but yet prevent thrombin-mediated collagen production and/or cell proliferation. Two primary human dermal fibroblast cell lines (HSF-1 and HSF-5, passage 8-10), isolated from the skin of the lower leg from healthy humans were used in this study following characterisation.

4.2.1 Procollagen production

Procollagen production was assessed in all three systems by measuring hydroxyproline levels by HPLC analysis after 24, 48 and 72 hours in the presence or absence of the pro-fibrotic mediator, TGF-β1. Measurement of procollagen levels was normalised for cell number to eliminate any potential effect of cell proliferation. Proliferation studies at 24, 48 and 72 hours showed that there was no increase in cell number in any of the culture systems with or without TGF-β1 (Figure 4.1).

There was a significant increase in procollagen production by both human dermal fibroblast cell lines in three-dimensional fibrin gels after 24 ($p<0.05$), 48 and 72 hours ($p<0.01$), compared with fibrin and plastic monolayer cultures (Figures 4.2 and 4.3). Procollagen levels were increased two-fold in the fibrin gels compared with plastic monolayer cultures at 72 hours in both fibroblast cell lines. There was no significant difference in procollagen production by dermal fibroblasts on fibrin monolayers compared with tissue culture plastic controls at any time-point.

A significant increase in procollagen production by dermal fibroblasts was observed in response to TGF-β1 in both three-dimensional and monolayer cultures at 48 and 72 hours ($p<0.05$). Dermal fibroblasts in fibrin gels supplemented with TGF-β1 demonstrated up to a 1.5-fold increase in procollagen production at 48 and 72 hours, compared to cultures without the growth factor ($p<0.01$). In contrast, fibroblasts on tissue culture plastic in the presence of TGF-β1 demonstrated a 2-fold increase in procollagen production at 48 and 72 hours compared with fibroblasts in the absence of TGF-β1.

To confirm that the effect on procollagen production was due to the three-dimensional nature of the fibrin matrix, individual components of the fibrin matrix were examined for their ability to stimulate procollagen production by dermal fibroblasts. There was no significant effect on collagen production following the addition of fibrinogen in solution at a physiological concentration (9μM), compared

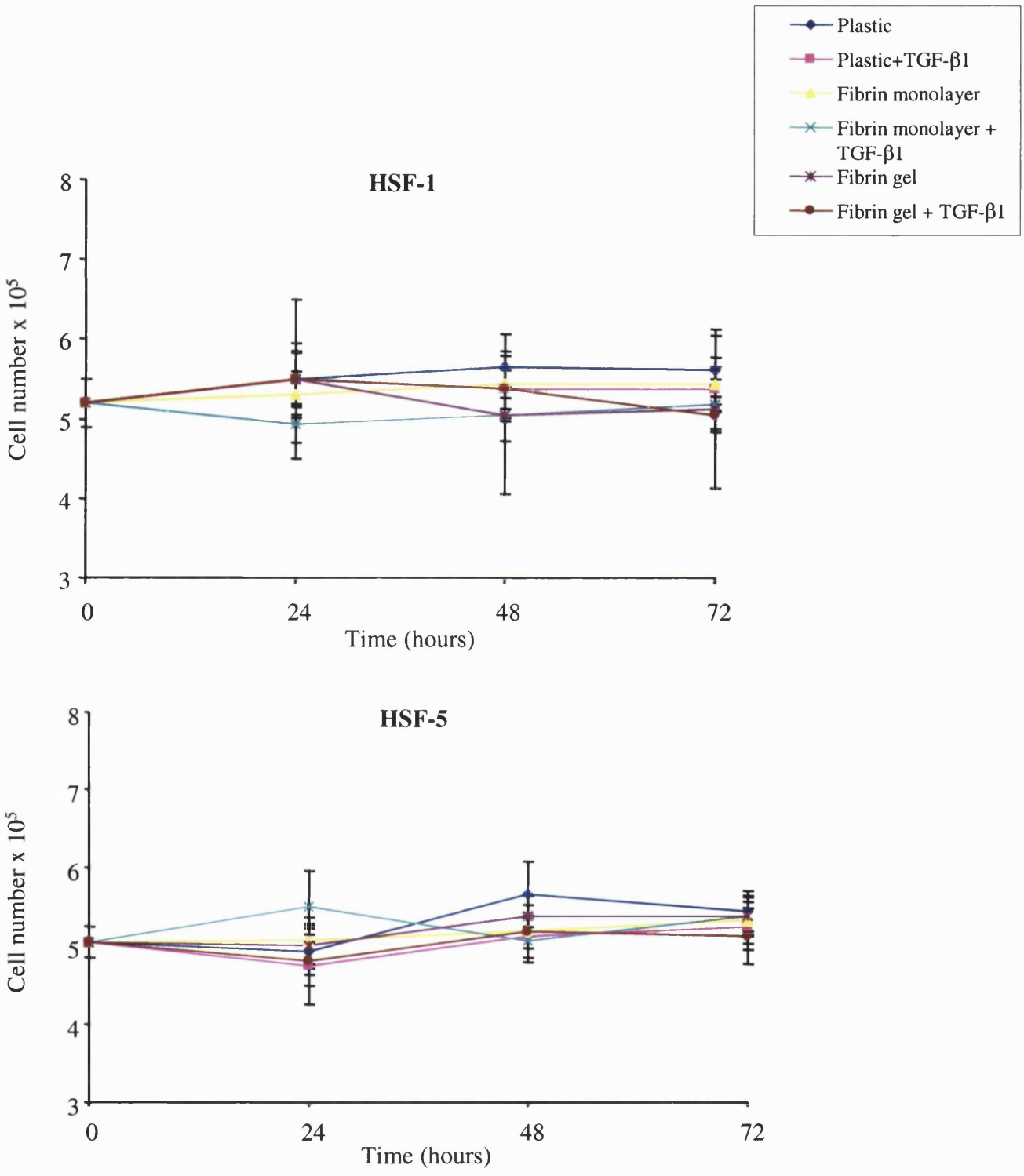


Figure 4.1. Proliferation of human dermal fibroblasts (HSF-1 and HSF-5) in three-dimensional fibrin gels, or on fibrin monolayers or tissue culture plastic controls after 24, 48 and 72 hours in the presence or absence of TGF-β1 (1ng/ml) (mean ± standard error, n=4).

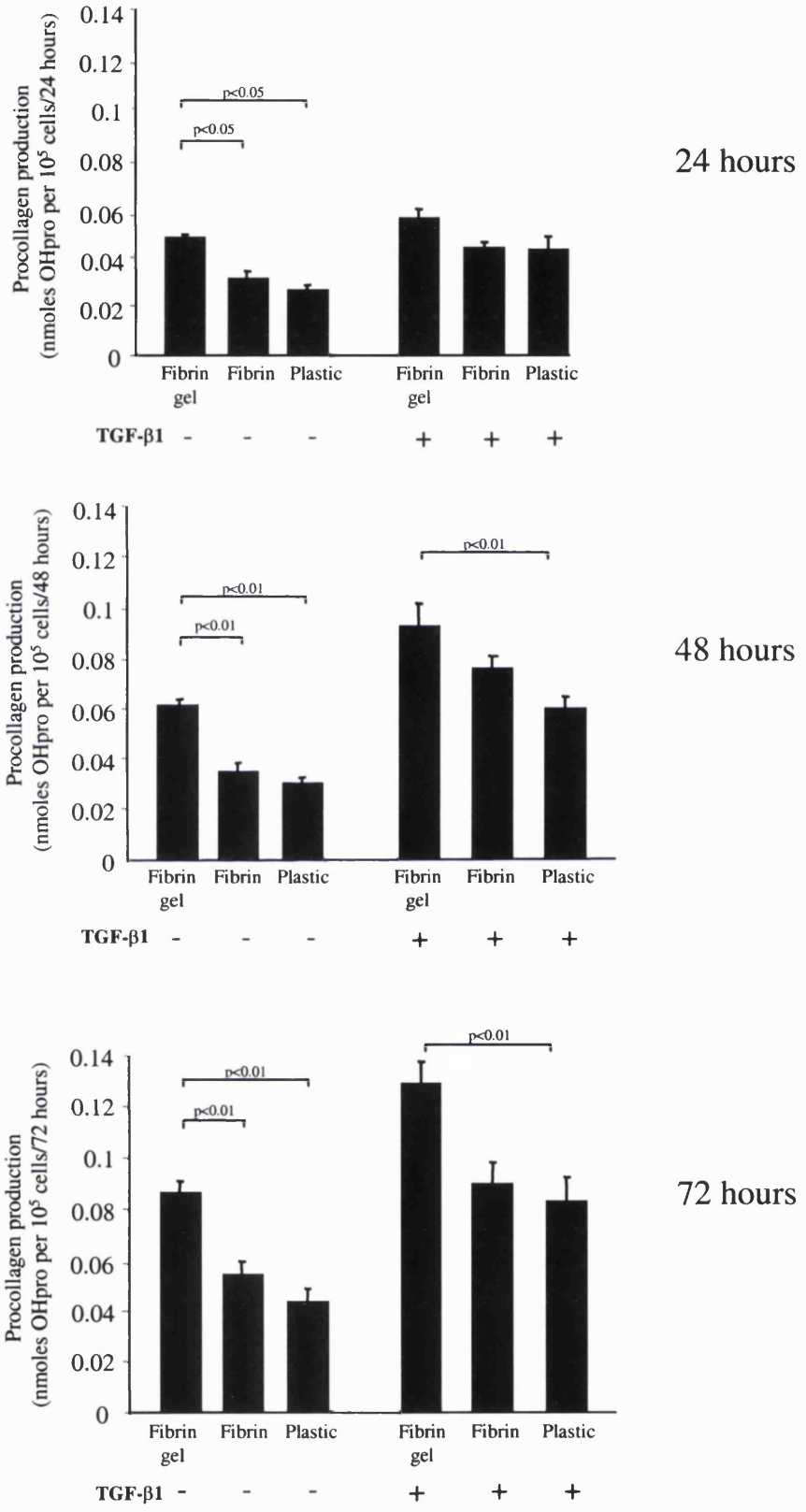


Figure 4.2. Procollagen production by human dermal fibroblasts (HSF-1) grown in three-dimensional fibrin gels compared with culture on fibrin monolayers or tissue culture plastic, in the presence and absence of TGF-β1(1ng/ml) (mean ± standard error, n=6).

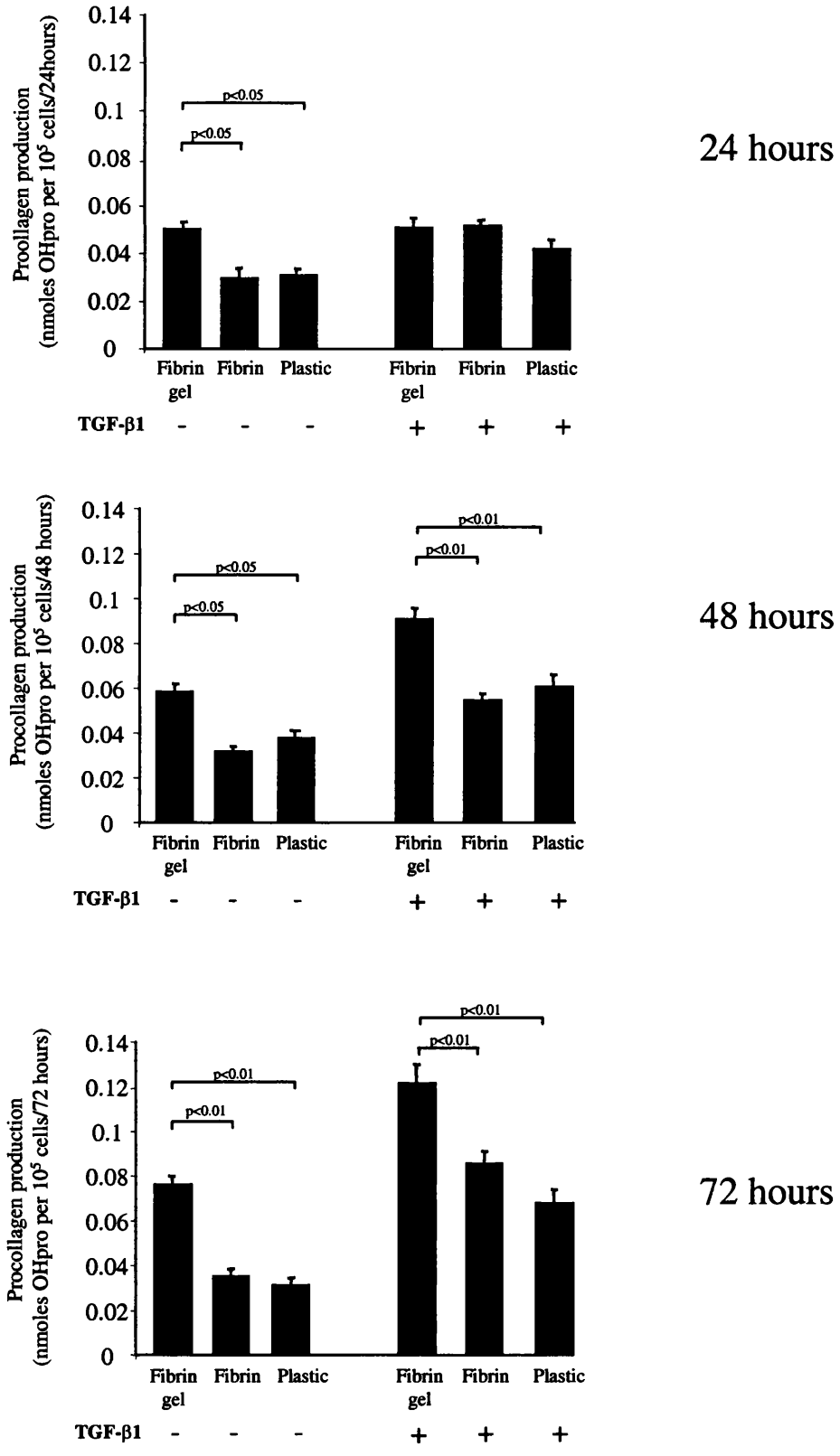


Figure 4.3. Procollagen production by human dermal fibroblasts (HSF-5) grown in three-dimensional fibrin gels compared with culture on fibrin monolayers or tissue culture plastic, in the presence or absence of TGF-β1(1ng/ml) (mean ± standard error, n=6).

with fibroblasts grown on plastic (Figure 4.4A). There was no significant increase in collagen production in response to thrombin alone (2nM) at 48 hours (Figure 4.4B). Similarly, there was no increase in collagen production in response to the fibrinogen cleavage products, fibrinopeptides A and B at physiological concentrations (10^{-6}M), which were calculated to be present in the fibrin gels (10^{-5} M - 10^{-7}M), compared with cultures without these factors (Figure 4.5).

4.3 *In vivo* results

The effect of fibrin matrix on collagen production was examined *in vivo* using a novel animal model of skin fibrosis. Fibrinogen and thrombin solutions were injected subcutaneously simultaneously in Balb-c mice, twice a week for 3 weeks. The number of injections and duration of the time-course were optimised for collagen deposition. Fibrinogen and thrombin were injected at physiological concentrations, 9μM and 140nM respectively. The same volume of sterile PBS was injected into the opposite flank of the same animal as a negative control. An additional group of mice was injected with thrombin (140nM) alone. No animals died during the course of the experiments and the consistency of the animal weights and behaviour showed that there were no adverse effects.

4.3.1 The effect of subcutaneous injections of fibrin on collagen deposition

One week following the final injection full thickness 8mm punch biopsies were removed and processed for collagen analysis by HPLC measurement of hydroxyproline. Multiple injections of fibrinogen and thrombin produced a two-fold increase in collagen deposition in the skin compared with skin injected with sterile PBS and untreated skin (Figure 4.6). Multiple injections of sterile PBS showed no increase in the levels of collagen compared with control skin. In addition, no increase in collagen was observed in skin repeatedly injected with thrombin alone (Figure 4.6).

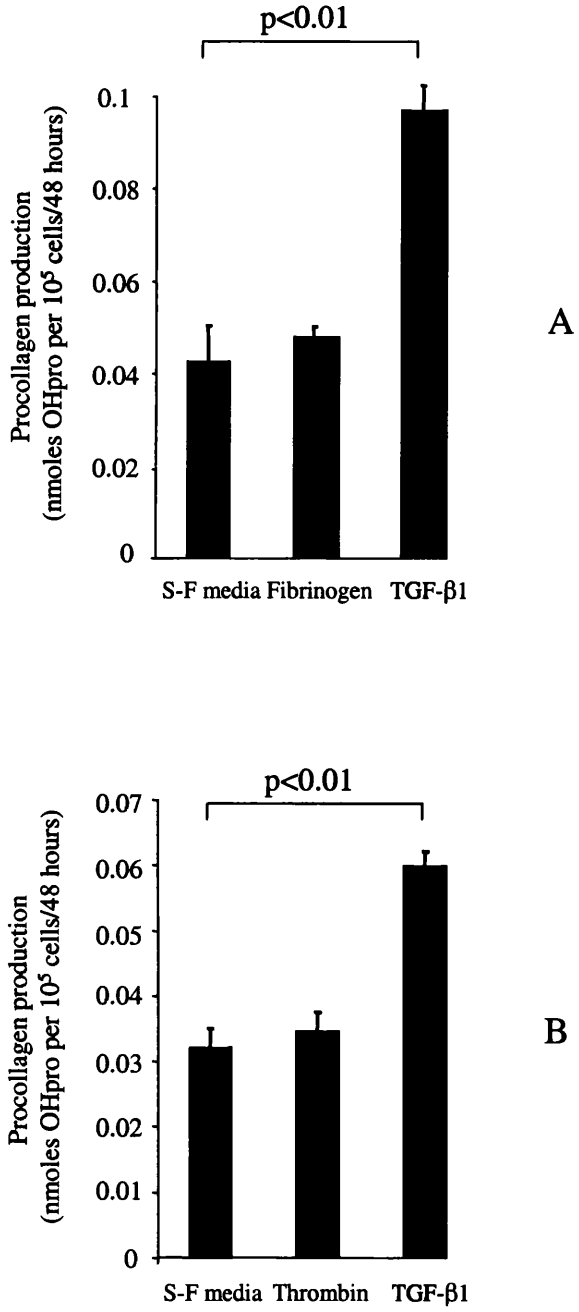


Figure 4.4. The effect of **A.** fibrinogen (9μM) and **B.** thrombin (2nM) on procollagen production by adult human dermal fibroblasts (HSF-1) after 48 hours (mean ± standard error, n=6). Similar results were obtained with HSF-5 fibroblasts (data not shown). TGF-β1(1ng/ml) acted as a positive control.

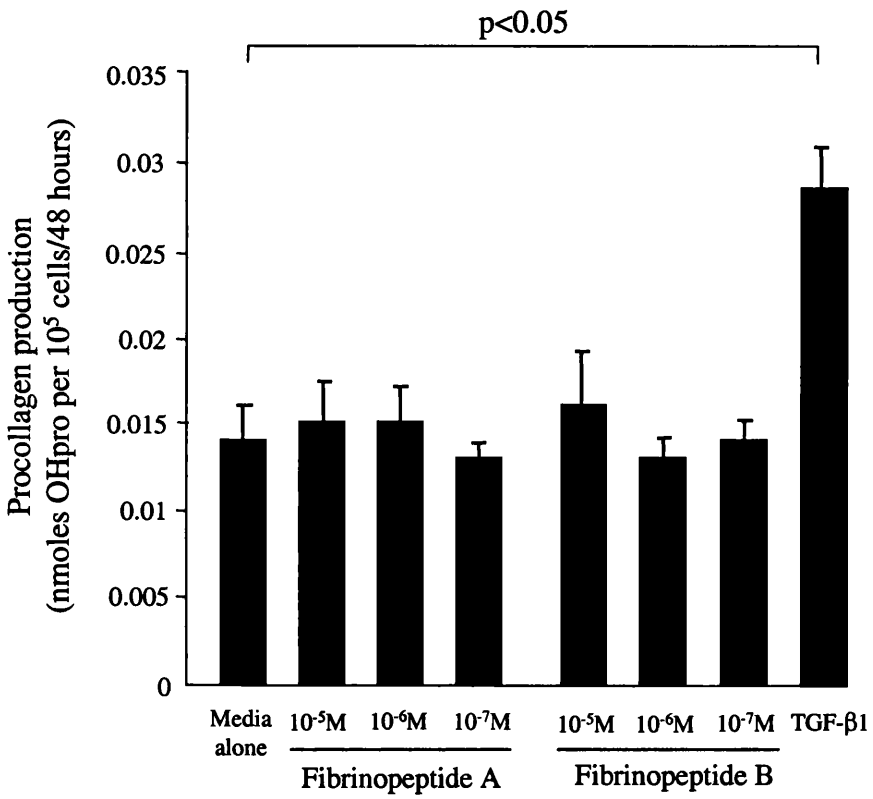


Figure 4.5. The effect of fibrinopeptide A or B on procollagen production by human dermal fibroblasts (HSF-5) after 48 hours (mean ± standard error, n=6). Similar results were obtained for HSF-1 fibroblasts (data not shown). TGF-β1(1ng/ml) acted as a positive control.

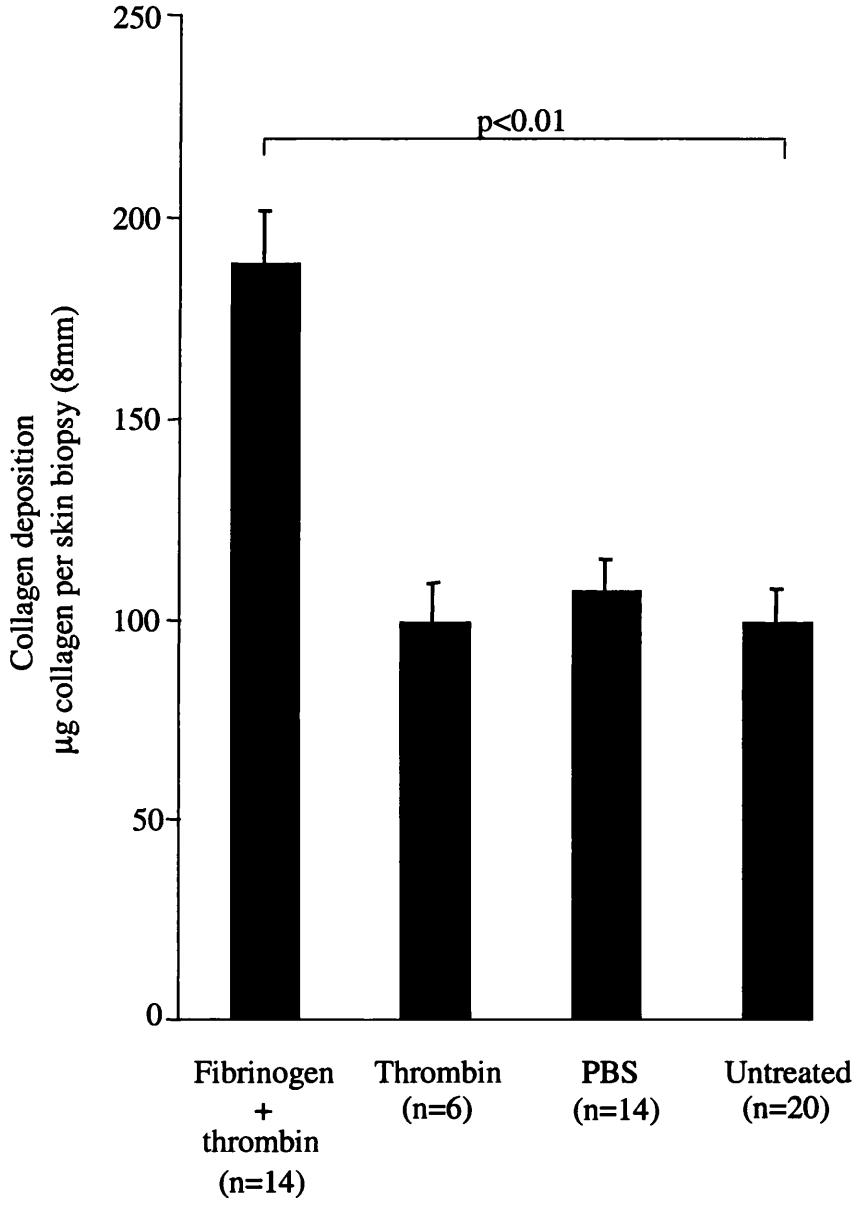


Figure 4.6. The effect of multiple subcutaneous injections of fibrinogen (9µM) and thrombin (140nM), thrombin alone (140nM) and PBS, on collagen deposition in the skin of Balb-c mice. Results show data from two combined experiments apart from thrombin which was from one experiment (mean ± standard error).

4.3.2 The effect of subcutaneous injections of fibrin on histological appearance

The effect of fibrin on the histological appearance of the skin was examined on days 1, 3, 5, 7 and 14 following the final injection. Briefly, full thickness 8mm punch biopsies were removed from fibrin-treated, PBS-treated and untreated sites and were processed for wax histology. Sections were stained by martius scarlet blue trichrome stain (Figure 4.7, 4.8 and 4.9). Normal mouse skin comprised of an epidermis, dermis containing numerous hair follicles, with a thin subcutaneous fat layer attached to the panniculus carnosus muscle layer. Below the muscle layer was a thin layer of connective tissue termed the 'fascia' layer, which divided the panniculus carnosus and the peritoneal muscle layer.

A number of differences in tissue architecture, extracellular matrix deposition, cellular infiltrate and angiogenesis were observed in fibrin-injected skin compared with PBS-injected and normal untreated skin. Since fibrin was repeatedly injected subcutaneously below the panniculus carnosus, the greatest changes were expected in the fascial layer. Measurement of the fascial layer depth by image analysis, from below the panniculus carnosus to the top of the peritoneum, demonstrated a three-fold increase in layer thickness in fibrin-injected skin compared with PBS-treated and normal skin at days 1 and 3 post-final injection (Figure 4.10A) ($p<0.001$). The thickness of this layer gradually declined over time, resulting in a two-fold increase by day 14 ($p<0.05$). An increase in fascial layer thickness was observed 1 day post-injury in PBS-injected skin, but no difference was observed from day 3 onwards. Measurement of total skin depth and dermal skin depth (data not shown) demonstrated that fibrin-injected skin was significantly thicker at all time points examined post-injury ($p<0.01$) (Figure 4.10B).

4.3.3 Collagen and fibrin deposition

Collagen in fibrin-injected skin was more densely packed and organised into parallel bundles across plane of the section, with numerous fibroblasts interspersed between the fibres (Figure 4.11A). In contrast, collagen deposition in PBS-treated sites appeared to be less organised, with randomly orientated bundles and swirls of

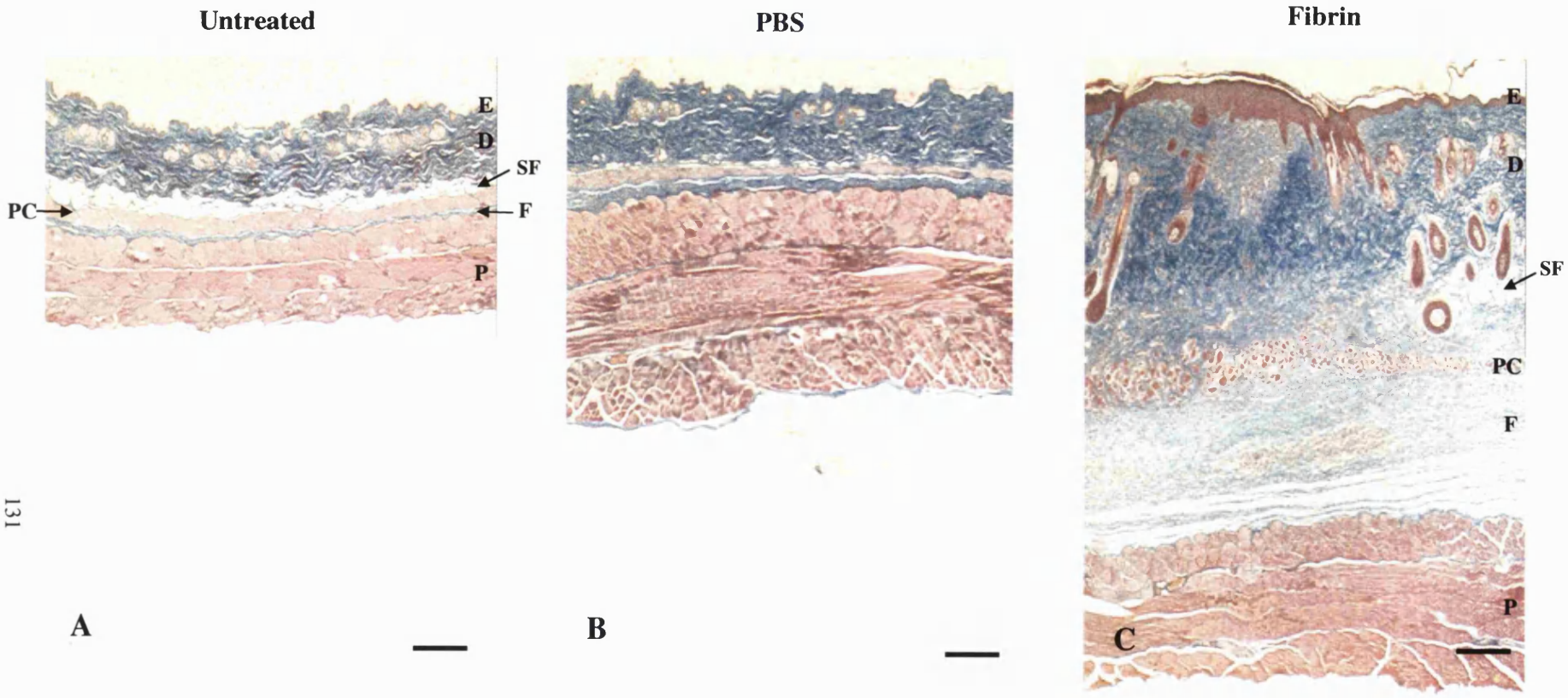


Figure 4.7. The effect of multiple subcutaneous injections of fibrinogen and thrombin on skin architecture.
A. untreated skin, **B**. PBS-injected skin, and **C**. fibrin-injected skin (E, epidermis, D, dermis, SF, subcutaneous fat, PC, panniculus carnosus, F, fascia, P, peritoneum), one day after the final injection. Fibrin injections resulted in marked thickening of the dermal and fascia layers, with increased amounts of collagen and replacement of the subcutaneous fat with fibrous tissue. Bar represents 100μm, Martius Scarlet Blue trichrome stain (collagen - blue, muscle and fibrin - red).

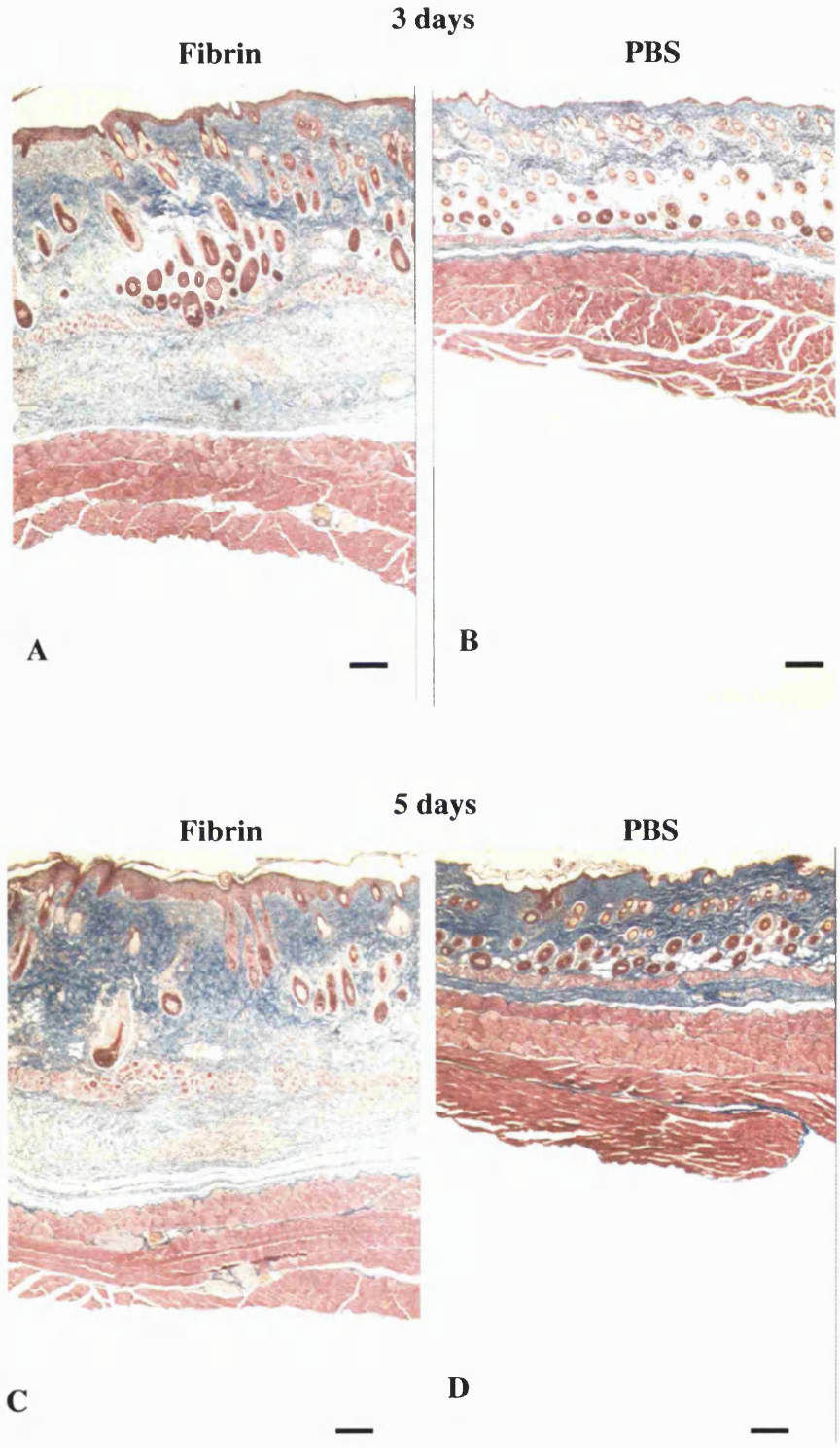


Figure 4.8. The effect of multiple subcutaneous injections of fibrinogen and thrombin (**A** and **C**) on skin architecture, compared with PBS-injected skin (**B** and **D**), 3 and 5 days after final injection. Bar represents 100μm. (Martius Scarlet Blue trichrome stain, collagen - blue, muscle and fibrin - red)

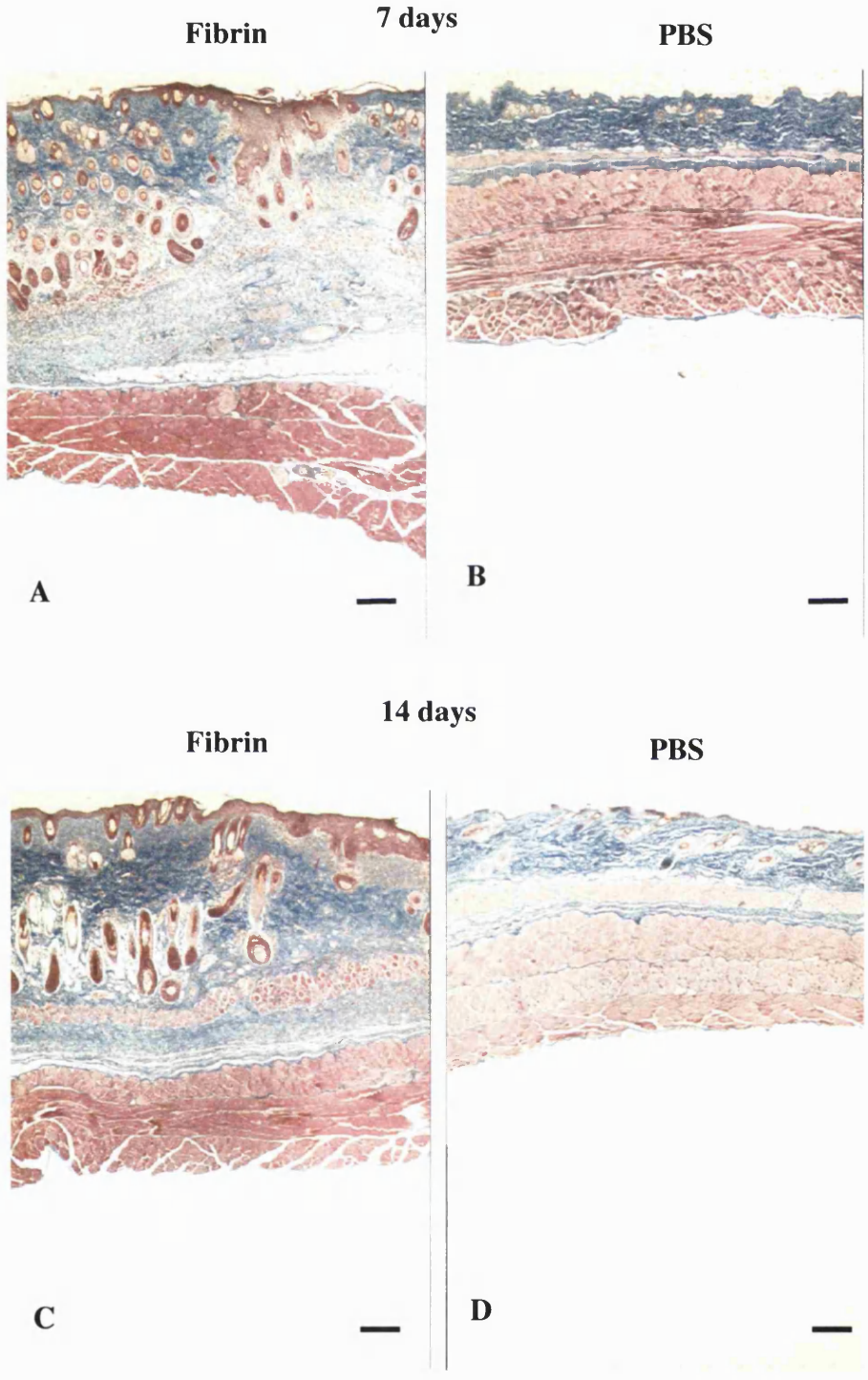


Figure 4.9. The effect of multiple subcutaneous injections of fibrinogen and thrombin (**A** and **C**) on skin architecture, compared with PBS-injected skin (**B** and **D**), 7 and 14 days after final injection. Bar represents 100μm. (Martius Scarlet Blue trichrome stain, collagen - blue, muscle and fibrin - red)

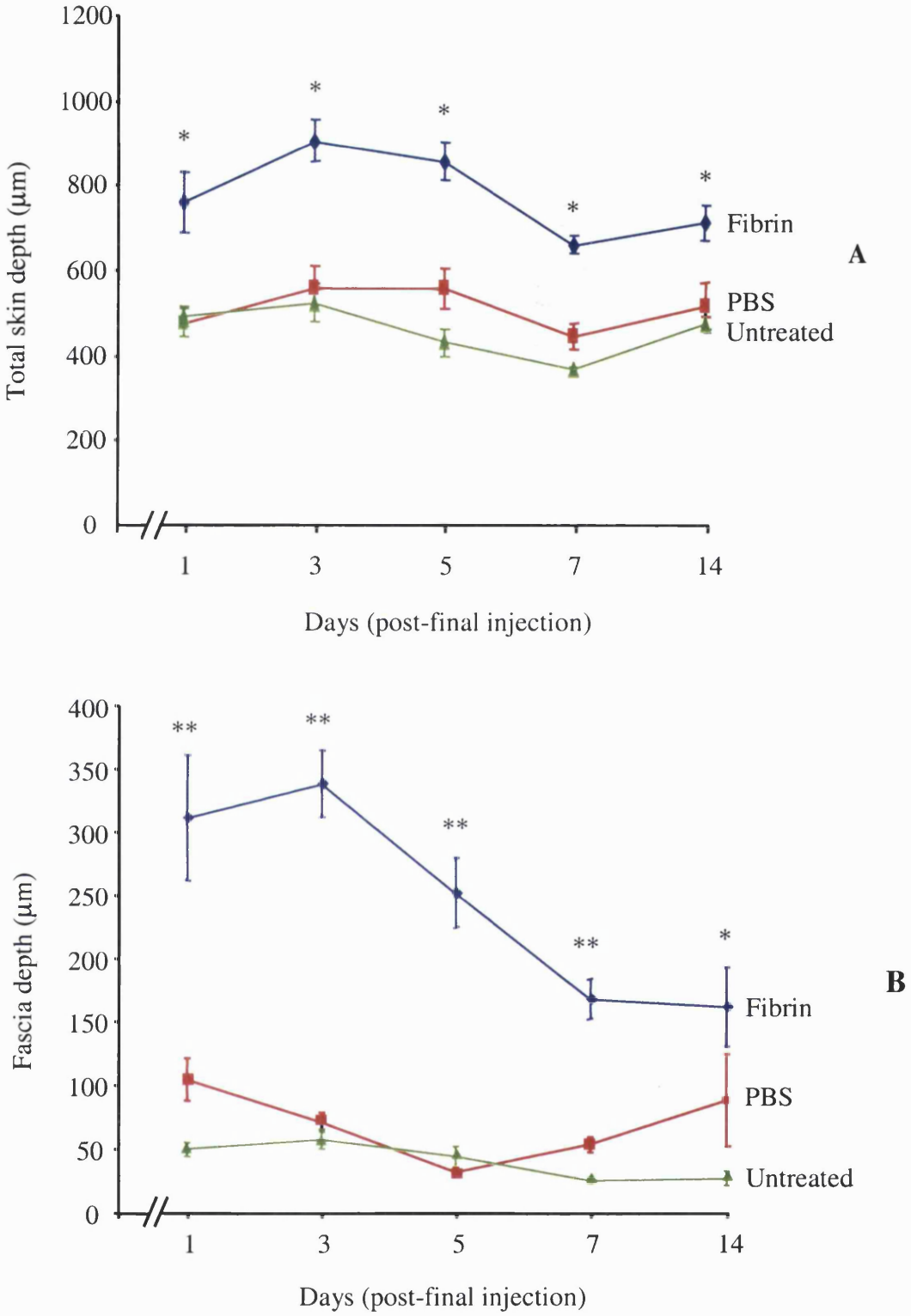


Figure 4.10. The effect of multiple subcutaneous injections of fibrinogen and thrombin on **A.** total skin depth, measured from base of epidermis to bottom of fascia and **B.** fascia depth in injected Balb-c mouse skin up to 14 days after the final injection (mean ± standard error, n=4, * = $p<0.05$, ** = $p<0.01$ compared with PBS control).

collagen in the fascial layer. Increased collagen was also apparent in the dermis in fibrin-treated skin, and there was a replacement of the subcutaneous fat layer by fibrotic tissue (Figure 4.11C). There was a significantly increased level of collagen in the fascial layer of fibrin-injected skin compared with PBS-treated and untreated skin at all time points post-injury (Figure 4.12A). At days 1 and 3 there was a 2.5-fold increase in collagen density in fibrin-injected skin which decreased to a 2-fold increase at days 5, 7 and 14 post-injury compared with PBS-treated skin. Collagen density was similar in PBS-treated skin and untreated skin. No significant difference was observed between PBS-injected and normal skin. Skin injected with thrombin alone also showed none of the histological features observed following fibrinogen and thrombin treatment (Figure 4.11D).

To examine the clearance of fibrin matrix in the fibrin-treated injection sites, fibrin density was examined by semi-quantitative image analysis (Figure 4.12B). Fibrin deposition was observed in all fibrin-injected skin samples days 1 and 3 after the final injection, however, fibrin deposits were found in only 2 of the four mice at 5 days, and no fibrin was seen in the skin taken from mice at days 7 or 14. Fibrin deposits were mainly observed in the fascial layer, although traces of fibrin were observed in the dermis at the injection site. The fibrin deposits in skin from days 1 and 3 post-final injection were large and often disrupted the normal architecture of the skin (Figure 4.11B).

4.3.4 Inflammation

Overall, injections of fibrin resulted in a mild inflammatory infiltrate. There were a large number of spindle-shaped fibroblasts arranged between collagen bundles in the surrounding matrix. The number of fibroblasts appeared to be greater at the earlier time-points (days 1, 3 and 5), and gradually decreased until few were remaining at day 14 post-injection. The inflammatory cell infiltrate was present at day 1, however, few inflammatory cells were present by day 7 post-final injection. The inflammatory infiltrate in the early time-points appeared to comprise mainly of macrophages and eosinophils, with relatively few neutrophils or lymphocytic cells, and were generally

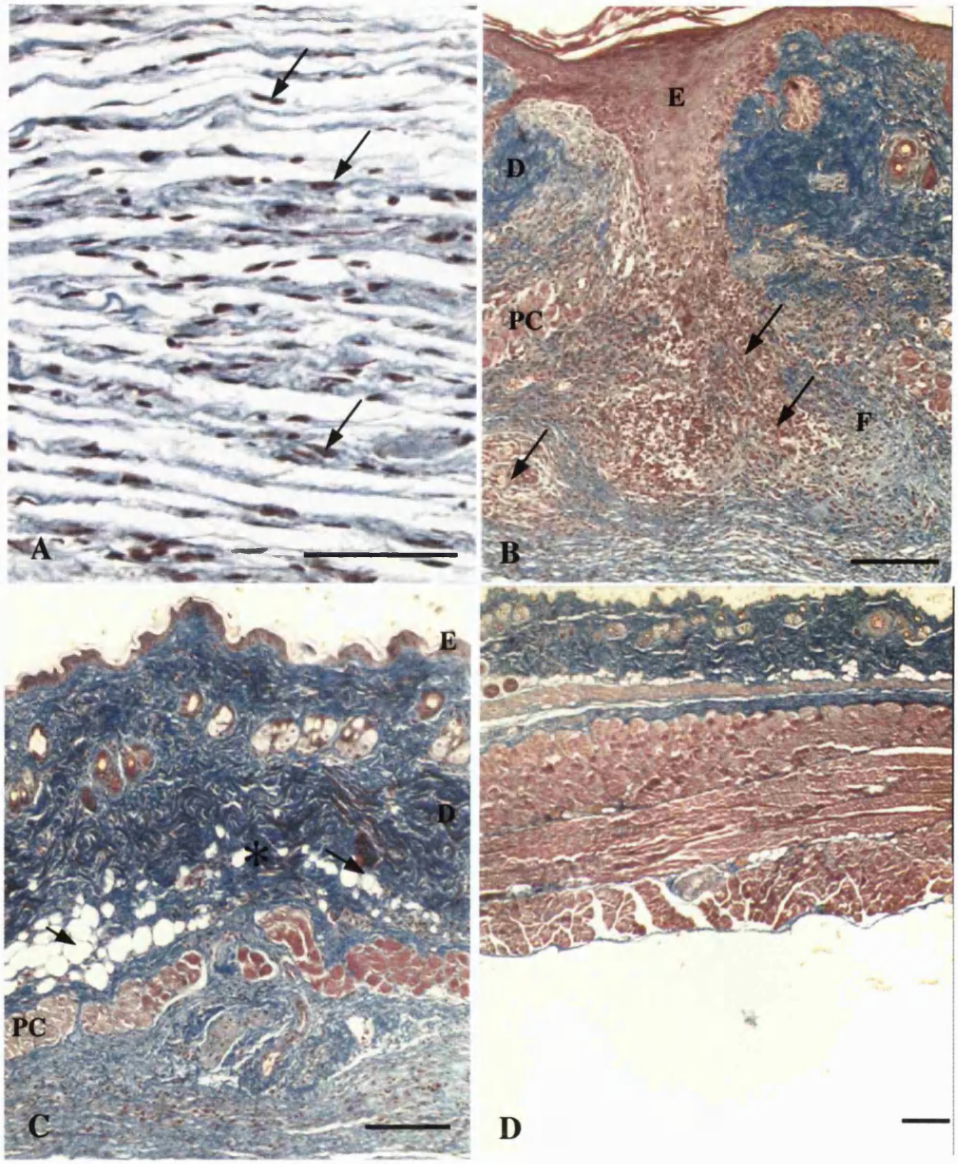


Figure 4.11. The effect of repeated subcutaneous injections of fibrinogen and thrombin on skin architecture. A. Parallel collagen bundles (blue) in the fascial layer with fibroblasts (arrows) aligned in parallel, 7 days post-final injection. **B.** Fibrin deposits (arrows, red) in the fascial layer (F) resulted in distortion of the dermis (D) and panniculus carnosus (PC) architecture, with epidermal hyperplasia (E), 3 days after the final injection. **C.** Replacement of subcutaneous fat layer (arrows) with collagen (*, blue) 7 days post-final injection. **D.** The effect of repeated subcutaneous injections of thrombin alone (140nM) 7 days after the final injection. No effect on histological appearance as observed. Bar represents 50μm. (Martius Scarlet Blue trichrome stain, collagen - blue, muscle and fibrin - red)

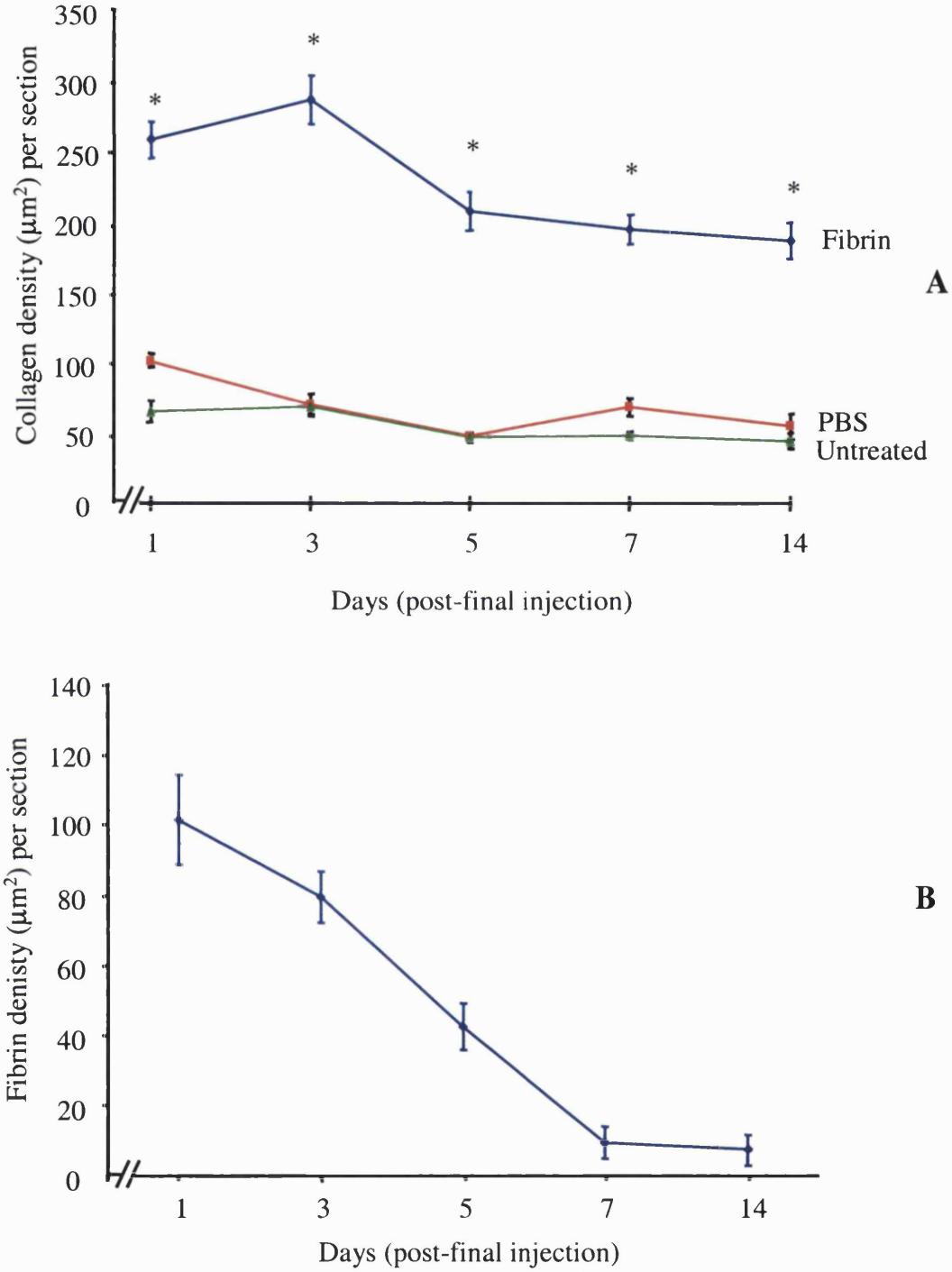


Figure 4.12. The effect of multiple subcutaneous injections of fibrinogen and thrombin on **A**. collagen deposition **B**. fibrin deposition in the fascia of injected Balb-c mouse skin up to 14 days after the final injection (mean ± standard error, n=4, * = $p<0.001$ compared with PBS control)

observed in the upper regions of the thickened fascial layer, in close proximity to large blood vessels.

4.3.5 Angiogenesis

The number of blood vessels was significantly increased in fibrin-treated skin compared with PBS-treated and normal skin at all time points post-final injection ($p<0.01$) (Figure 4.13). The greatest number of blood vessels was observed on day 1, but had declined to a two-fold increase by day 14. No significant difference in the number of blood vessels was observed in PBS-treated skin compared with untreated controls at any time point. The matrix around the blood vessels was densely arranged with concentric rings of collagen fibres surrounding arterioles and venules.

In summary, this chapter has demonstrated that fibrin deposition was associated with increased collagen production both *in vitro* and *in vivo*. Dermal fibroblasts in three-dimensional fibrin gels produced significantly increased levels of collagen compared with monolayer cultures. The increase in collagen production appeared to be the result of a fibroblast-matrix interaction, and did not appear to be due to direct stimulation by individual matrix components, such as fibrinogen, thrombin or fibrinogen degradation products. *In vivo*, repeated subcutaneous injections of fibrinogen and thrombin caused a number of fibrotic skin changes, including a significant increase in collagen deposition in the skin, with increased skin thickening up to 14 days after the final injection.

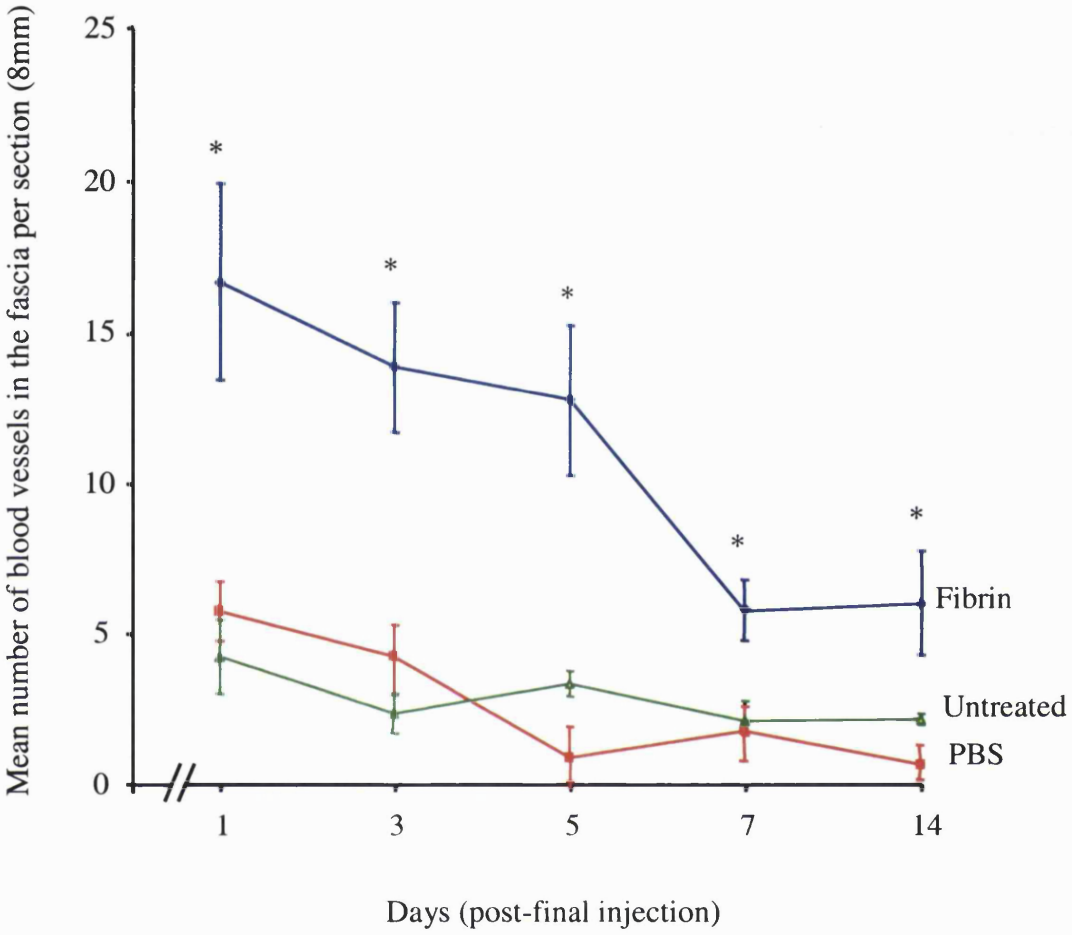


Figure 4.13. The effect of subcutaneous injections of fibrinogen and thrombin on the number of blood vessels in the fascia of injected Balb-c skin up to 14 days following the final injection (mean ± standard error, n=4, * = $p<0.01$ compared with PBS control).

4.4 Discussion

Fibrin deposition in the dermis is a prominent feature of LDS. Fibrin is the most abundant protein during the initial tissue repair process (Tuan et al., 1996) and its deposition provides a provisional matrix promoting inward migration of tissue repair cells and preventing excessive blood loss. Gradually, the fibrin matrix becomes replaced by collagen. However, few studies have defined the role of fibrin in mediating collagen production by dermal fibroblasts. In this chapter, the effect of fibrin matrix on the production of collagen by human dermal fibroblasts was examined both *in vitro* and *in vivo* using novel approaches.

The *in vitro* model of a 'fibrin matrix' used in this study, consisted of a three-dimensional fibrin gel culture system with fibroblasts seeded in the gel. The three-dimensional matrix is thought to provide a more physiological environment for fibroblasts compared with monolayers (Gillery et al., 1989; Tuan et al., 1996). There was a significant increase in collagen production by dermal fibroblasts in three-dimensional fibrin gels compared to fibrin and plastic monolayer cultures at 24, 48 and 72 hours. The effect of fibrin matrix on collagen synthesis was further enhanced by the addition of the pro-fibrotic mediator, TGF-β1, demonstrating that fibroblasts in the fibrin matrix alone were not maximally stimulated. These results parallel previous studies showing that fibroblasts in a fibrin matrix synthesise a significantly greater amount of collagen compared with cells in three-dimensional collagen gel or monolayer cultures, measured by incorporation of radio-labelled proline (Gillery et al., 1989, Coustry et al., 1990).

Collagen levels in this study were normalised for cell number to ensure increases in collagen were not due to cell proliferation. No significant change in cell number was observed in all three culture systems at all time points studied, as fibroblasts were plated at a high cell number to optimise collagen production (Booth et al., 1980) and to limit further proliferation. Tuan and colleagues (1996), demonstrated no increase in cell number at 24, 48 or 72 hours by fibroblasts in fibrin gels or on fibrin monolayers compared to plastic. However, both of these systems showed a significant increase in cell proliferation after 5 days culture. Therefore, the observed two-fold increase in collagen

production in fibrin gels compared to monolayer controls was not due to changes in cell number.

Other studies have suggested that a fibrin matrix may decrease collagen synthesis by dermal fibroblasts (Pardes et al., 1995, Tuan et al., 1996). Pardes and colleagues (1995) reported a decrease in procollagen type I gene expression by dermal fibroblasts grown on the surface of a three-dimensional fibrin matrix compared with tissue culture plastic at 24 and 48 hours. The culture system used in their study was similar to the fibrin monolayer system of the present study, in which no significant increase in collagen production was observed compared to tissue culture plastic. This suggests that fibroblasts need to be surrounded by a three-dimensional matrix to induce an increase in collagen production in response to fibrin. Furthermore, Gillery and colleagues (1989) showed that the rate of collagen synthesis by dermal fibroblasts was significantly enhanced in fibrin gels similar to those used in the present study compared with free-floating, tensionless fibrin gels. Remodelling of fibrin matrix may permit fibroblasts to establish mechanical tension throughout the gel. This Centre has shown previously that these mechanical forces may potentially play a role in the up-regulation of collagen gene expression (Butt et al., 1995) and thus may result in increased collagen deposition as observed.

Tuan and colleagues (1996) suggested that the rate of collagen synthesis was decreased by dermal fibroblasts grown in three-dimensional fibrin gels compared with plastic monolayer cultures, measured by the rate of incorporation of radio-labelled proline. These findings contradict our own results and those of several other studies (Gillery et al., 1989; Coustry et al., 1990). However, this method of collagen analysis does not take into account the rate of intracellular collagen degradation, and thus is not an indicator of net collagen production. Studies from this Centre have demonstrated that the results obtained with the HPLC method of collagen analysis are highly reproducible, with coefficient variation of less than 2% for hydroxyproline measured in the same sample hydrolysate assayed on different days (Campa et al.,1990), suggesting that the results of our study are reliable. These data account for procollagen levels in both the media and gel/fibroblast layer, measuring both soluble and insoluble collagen, and show an increase in collagen production in the three-dimensional model system. In addition, the concentration of fibrin

used by Tuan and colleagues was lower than that of the present study (2mg/ml and 3mg/ml respectively). Physiological concentrations (3mg/ml) of fibrin were used to optimise the environment for fibroblast collagen production, and may account for the reduced protein synthesis by fibroblasts in fibrin gels of lower concentrations.

Fibrin matrices were produced using physiological concentrations of fibrinogen, and were 98% pure (Sigma, Poole, UK). Therefore, other factors in the fibrin matrix could have played a role in regulating collagen production, for example thrombin, fibrinopeptides A and B, fibronectin and growth factors and cytokines. Thrombin has been shown to be a potent inducer of procollagen type I gene up-regulation and is a potent mitogen for lung fibroblasts, via proteolytic activation of protease activated receptor-1 (PAR-1) (Chambers et al., 1998), but at concentrations 10-fold higher than were used in this study. The concentration of thrombin used (2nM) was sufficient to allow clot formation but had no effect on fibroblast proliferation and collagen production. However, the effect of thrombin bound to a three-dimensional fibrin matrix or following its release into the media were not investigated, but may play a role in mediating collagen production. Pardes and colleagues (1995) analysed procollagen type I gene expression in response to fibrinopeptides A and B (10^{-6}M) and demonstrated a significant increase in procollagen mRNA in response to fibrinopeptide A. The results presented by the current study examining hydroxyproline levels suggest there was no effect on collagen production in response to either fibrinopeptides A or B at physiological concentrations (10^{-6}M) or at the concentrations used in the previous study. This discrepancy may again be due to different methods of collagen synthesis analysis. Furthermore, although our findings suggest that the fibrinopeptides alone do not play a role in regulating collagen production, they may play a role when incorporated in a three-dimensional lattice, perhaps in association with other matrix components.

Collagen production by dermal fibroblasts was significantly enhanced in response to TGF-β1 in all three-culture systems at 48 and 72 hours. TGF-β1 exerts its effects on collagen deposition in a number of ways, including increased procollagen gene expression, increased mRNA stability and through reducing the proportion of newly synthesised procollagen degraded intracellularly (McAnulty et al., 1995). Coustry and

colleagues (1990) demonstrated that fibroblast responsiveness to TGF-β1 was mainly dependent on the nature of the extracellular matrix. The group showed that fibroblasts in collagen gels produced significantly less collagen in response to TGF-β1 than fibroblasts in fibrin gels, suggesting that fibrin provides a more suitable matrix for the availability of growth factors. The synergistic effects of the fibrin matrix and growth factors on collagen synthesis by fibroblasts may explain the increased collagen production in three-dimensional fibrin gels, since monolayer cultures may not provide the same 'reservoir' effect.

The mechanisms by which fibroblasts interact with the fibrin matrix are incompletely understood. Fibrinogen has been shown to bind to integrin receptors, αvβ3 and α5β1 on fibroblasts (Gailit and Clark, 1996) which may affect gene expression via an intracellular signalling cascade. Other non-integrin fibrinogen receptors have been recognised on fibroblasts, including ICAM-1 (Languino et al., 1995). Potentially, the number of receptor-ligand interactions would be greater in the three-dimensional fibrin matrix resulting in increased production and accumulation of collagen. Other key components of the fibrin matrix are ECM molecules such as fibronectin and vitronectin (Greiling and Clark, 1997) derived from contamination of the fibrinogen solution or endogenously produced (Clark et al., 1995). Fibronectin can directly interact with fibroblasts through integrins such as α5β1, αvβ3, α4β1 and α3β1, causing downstream intracellular signalling responses via MAPK/ERK pathways which could potentially lead to enhanced collagen synthesis (Gailit and Clark, 1996; Palcy and Goltzman, 1999). Indeed, studies in our laboratory suggest that fibronectin may affect collagen regulation (Reynolds, PhD thesis, 2000). Mechanical-load induced procollagen synthesis by cardiac fibroblasts was inhibited by the addition of blocking antibodies specific for integrins αvβ3 and α5β1. The direct effect of fibronectin on collagen production by fibroblasts in fibrin gels was not examined in this study, however, evidence that αvβ3 binds fibrin(ogen) strongly (Gailit and Clark, 1996) lends support the theory that fibrin may directly stimulate collagen synthesis. In summary, *in vitro* results from these studies show that collagen production was enhanced in three-dimensional fibrin matrix compared with monolayer controls. This effect was not likely to be due to the action of thrombin or fibrinopeptides,

indicating that the three-dimensional fibrin matrix may directly regulate the production of collagen by dermal fibroblasts.

To investigate the effect of fibrin deposition on collagen accumulation *in vivo*, a novel fibrotic skin model was established by administering repeated subcutaneous injections of fibrinogen and thrombin in Balb-c mice. Moreover, the model mimics the chronic deposition of fibrin in the interstitium of LDS-affected skin. Each animal was repeatedly injected with fibrinogen and thrombin solutions, and sterile PBS alone. The injection of PBS provided a control for the injury caused by the injection process. Mice were sacrificed at days 1, 3, 5, 7 and 14 after the final injection to examine the processes of fibrin matrix degradation and the associated accumulation of collagen over time. Histological analysis of fibrin-treated skin showed fibrotic changes, including skin thickening, increased collagen deposition and a large number of fibroblasts interspersed between collagen fibres arranged in parallel bundles. Since the fibrinogen and thrombin mixture was injected subcutaneously and below the panniculus carnosus, the most dramatic effect was expected in the fascial layer. Indeed, repeated injection of fibrin resulted in a significant increase in the depth of this layer and in the amount of collagen, demonstrated by image analysis.

Collagen deposition in the skin of fibrin-treated mice was examined by HPLC analysis of hydroxyproline, one week post-final injection. Biochemical analysis confirmed the histological data, showing a 2-fold increase in the amount of collagen present in fibrin-injected skin compared with PBS-treated or normal controls. Furthermore, these data correlated with a gradual decline in the distribution of fibrin over time. In parallel with increased collagen levels was a significant increase in the number of blood vessels in the fascia, with little inflammatory cell infiltrate that also declined over the time-course. Skin injected with PBS and normal skin, distant from the fibrin-injected sites, failed to show any signs of fibrotic skin changes.

This novel animal model of skin fibrosis provided reproducible fibrotic lesions in the skin, often clinically palpable after two weeks (4 injections). The number of injections and the duration of the time-course were optimised to provide maximal collagen

deposition within a convenient time-frame for repeated experiments. This provides a number of advantages over alternative animal models of excessive collagen accumulation in the skin. For example, collagen deposition was significantly increased greater than 14 days after the final injection, following only six repeated injections. Alternative skin fibrosis models in newborn mice following daily injections of TGF-β1 subcutaneously for up to 4 weeks (Roberts et al., 1986; Shinozaki et al., 1997; Mori et al., 1999) showed that fibrotic tissue completely resolved three days following the last injection, regardless of the number of injections. Persistent subcutaneous fibrosis could only be maintained up to a week later following repeated daily injections of recombinant TGF-β1 and PDGF at high concentrations for an additional week (Shinozaki et al., 1997). No other study has administered fibrin directly into the skin of mice, however, various effects of fibrin matrix on the tissue repair process have been reported in several other models. Knighton and colleagues (1982) showed both neovascularisation and fibroblast proliferation in fibrin gels implanted into the cornea of rabbits. Indeed, more recent evidence showed that fibrin-filled 'plexiglass' chambers implanted into the subcutaneous tissue of rats, demonstrated invasion of the fibrin chambers by various cell types, including macrophages, fibroblasts and endothelial cells, with numerous new blood vessels (Dvorak et al., 1987). It was proposed that fibrin matrix provides a provisional scaffold for granulation tissue formation, encouraging new blood vessel generation and fibroblast proliferation prior to matrix production.

Although results of the current study as well as a number of other reports suggest that the fibrin matrix provides a stimulus for ECM production, several other studies have proposed that fibrin(ogen) is not required for collagen deposition and fibroblast proliferation in fibrotic disease (Hattori et al., 2000, Ploplis et al., 2000,). Using a bleomycin model of lung fibrosis in fibrinogen-deficient animals, Hatorri et al., (2000) and Ploplis and colleagues (2000) found that collagen levels were similar in fibrinogen-deficient mice and wild-type mice, 7 days and 14 days post-injury. However, both these studies could be criticised for their experimental design, as in one study only two knock-out mice were in the treatment group, and secondly, collagen levels in homozygous fibrinogen-deficient animals were compared with animals which were heterozygous and possessed fibrinogen levels that were only 30% of their wild-type counterparts. Although

no increase in collagen deposition was observed in heterozygous fibrinogen-deficient animals, the additional 70% fibrinogen that would have been present may have resulted in a significant increase in collagen following bleomycin instillation. However, the finding that plasminogen- or plasminogen activator-deficient mice have increased levels of lung collagen compared to wild-type animals after bleomycin treatment suggests that fibrin deposition is a common consequence of bleomycin-induced pathologies (Swaisgood et al., 2000). Furthermore, Yamamoto and colleagues (1999) demonstrated that repeated subcutaneous injections of bleomycin in BALB-c mice resulted in skin thickening and enhanced collagen production. It is possible that the fibrotic skin changes may be the result of vascular damage and chronic leakage of fibrin(ogen) in the bleomycin-treated tissue.

Several studies have attempted to develop a model of the skin changes associated with LDS, including a canine model of venous hypertension (Burnand et al., 1982) and lipase-induced panniculitis in rats (Naschitz et al., 1999). An early study by Burnand and colleagues failed in their attempt to mimic skin changes ascribed to LDS, although there was an increase in the number of capillaries in the skin. Recently, Naschitz and colleagues showed that subcutaneous injections of lipase caused a panniculitis-like syndrome of the subcutaneous fat layer and chronic inflammatory and sclerotic response in the fat layer, muscle fascia and muscle layers. However, ligation of the rat hind-limbs and subsequent venous hypertension had no effect on the formation or resolution of the skin changes. The group concluded that the animal model did not duplicate lipodermatosclerosis in man. There are problems associated with creating an experimental animal model of venous hypertension, presumably due to humans being bipedal, in contrast to quadrapedal laboratory animals, which have different venous system anatomy and venous pressures. The effects of venous hypertension in animals such as mice, rats or dogs, are clearly less severe than those effects in the human. Although the model used in this thesis did not reproduce the effects of chronic venous hypertension, it did represent the downstream effects of chronic fibrin deposition.

In contrast to these previous attempts to duplicate the skin changes of LDS, histological analysis of our skin fibrosis model showed many similarities to LDS skin. Firstly, fibrin-

injected skin was markedly thicker compared to untreated and PBS-treated skin. This thickening was due to accumulation of parallel-aligned collagen bundles, with numerous fibroblast-like cells, similar to the fibrotic dermis of human LDS skin. Secondly, there was little inflammation in the skin of patients with LDS compared with controls, and fibrin-treated skin also showed no inflammatory cell infiltrate one week after the final injection. Moreover, fibrin-treatment resulted in extension of the dermis and replacement of the subcutaneous fat layer with dermal-like tissue, a hallmark of LDS. Finally, HPLC analysis of collagen production in fibrin-treated skin demonstrated a two-fold increase compared with PBS-treated and untreated controls, which compares favourably to the enhanced collagen production demonstrated in LDS skin by Brinckmann et al., (1999). These features make this novel, reproducible, animal model system more representative of fibrotic LDS skin changes than previous animal models.

Fibrosis is usually associated with an injury, which in turn causes an inflammatory response and prolonged tissue repair (Trojanowska et al., 1998). Secretion of growth factors and cytokines from invading inflammatory cells or activated platelets may all contribute to the enhanced deposition of collagen in the skin. To examine the role of inflammatory cell infiltrate in the development of skin fibrosis following repeated injections of fibrin, the number of cells in the skin samples was measured by image analysis techniques. The number of cells infiltrating fibrin-injected skin appeared to be greater in the earlier time-points, days 1 and 3 after the final injection. Fibrin(ogen) cleavage products have been shown to act as chemoattractants for monocytes, neutrophils and fibroblasts (see Herrick et al., 1999 for review). Indeed, fibrinopeptide B and plasmic-derived fibrinogen peptide (Bβ1-42), fragment D and fragment E, have all been shown to stimulate the migration of neutrophils and monocytes with an activity comparable to that of PDGF (Skogen et al., 1988). The presence of several chemotactic domains in the fibrinogen molecule indicates that proteolytic cleavage by thrombin or plasmin may release fragments capable of mobilising leukocytes and fibroblasts to the site of injury and thus may potentiate the inflammatory response. In addition, fibrin deposits which occur *in vivo* are rich in platelets (Knighton et al., 1982), and these in turn possess numerous growth factors and cytokines, including TGF-β and PDGF. Growth factors such as these have been shown to up-regulate procollagen synthesis by fibroblasts

and are potent fibroblast mitogens (McAnulty et al., 1995; Stavenow et al., 1981). The profile of inflammatory cells infiltrating the fibrin-treated tissues, and growth factor and cytokine distribution involved in the up-regulation of collagen production in our current model warrants further study.

In conclusion, the findings of this chapter have shown that fibrin deposition is associated with collagen accumulation both *in vitro* and *in vivo*. Dermal fibroblasts in three-dimensional fibrin matrix produce more collagen than monolayer cultures, and chronic fibrin deposition in the skin resulted in excessive collagen accumulation. Fibrin may directly mediate collagen production by fibroblasts, however, the mechanisms by which the fibrin matrix induces these effects are not known. Fibrin deposition in fibrotic disorders such as LDS is both chronic and persistent, due to reduced levels of fibrinolytic proteases and increased levels of their inhibitors (Margolis et al., 1996; Rogers et al., 1999; Peschen et al., 2000). The following chapter will address the hypothesis that the persistence of fibrin, due to reduced fibrinolysis, leads to further collagen production, and may play a role in the fibrotic skin changes of LDS.

Chapter 5
RESULTS AND DISCUSSION

Persistent fibrin matrix and reduced fibrinolysis are associated with excessive collagen accumulation

5.1 Introduction

The previous chapter showed that three-dimensional fibrin matrix was associated with collagen production both *in vitro* and *in vivo*. Fibrin deposition in fibrotic disorders such as LDS is both chronic and persistent, due to chronic leakage of plasma fibrinogen from the vasculature with reduced levels of fibrinolytic proteases as well as increased levels of their inhibitors (Margolis et al., 1996; Rogers et al., 1999; Peschen et al., 2000). If normal fibrin deposition is associated with collagen production, then fibrin persistence may lead to an even greater accumulation of collagen. This chapter addresses the hypothesis that persistent fibrin matrix and reduced fibrinolysis leads to enhancement of further collagen production. This concept is examined both in *vitro* and *in vivo*.

To examine the effect of fibrin persistence on collagen production *in vitro*, two methods have been used, one involving the addition of a specific inhibitor of plasmin, α_2-antiplasmin, to human dermal fibroblast-populated fibrin gels, and a second, incorporating dermal fibroblasts isolated from plasminogen activator-deficient mice into three-dimensional fibrin gels. Reduced fibrinolysis in the three-dimensional fibrin gel system was confirmed using a novel, fluorescently-labelled fibrin degradation assay. Cell number and procollagen production were assessed by cell counting and HPLC analysis of hydroxyproline respectively at 24, 48 and 72 hours. To investigate the effects of persistent fibrin deposition with reduced fibrinolysis *in vivo*, the novel, characterised model of skin fibrosis was used in plasminogen activator-deficient mice. Collagen deposition in the skin following repeated subcutaneous injections of fibrin was measured by HPLC analysis of collagen and by semi-quantitative image analysis of histological tissue sections.

5.2 Results

5.2.1 Characterisation of the fibrin-FITC fibrinolysis assay

Fibrinolysis by dermal fibroblasts in three-dimensional fibrin gels was examined using fibrinogen conjugated to a fluorescent marker, FITC (fluoroscein isothiocyanate). A standard curve of fibrin-FITC degradation was established by adding increasing concentrations of plasmin (25nM to 500nM in DMEM media) to cell-free fibrin gels for 1 hour at 37°C (Figure 5.1). The standard curve showed a linear distribution at concentrations up to 200nM plasmin, at which point total fibrin gel degradation was observed and FITC-fibrin degradation products were present in the supernatant. To assess the efficiency of α_2-antiplasmin (0.25μM to 2μM in DMEM media) at inhibiting plasmin-mediated fibrinolysis, the inhibitor was added in combination with plasmin to the FITC-labelled fibrin gels. Results showed that 1μM α_2-antiplasmin was sufficient to completely inhibit fibrinolysis by plasmin at concentrations up to 500nM (Figure 5.1). No further inhibition of fibrinolysis was observed at higher concentrations of the inhibitor (2μM), therefore, in future experiments only 1μM α_2-antiplasmin concentrations were used.

5.2.2 The effect of α_2-antiplasmin on fibrinolytic activity of dermal fibroblasts

The effect of α_2-antiplasmin on fibrinolysis by dermal fibroblasts in fibrin gels was examined using the FITC-labelled fibrin gel system. Plasmin (200nM) was added to FITC-labelled fibrin gels without cells to determine the total amount of FITC-degradation products released following complete lysis of the gel. The data showed that total FITC levels in the fibrin gels remained identical, even after total degradation, and that there was no decay of the fluorescent FITC marker over the 72 hour time course. The level of plasminogen contaminating the fibrinogen solution was estimated to be 200nM (manufacturer's information, Sigma, Poole, UK). Therefore, with the assumption that all plasminogen in the fibrin gels was activated by fibroblasts, then total fibrin degradation would occur. Dermal fibroblasts embedded within fibrin gels caused approximately 70% lysis of the original gel after 72 hours, suggesting that plasminogen was present in the gels at sufficient quantities to not be rate-limiting for fibrinolysis (Figure 5.2). Addition of α_2-antiplasmin to the cultures significantly reduced fibrinolysis compared with gels

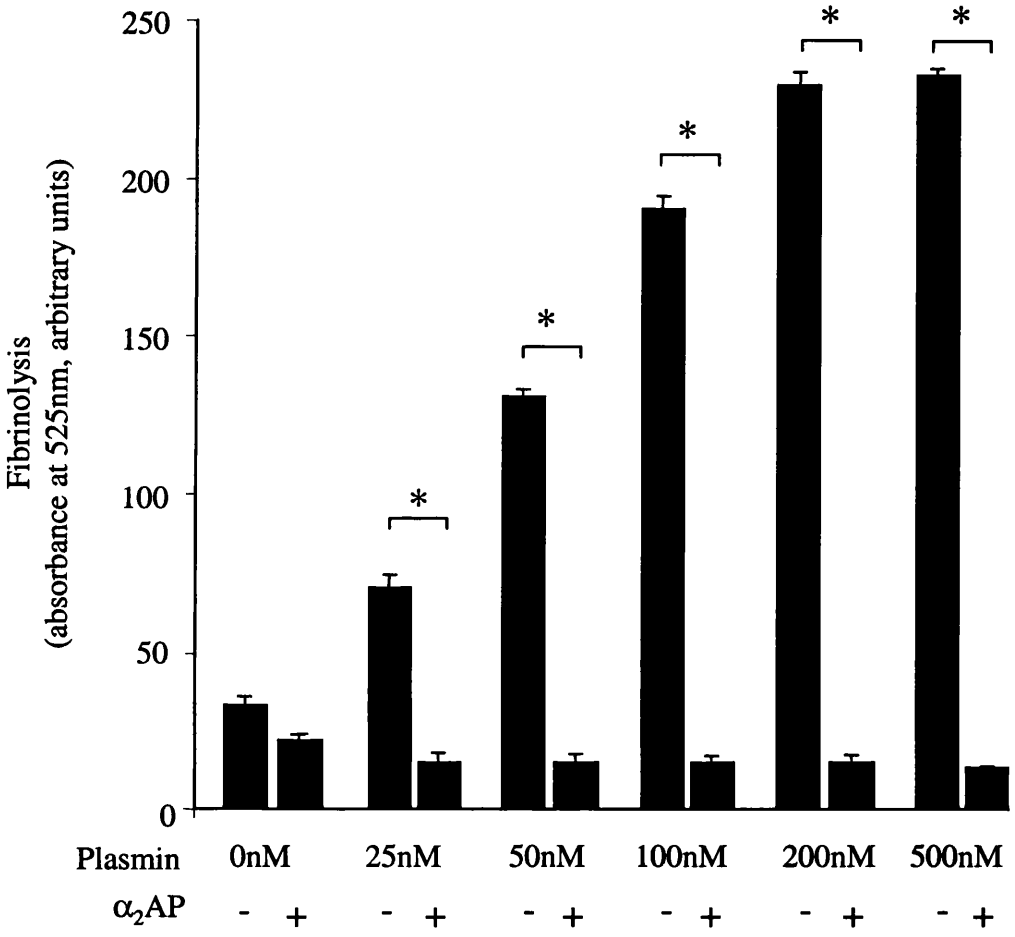


Figure 5.1. α_2-antiplasmin significantly reduced fibrinolysis by exogenously added plasmin in fibrin gels. Degradation of FITC-labelled fibrin gels by plasmin with and without α_2-antiplasmin (α_2AP) (1μM) after 1 hour incubation at 37°C. The amount of FITC-labelled fibrin degradation products released into the supernatant was measured at 525nm (mean ± standard error, n=12, * = $p<0.001$)

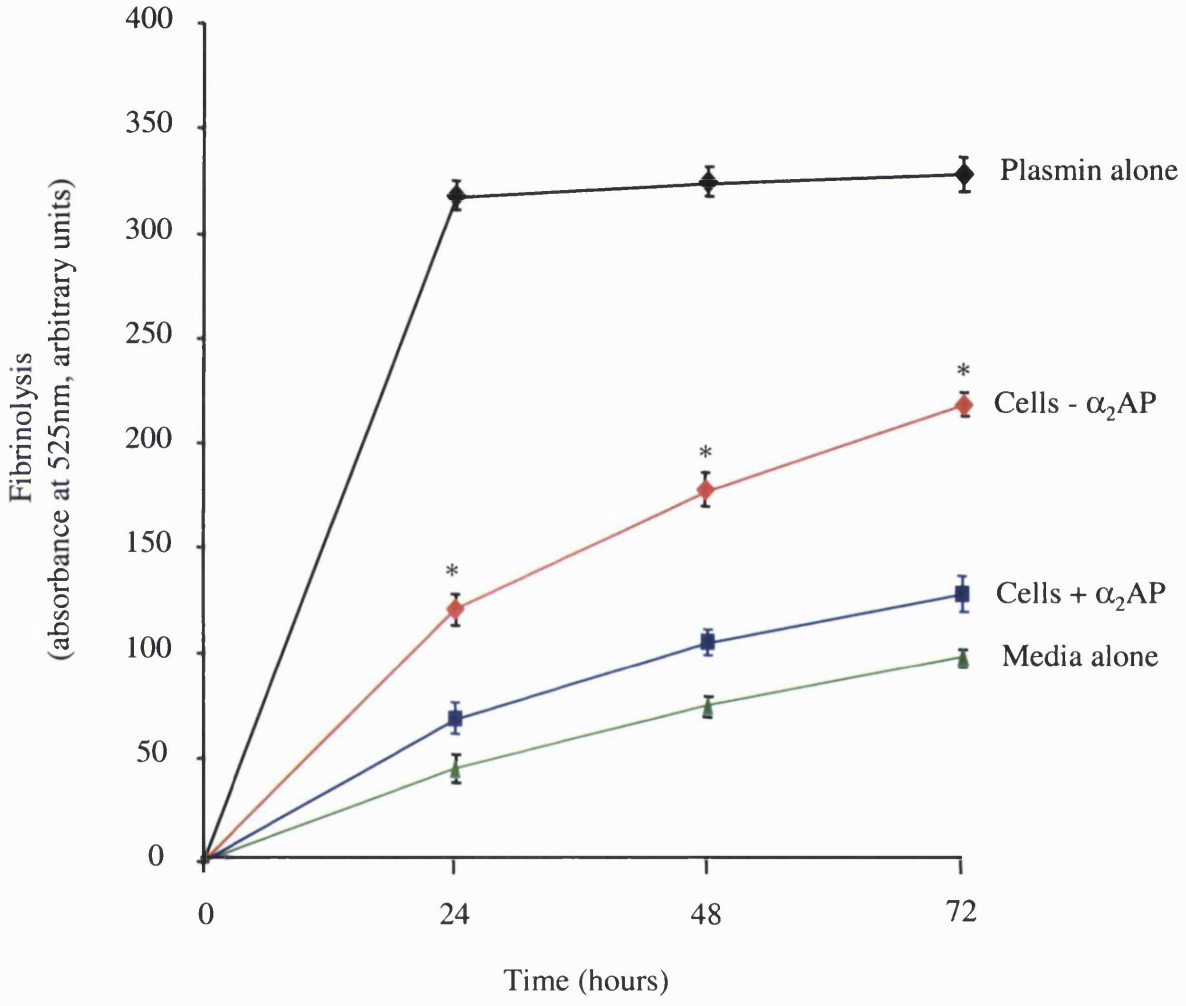


Figure 5.2. α_2-antiplasmin significantly reduced fibrinolysis by human dermal fibroblasts in fibrin gels. Degradation of FITC-labelled fibrin gels by human dermal fibroblasts with and without α_2-antiplasmin (α_2AP) (1μM). Plasmin (200nM) and media alone were positive and negative controls without cells respectively (mean ± standard error, n=6, * = $p<0.01$)

without the inhibitor at all time-points examined. Indeed, fibrinolysis was impaired by approximately 70% in the presence of α_2-antiplasmin at 24, 48 and 72 hours, compared with cells without the inhibitor. Fibrin degradation also occurred when serum-free media alone was added to fibrin gels without fibroblasts, and increased at a constant low rate throughout the experiment, however, this was lower than the level of fibrinolysis by fibroblasts in the presence of α_2-antiplasmin.

5.2.3 The effect of reduced fibrinolysis by α_2-antiplasmin on procollagen production by dermal fibroblasts

Having established that addition of α_2-antiplasmin inhibited the degradation of fibrin gels by fibroblasts, the effect of reduced fibrinolysis on procollagen production was subsequently investigated. Human dermal fibroblasts grown in fibrin gels in the presence of 1μM α_2-antiplasmin, produced significantly increased levels of procollagen compared with cells in fibrin gels without inhibitor after 48 hours and 72 hours in culture ($p<0.01$, Figure 5.3). Procollagen levels were increased by approximately 30% after 48 hours in the presence of the inhibitor, whilst the maximal increase was observed at 72 hours with a 40% rise in procollagen production, compared to without inhibitor. There was no significant increase in procollagen production at 24 hours. Fibroblasts in fibrin gels produced significantly more procollagen with and without the inhibitor compared with fibroblasts on tissue culture plastic at all time-points ($p<0.01$). Furthermore, α_2-antiplasmin alone showed no effect on procollagen production by fibroblasts grown on plastic at any time point examined, suggesting that increased procollagen levels were due to reduced fibrinolysis and fibrin persistence and not the peptide itself. Moreover, cell count studies showed that there was no fibroblast proliferation in the presence of α_2-antiplasmin in either fibrin gels or on tissue culture plastic (data not shown).

5.2.4 The effect of plasminogen activator-deficiency on the fibrinolytic activity of dermal fibroblasts

The effect of reduced fibrinolysis on collagen production was also examined using plasminogen activator-deficient fibroblasts, isolated from tPA- and uPA-deficient mice. This method allowed analysis of the roles of the individual plaminogen activators in

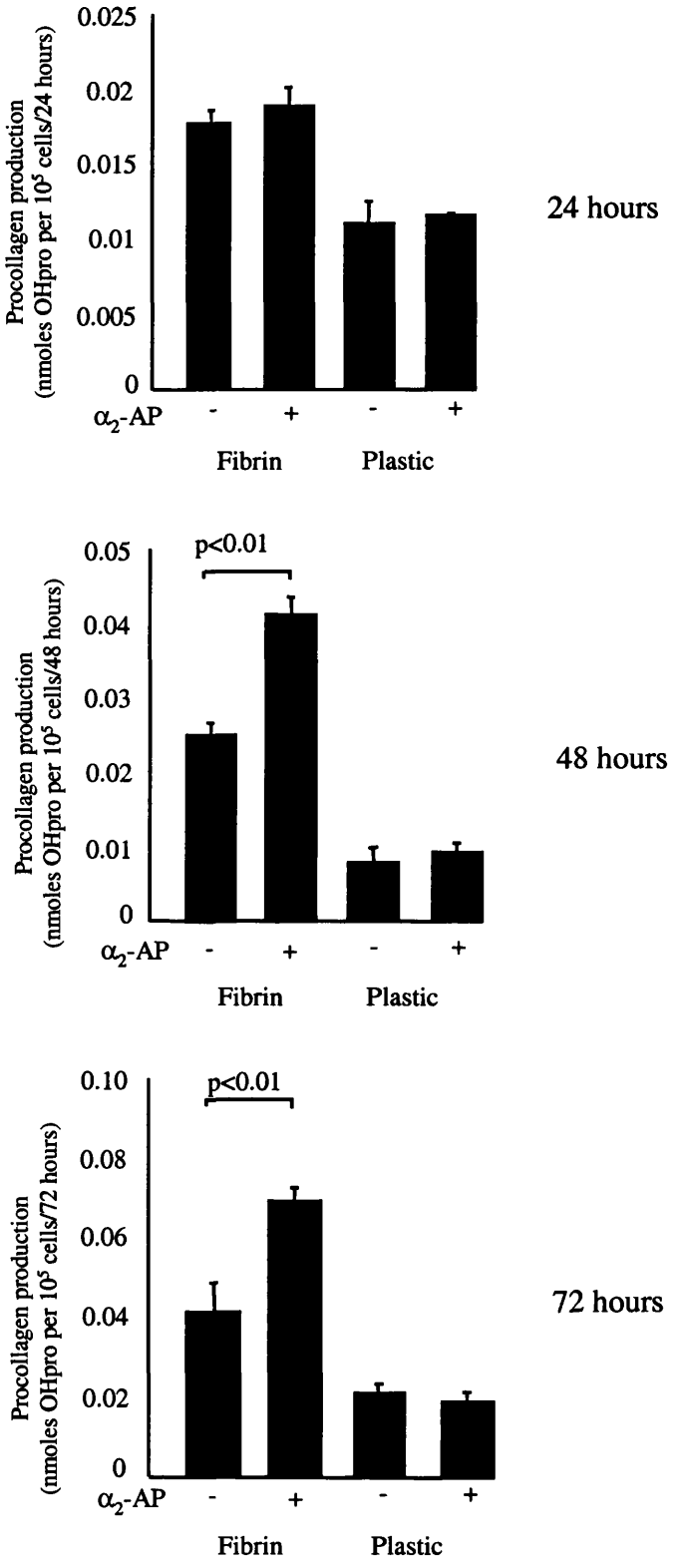


Figure 5.3. Procollagen production by human dermal fibroblasts grown in fibrin gels with and without α_2-antiplasmin (α_2AP) (1μM), compared with cells grown on tissue culture plastic, measured by HPLC analysis of hydroxyproline (mean ± standard error, n=6).

fibroblast-mediated fibrinolysis and procollagen production. Fibrin gel degradation by dermal fibroblasts isolated from tPA-deficient mice was significantly reduced compared with wild-type and uPA-deficient fibroblasts at 24, 48 and 72 hours ($p<0.01$, Figure 5.4). Similarly, uPA-deficient fibroblasts showed a significant decrease in fibrinolytic activity compared with wild-type fibroblasts after 48 and 72 hours ($p<0.01$). The fibrinolytic activity of tPA-deficient fibroblasts was approximately 40% less at 24 and 72 hours, and 50% less at 48 hours, than that of wild-type fibroblasts. Fibrinolytic activity of uPA-deficient fibroblasts was only 10% less at 48 hours and approximately 35% less at 72 hours. Degradation of the FITC-labelled fibrin gel with serum-free media alone accounted for approximately 20% of the fibrinolytic activity of wild-type fibroblasts at 72 hours. Total FITC-fibrin gel degradation was examined following the addition of plasmin to fibrin gels without fibroblasts. Wild-type fibroblasts degraded approximately 60% of the fibrin gel by 72 hours, compared with 38% by uPA-deficient fibroblasts and 32% by tPA-deficient fibroblasts.

5.2.5 The effect of plasminogen activator-deficiency on procollagen production by dermal fibroblasts

The effect of reduced fibrinolysis and fibrin persistence on procollagen production by plasminogen activator-deficient fibroblasts was assessed in three-dimensional fibrin gels by HPLC analysis of hydroxyproline. Procollagen production was significantly increased by tPA- and uPA-deficient and wild-type fibroblasts in fibrin gels compared with fibroblasts on tissue culture plastic. There was no significant difference in basal levels of procollagen production between the different types of fibroblasts. Similar levels of procollagen produced on tissue culture plastic at all time points examined. The level of procollagen production by tPA-deficient fibroblasts was significantly greater than that produced by uPA-deficient and wild-type dermal fibroblasts in fibrin gels at 48 and 72 hours (Figure 5.5). However, there was no significant increase in procollagen production by uPA-deficient fibroblasts compared with wild-type fibroblasts at any of the time-points examined even though they showed significantly reduced levels of fibrinolysis.

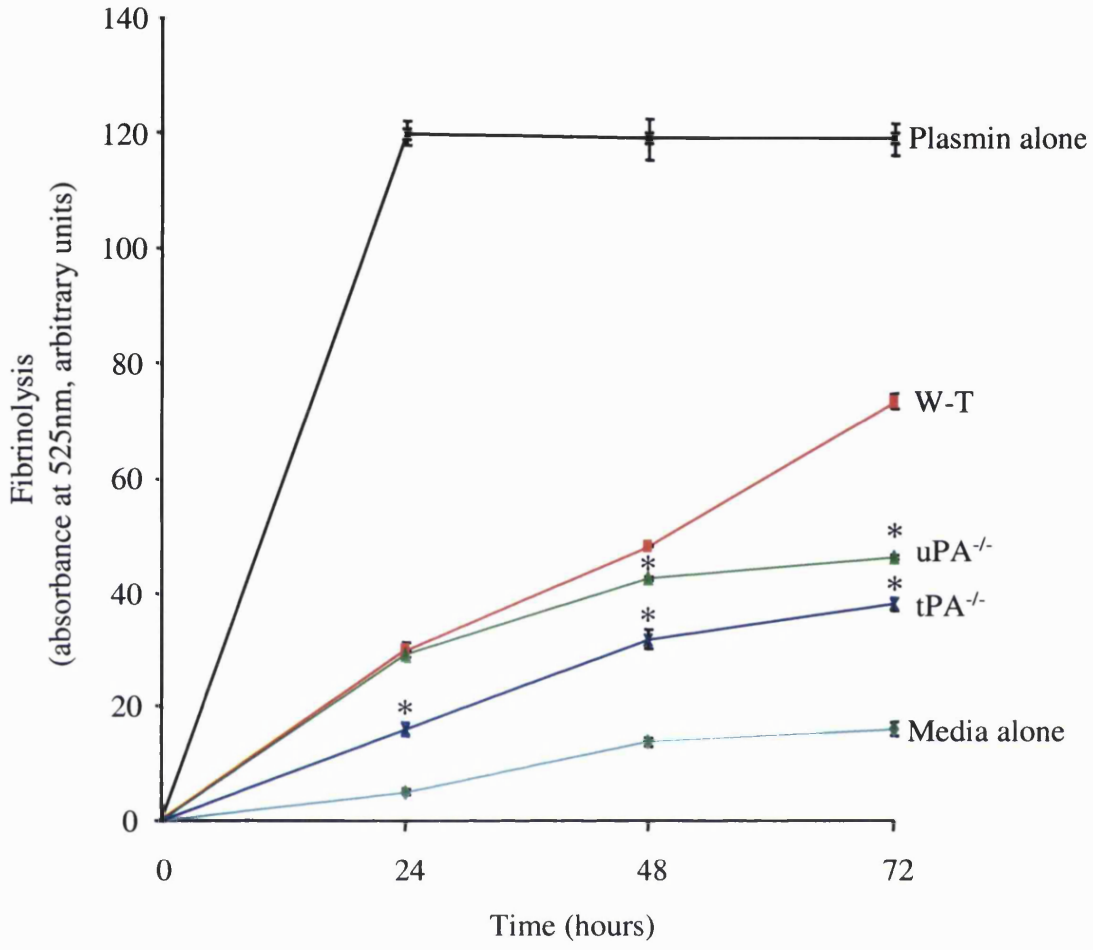


Figure 5.4. Degradation of FITC-labelled fibrin gels by plasminogen activator-deficient dermal fibroblasts (tPA-/- or uPA-/-) or wild-type dermal fibroblasts (W-T). Plasmin (200nM) and media alone represented positive and negative controls, without cells (mean ± standard error, n=6, * = $p<0.01$ compared with wild-type)

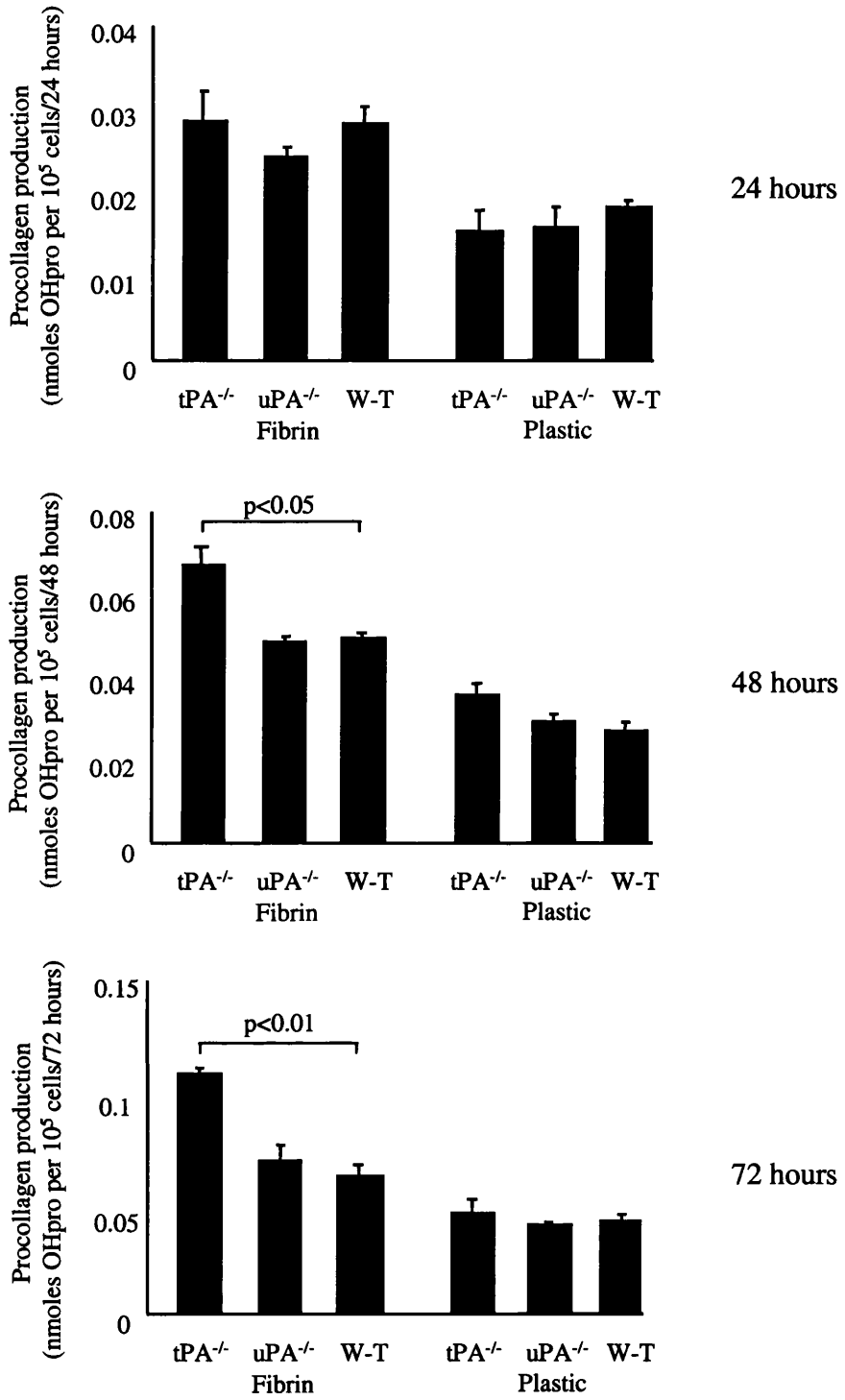


Figure 5.5. Procollagen production by plasminogen activator-deficient fibroblasts ($tPA^{-/-}$ or $uPA^{-/-}$) or wild-type fibroblasts (W-T) grown in fibrin gels, or on tissue culture plastic, measured by HPLC analysis of hydroxyproline (mean ± standard error, n=6).

5.2.6 The effect of subcutaneous injections of fibrin on collagen production in plasminogen activator-deficient animals

The effect of persistent fibrin deposition and reduced fibrinolysis on collagen production was also examined *in vivo* by injecting plasminogen activator-deficient mice with subcutaneous fibrinogen and thrombin. The same volume of sterile PBS was injected into the opposite flank of each animal to act as a negative control. One week after the final injection, the two injection sites and an area of untreated skin were harvested. Full thickness skin biopsies were processed for histological analysis (n=4) and total collagen content by HPLC analysis of hydroxyproline (n=10). No animals died following any of the treatments and the consistency of the animal weights showed that they remained healthy throughout the experimental period.

5.2.7 Histological appearance

All tissue sections were stained with Haemotoxylin and Eosin and Martius Scarlet Blue and skin thickness, collagen density and fibrin density were assessed by semi-quantitative image analysis (Figure 5.6). All animals had a similar thickness of untreated skin, when measuring from the base of the epidermis to the top of the peritoneum (Figure 5.7A). The skin of both plasminogen activator-deficient animals was thicker than wild-type skin injected with fibrin, and fibrin-injected skin from all animal groups was significantly thicker than their respective PBS-treated and untreated controls. There was no significant difference in total thickness between tPA-deficient and uPA-deficient animals one week after the final injection. Plasminogen activator-deficient mice fibrin-injected sites had significantly thicker fascia compared with wild-type animals, although there was no significant difference between tPA- and uPA-deficient animals (Figure 5.7B). Repeated PBS injections had no effect on total or fascial layer thickness and was similar to untreated skin in all three groups.

Semi-quantitative analysis of Martius Scarlet Blue-stained sections demonstrated that there was a significantly greater amount of residual fibrin in the skin of tPA-deficient animals, compared with uPA-deficient and wild-type mice one week after the final injection ($p<0.05$, Figure 5.8A). In contrast, there was no significant difference in the amount of fibrin observed in the skin of uPA- deficient and wild-type mice. Fibrin

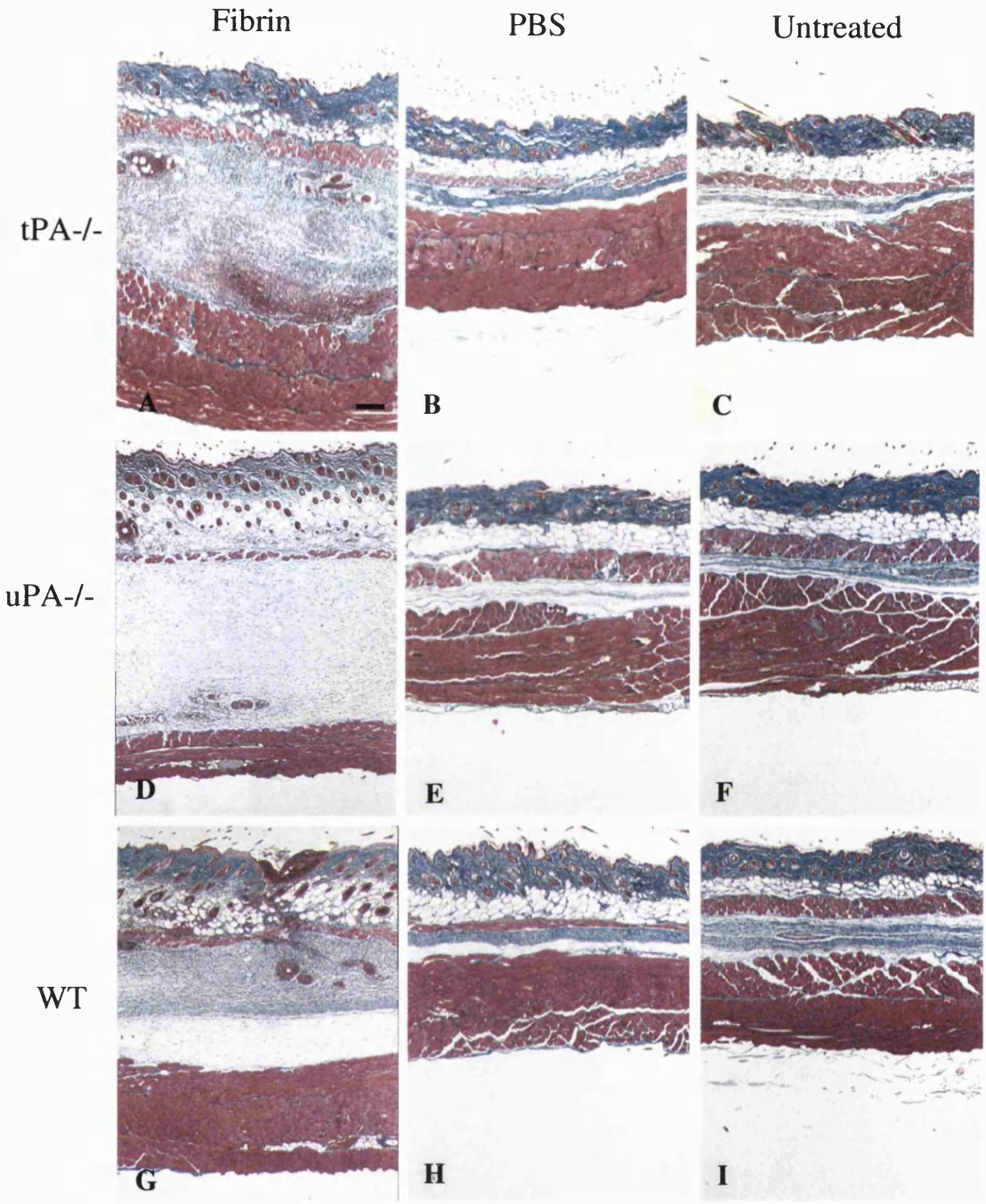


Figure 5.6. The effect of multiple injections of fibrin (A, D, G), PBS (B, E, H) or no treatment (C, F, I) on the skin of plasminogen activator-deficient and wild-type mice, 7 days after the final injection. (Bar represents 100μm, Martius Scarlet Blue stain.)

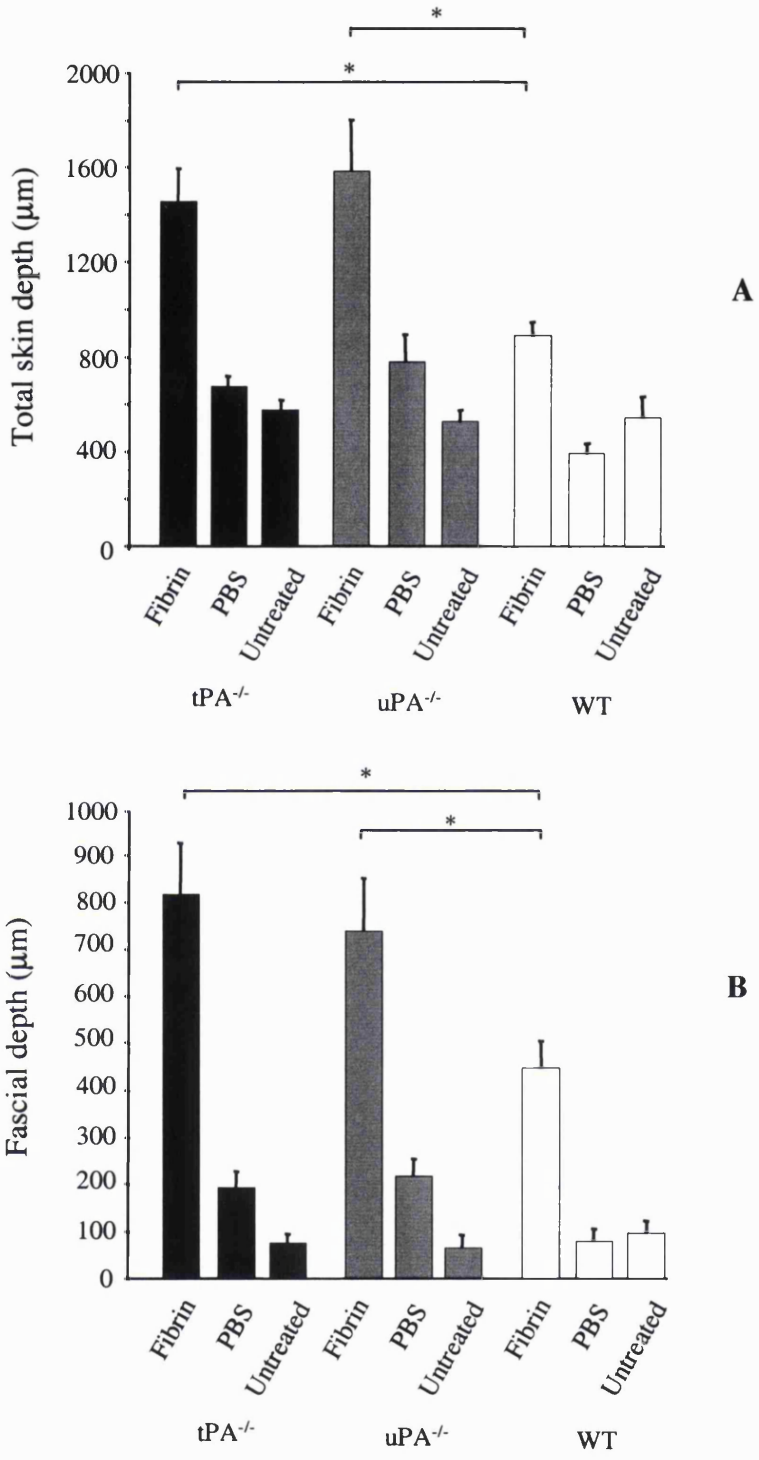


Figure 5.7. The effect of multiple subcutaneous injections of fibrinogen and thrombin or PBS alone on **A**. total skin depth and **B**. fascial layer depth one week after the final injection in plasminogen activator deficient mice. Each bar represents five measurements from nine sections from each 8mm diameter biopsy, taken from each of four mice by two independent scorers (mean ± standard error, n=4, * = $p<0.05$)

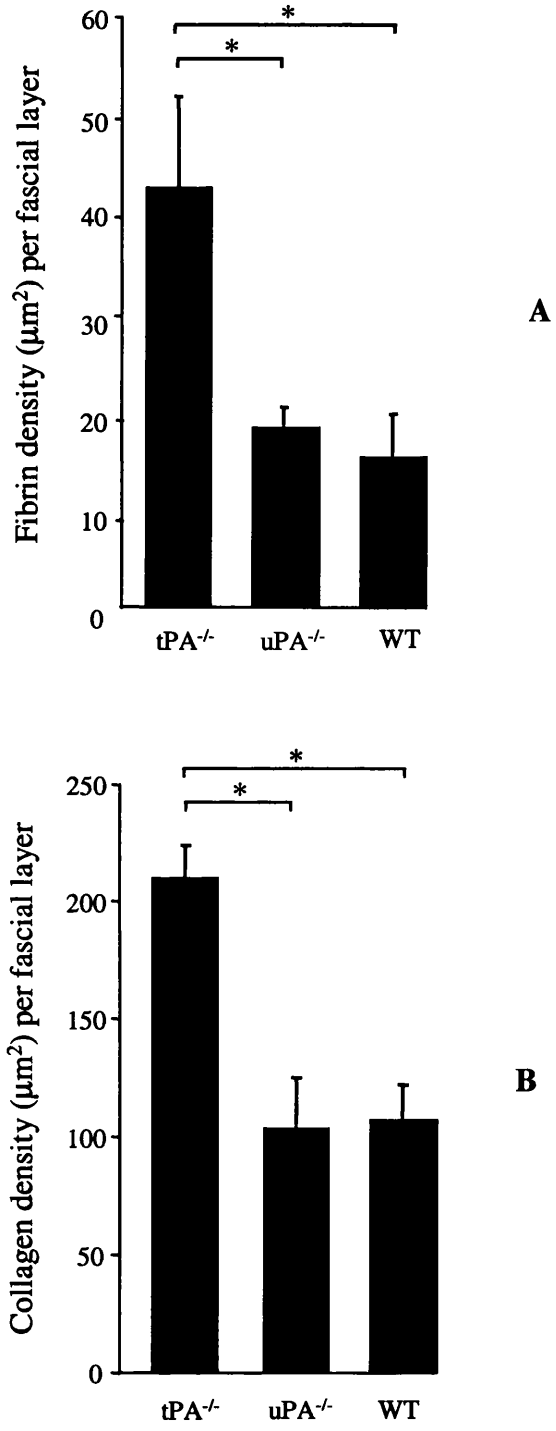


Figure 5.8. The effect of multiple subcutaneous injections of fibrinogen and thrombin on **A.** fibrin density and **B.** collagen density in the fascial layer one week after the final injection in plasminogen activator deficient mice. Five measurements from nine sections from each injection site (8mm biopsy) were taken from each of four mice by two independent scorers (mean ± standard error, n=4, * = $p<0.05$)

deposits in wild-type biopsies were scarce and any residual fibrin was diffusely distributed. Fibrin deposition in the skin of tPA-deficient animals was more widespread and concentrated in large deposits, mainly in the fascia, although numerous lesions were observed in the dermis. There were numerous spindle-shaped fibroblasts that appeared to invade the fibrinous lesions and were depositing dense collagen bundles (Figure 5.9A and B). Indeed, the amount of collagen in the fascial layer was significantly enhanced in tPA-deficient animals compared with uPA-deficient and wild-type animals, semi-quantified by image analysis ($p<0.05$, Figure 5.8B). In areas with minor amounts of residual fibrin, collagen was observed in parallel bundles interspersed between fibrin fibres (Figure 5.9C). In regions of the fascial layer the collagen bundles were more disorganised and random in orientation, and were often surrounded by areas of parallel aligned collagen bundles that were more reminiscent of scar formation (Figure 5.9D). Fibrin deposits in all skin samples had numerous inflammatory cells, in particular macrophages and eosinophils (Figure 5.10A). Increased collagen accumulation and an inflammatory cell infiltrate was also observed in the subcutaneous fat tissue, with a mild fibrotic response in the fat septae and around blood vessels (Figure 5.10B). However, inflammatory cell accumulation was most severe in the uPA-deficient mice, and often blood vessels were 'cuffed' by the inflammatory cell infiltrate with erythrocyte extravasation (Figure 5.10C).

Numerous blood vessels were observed in close proximity to or within fibrin deposits in skin samples from all three strains of mice. There appeared to be no difference in the number of blood vessels in the skin of plasminogen activator-deficient and wild-type mice following administration of fibrin, but there appeared to be more blood vessels in fibrin-injected sites compared with PBS-injected sites in all three strains (data not shown).

5.2.8 Collagen production

Collagen deposition was analysed by HPLC analysis of hydroxyproline in fibrin-injected, PBS-injected and untreated sites one week after the final injection (Figure 5.11). Collagen deposition in the skin of tPA-deficient mice was significantly increased compared with uPA-deficient and wild-type mice in response to subcutaneous injections

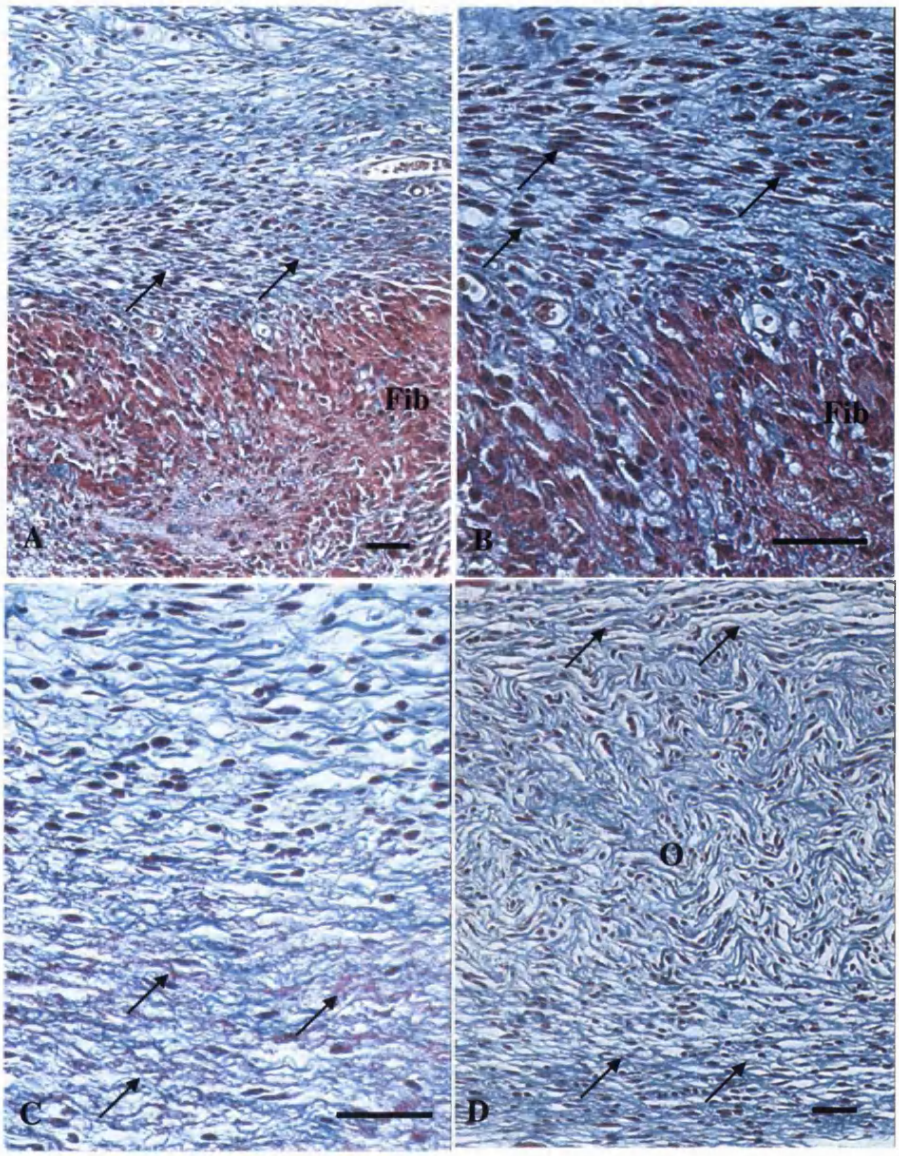


Figure 5.9. Changes in collagen and fibrin distribution following multiple subcutaneous injections of fibrin in plasminogen activator-deficient mice, one week after the final injection. **A.** Parallel collagen bundles (blue) at the edge of fibrinous deposits (Fib, red) interspersed with fibroblasts (arrows) in the fascia of tPA-deficient skin (**B**. High power magnification of A). **C.** Fibrin fibres (arrows, red) interspersed between parallel collagen bundles (blue) in the fascial layer of tPA-deficient skin. **D.** Different orientation of collagen bundles in the fascia. Parallel bundles of collagen (arrows, blue) surround randomly orientated collagen 'swirls' (O, blue) in the fascia of tPA-deficient skin. (Bar represents 50μm, Martius Scarlet Blue trichrome stain)

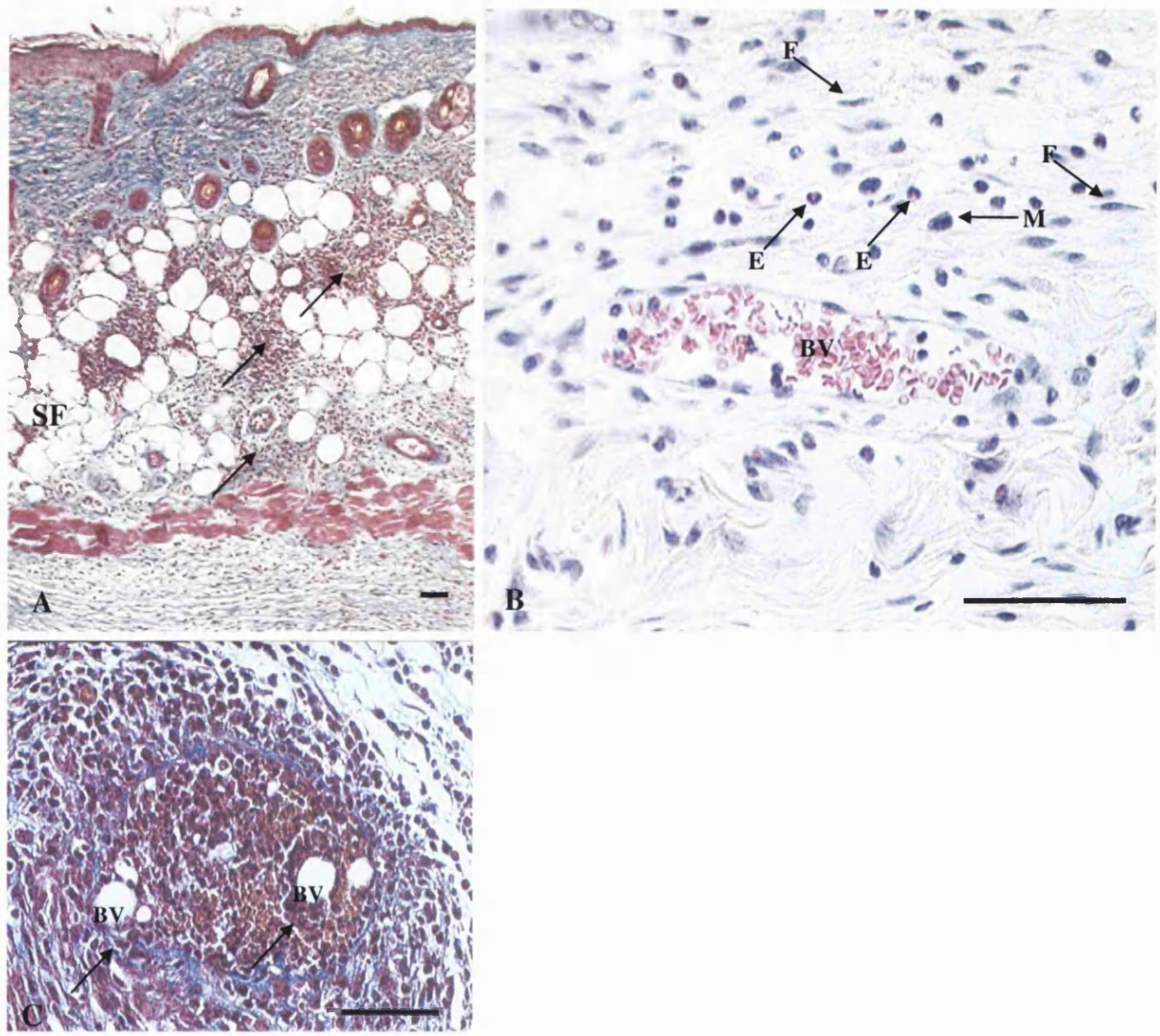


Figure 5.10. The effect of multiple subcutaneous injections of fibrin on inflammatory cell distribution in plasminogen activator-deficient mice, one week after the final injection. **A.** Infiltration of subcutaneous fat (SF) by inflammatory cells with collagen deposition (blue) in tPA-deficient fibrin-injected skin. **B.** Macrophages (M), eosinophils (E) and fibroblasts (F) in the fascial layer of fibrin-injected uPA-deficient skin. **C.** Inflammatory cell 'cuffing' (arrows) of blood vessels (BV) with erythrocyte extravasation (orange) in the fascia of uPA-deficient skin. (Bar represents 50μm, Martius Scarlet Blue trichrome stain.)

of fibrinogen and thrombin ($p<0.05$). Indeed, tPA-deficient mice demonstrated nearly a 2-fold increase in collagen deposition above PBS injections. Collagen deposition in fibrin-injected sites was significantly increased in all three strains compared with their respective PBS-injected controls. However, there was no difference in the amount of collagen deposited in uPA-deficient and wild-type mice, supporting histological measurements of skin thickness and collagen density. The levels of collagen in PBS-injected and normal untreated skin were similar in the three strains of mice (Figure 5.11).

In the previous chapter, fibrin injected subcutaneously into Balb-c mice resulted in a significant increase in collagen production compared with PBS-injected and untreated skin. Here, fibrin injected into tPA-deficient mice showed a further increase in production of collagen compared with uPA-deficient and wild-type mice. This chapter confirmed both *in vitro* and *in vivo*, that reduced fibrinolysis and persistence of fibrin matrix are associated with excessive accumulation of collagen by dermal fibroblasts.

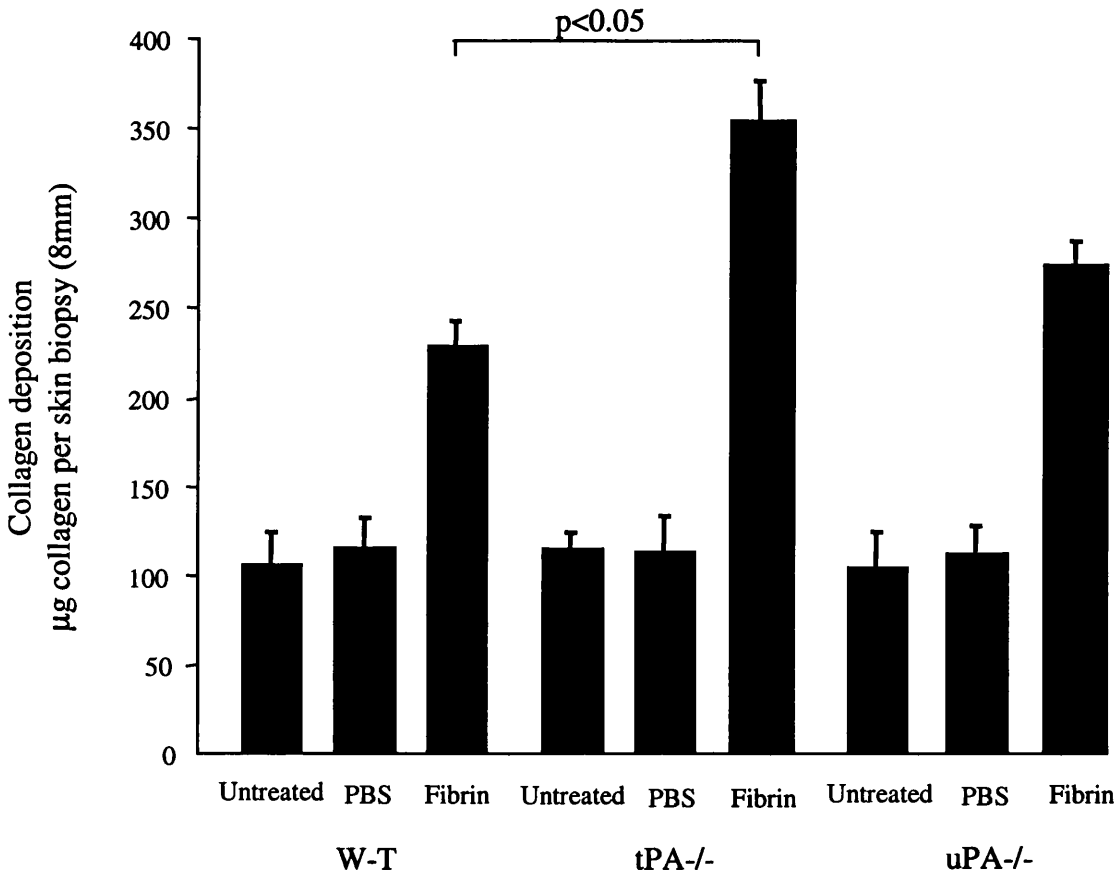


Figure 5.11. The effect of multiple subcutaneous injections of fibrinogen (9µM) and thrombin (140nM) on collagen deposition in the skin of plasminogen activator-deficient and wild-type mice by HPLC analysis of hydroxyproline, one week after the final injection (mean ± standard error, n=10).

5.3 Discussion

To examine the hypothesis that persistent fibrin deposition and reduced fibrinolysis are associated with an enhanced accumulation of collagen in the skin, the *in vitro* and *in vivo* systems developed in the previous chapter were modified to encompass reduced fibrinolysis. Collagen production by human dermal fibroblasts in fibrin gels was assessed in the presence of the plasmin inhibitor, α_2-antiplasmin. Furthermore, collagen production by dermal fibroblasts isolated from plasminogen activator-deficient animals was also examined. Reduced fibrinolysis in these systems was confirmed using a fibrin degradation assay. These studies were extended *in vivo*, by examining the effect of persistent fibrin and reduced fibrinolysis on collagen deposition in the skin, performing the skin fibrosis model in plasminogen activator-deficient mice.

Several studies have shown that fibroblasts produce plasminogen activators (Rijken, 1995; Lijnen and Collen, 1995) *in vitro* and *in vivo*, which in turn, catalyse the conversion of plasminogen into plasmin, resulting in the degradation of the insoluble fibrin matrix into soluble fibrin degradation products. Addition of the plasmin inhibitor, α_2-antiplasmin, to dermal fibroblasts in fibrin gels resulted in a decrease in fibrinolysis compared with cells in gels without the inhibitor. α_2-antiplasmin is considered one of the most important circulating fibrinolytic inhibitors. It is a rapid and effective inhibitor of plasmin, and also inhibits the activity of tPA (Mossesson, 1990). Studies have shown that α_2-antiplasmin becomes reversibly cross-linked to the Aα chain of fibrinogen or fibrin (Ichinose and Aoki, 1982) resulting in the half-life of plasmin activity in a fibrin matrix of 0.1 seconds (Lijnen and Collen, 1995). Titration of α_2-antiplasmin against exogenously added plasmin demonstrated that 1μM of the inhibitor was sufficient to inhibit up to 500nM of exogenously added plasmin. Plasminogen levels contaminating the fibrinogen solution were 200nM (from manufacturer's information), thus 1μM α_2-antiplasmin should have been capable of inhibiting all plasmin activated by embedded fibroblasts. However, not all the plasminogen in the fibrin matrix appeared to be activated by the fibroblasts, even after 72 hours. This suggests that plasminogen levels were sufficient to allow complete fibrinolysis and were not rate-limiting, as confirmed by the addition of exogenous plasmin at 200nM, which showed complete degradation of gel

within 1 hour. Degradation of the fibrin gel was also observed in the absence of cells, suggesting FITC was released into the media due to unstable association of the fibrin monomers due to the absence of covalent cross-linking by transglutaminase factor XIII, or the presence of other fibrin-degrading proteases. Contamination of the fibrinogen preparation with bacterial proteases, or indeed proteases cross-linked to the fibrinogen is possible, and studies have shown that proteases such as matrix metalloproteinases and cathepsins D and G are also able to degrade fibrin (reviewed by Herrick et al., 1999), which are not inhibited by α_2-antiplasmin. The presence of such proteases in this system was not examined.

Dermal fibroblasts in fibrin gels in the presence of α_2-antiplasmin, produced significantly more collagen compared with cells in gels without inhibitor at 48 and 72 hours. The inhibitor alone had no effect on collagen production as demonstrated by fibroblasts on plastic, suggesting that the increase in collagen deposition was due to the effect of fibrin persistence and reduced fibrinolysis. Collagen production by dermal fibroblasts in fibrin gels following the addition of fibrinolytic inhibitors has not been demonstrated previously. As discussed in the previous chapter, the mechanism by which fibroblasts interact with fibrin matrix remains incomplete, although evidence suggests that integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ play a role (Gailit and Clark, 1996). Fibrin matrix remodelling is a fundamental event during tissue repair leading to up-regulation of matrix protein synthesis and deposition (Dvorak, 1986). Disruption of these remodelling events, through the addition of an inhibitor of fibrinolysis is likely to alter the deposition of matrix components, in particular collagen. One consequence of remodelling is a change within the three-dimensional organisation of the matrix to reveal cryptic sites previously unrecognisable by cell surface receptors (Streuli, 1999). Fibrin persistence in this system may result in a continued exposure of such sites to fibroblast receptors that induce enhanced collagen deposition or the down-regulation of matrix-degrading enzymes. Matrix remodelling also has the potential to conceal receptor-ligand interaction sites, that may signal the down-regulation of matrix protein gene expression. Thus, subtle changes within the extracellular matrix by specific protease degradation may influence how the

matrix is remodelled. A persistent fibrin matrix may regulate procollagen gene expression differently from a provisional fibrin matrix that is cleared normally.

Lorimier and colleagues (1996) added inhibitors of plasmin to dermal and gingival fibroblasts in fibrin gels, to investigate differences in gel remodelling. The group supplemented dermal and gingival fibroblasts in fibrin gels with synthetic inhibitors of plasmin, aprotinin and ε-aminocaproic acid, and found an inhibition of fibrinolysis in a dose-dependent manner. Although reducing fibrinolysis resulted in significantly slower remodelling of fibrin gels by both cell types, more fibrin was degraded by the gingival fibroblasts than dermal fibroblasts. This difference was due to significantly increased levels of tPA produced by gingival fibroblasts and it was proposed that this difference may explain why wounds of the oral cavity heal faster and with less collagen deposition than dermal wounds. In addition, other studies have demonstrated fundamental differences associating fibroblasts with reduced fibrinolysis to excessive collagen production. PAI-1 levels produced by dermal fibroblasts isolated from patients with the fibrotic skin conditions keloids and Werner's syndrome, were shown to be significantly increased (Tuan et al., 1996, Higgins et al., 1999). Tuan and others (1996) associated this up-regulation of the fibrinolytic inhibitor with a reduced ability of the fibroblasts to degrade fibrin gels, and proposed that this may lead to prolonged tissue repair and excessive connective tissue production. Higgins and colleagues (1999) confirmed that the increase in PAI-1 levels was associated with fibroblasts predisposed to a fibrotic phenotype, producing excessive quantities of both collagen and fibronectin.

An alternative method for examining the effect of reduced fibrinolysis and fibrin persistence on collagen production was by using fibroblasts from genetically modified mice, deficient in plasminogen activators, tPA or uPA. The development of these mice has provided the means to examine the role of the fibrinolytic components, tPA, uPA and plasmin in various physiological and pathological processes. Studies in mice deficient for components of the fibrinolytic system have confirmed the importance of the fibrinolytic pathway in inflammation, reproduction, angiogenesis, thrombolysis, tumour metastasis, macrophage function and in the maintenance of vascular patency (reviewed by Carmeliet

et al., 1994). Reduced fibrinolysis by dermal fibroblasts isolated from tPA and uPA-deficient mice was examined using the FITC-fibrin degradation assay. Both tPA- and uPA-deficient fibroblasts demonstrated significantly lower fibrinolysis than wild-type fibroblasts. Fibrinolysis was significantly more diminished in tPA-deficient fibroblast cultures compared with uPA-deficient fibroblasts, indicating a physiological distinction between tPA and uPA in the remodelling of fibrin by fibroblasts. Tissue PA is a key enzyme in the fibrinolytic cascade, responsible for the efficient removal of fibrin from the intravascular system and tissues after injury primarily through the activation of plasmin (Dano et al., 1985). The significance of this enzyme to the fibrinolytic system is indicated by its high affinity for fibrin matrix (Rijken et al., 1982), resulting in an increase in its activity by several hundred-fold when bound (Hoylaerts et al., 1982). In contrast, uPA acts in a fibrin-independent cell-bound manner, the main role of which is thought to be the generation of plasmin in events involving pericellular extracellular matrix degradation, including cell migration, matrix remodelling and inflammatory cell infiltration (Andreasen et al., 1997).

The consequence of impaired fibrinolysis on procollagen production was further examined in dermal fibroblasts isolated from tPA-deficient mice. Procollagen production was significantly increased compared with fibroblasts from uPA-deficient and wild-type mice. Collagen production by uPA deficient and wild-type fibroblasts was similar. These data suggest tPA-deficiency results in enhanced collagen production under conditions of persistent fibrin deposition and/or reduced fibrinolysis, and the increase in collagen was not due to an increased basal rate of collagen production as shown by fibroblasts on tissue culture plastic. In contrast, uPA-deficient fibroblasts also exhibited significantly reduced fibrinolysis in FITC-fibrin gels but showed no concomitant increase in collagen production. This evidence suggests a major role for tPA in the regulation of collagen by fibroblasts in fibrin matrix, and emphasises differences in physiological activity between tPA and uPA. Although little is known about fibroblast-fibrin interactions in the synthesis of collagen, these observations suggest that tPA-mediated plasmin activation and fibrinolysis plays an important role in the regulated deposition of collagen by fibroblasts

in fibrin matrix. In the absence of this pathway, collagen accumulation becomes excessive.

Although the principal role of plasmin is thought to be in the degradation of fibrin into soluble degradation products (Mossesson, 1990), plasmin is also a potent activator of MMPs, in particular, the interstitial collagenase MMP-1 (Rao et al.,1999, Murphy and Gavrilovic 1999, Brassart et al., 2001). MMP-1 denatures fibrillar collagens by cleaving the α1 and α2 chains at specific amino acid recognition sequences and is fundamental for regulated deposition of collagen in the extracellular matrix (Nagase 1991). Moreover, there is considerable evidence that uPA-mediated plasmin generation and MMP activation occurs at the cell surface (Murphy and Gavrilovic, 1999). The absence of this interaction in uPA-deficient fibroblasts in fibrin matrix may prevent pericellular collagenolysis and thus collagen accumulates around the cell. Studies with fibroblasts in collagen gels demonstrated down-regulation of collagen gene expression following interaction of α1β1 and α2β1 integrins with the collagen-rich matrix (Riikonen et al., 1995). This mechanism may reduce collagen production by uPA-deficient fibroblasts compared with tPA-deficient cells. Other *in vitro* studies have shown that interstitial collagenases degrade collagen only after uPA-activated plasmin-dependent removal of the surrounding glycoproteins (Montgomery et al., 1993). The lack of glycoprotein degradation by uPA-deficient fibroblasts, may also contribute to the accumulation of collagen. It is clear that the interaction of fibrinolytic and MMP pathways may play an important role in both the degradation of fibrin and regulation of collagen deposition in the same *in vitro* system. The effect of inhibiting fibrinolysis on the activation MMP-1, and the accumulation of collagen by dermal fibroblasts in fibrin gels will be investigated in the subsequent chapter.

The effect of reduced fibrinolysis and persistent fibrin deposition was examined *in vivo* using the model of skin fibrosis described in the previous chapter. Collagen production was significantly increased in the skin of tPA-deficient animals compared with both uPA-deficient and wild-type mice, one week after the final injection of fibrin. Indeed, this increase in hydroxyproline levels in tPA-deficient skin was reflected by a significant

increase in skin depth and fascial layer depth. Collagen density in the fascia was significantly increased as measured by semi-quantitative image analysis. In addition, persistent fibrin deposition was a prominent histological feature of fibrin-treated tPA-deficient skin, and was observed primarily in the fascia. This is supported by evidence showing fibrin deposition in plasminogen activator-deficient mice and other mice with impaired fibrinolysis following experimental injury (Swaisgood et al., 2000). Bleomycin-instillation resulted in approximately a 2-fold increase in the level of collagen in the lungs of tPA-deficient, plasminogen-deficient and PAI-1 overexpressing mice compared with wild-type mice. Furthermore, Eitzman and others (1996) also demonstrated that overexpression of PAI-1 resulted in increased deposition of collagen in the lungs of bleomycin treated mice, whereas PAI-1-deficient mice produced levels of collagen comparable to wild-type mice. These results confirm those of the present study suggesting that the persistence of fibrin is directly associated with enhanced accumulation of collagen. Fibrin has been shown to serve as a provisional scaffold into which inflammatory cells and fibroblasts migrate, proliferate and deposit extracellular matrix components, in particular collagen (Dvorak, 1986). Therefore, the inability of tPA-deficient mice to clear repeated injections of fibrin deposited in the fascia would result in the continuous migration and proliferation of fibroblasts into the provisional matrix. This may lead to the development of skin fibrosis, characterised by excessive accumulation of collagen and disruption of skin architecture. Repeatedly injecting fibrin into the skin of tPA-deficient animals would appear to be a more accurate model of human fibrotic conditions such as LDS. Several studies have shown that levels of tPA are significantly reduced in both the blood and skin of patients with LDS (Margolis et al., 1996, Rogers et al., 1999). These results support the observation of increased collagen accumulation in the skin of patients LDS with extensive and chronic fibrin leakage.

Although enhanced collagen accumulation is associated with persistent fibrin deposition as hypothesised, it is clear that the fibrinolytic proteases tPA and uPA exert different effects on the deposition of collagen both *in vitro* and *in vivo*. The observation that collagen production is increased only by tPA-deficient dermal fibroblasts in fibrin matrix *in vitro* and *in vivo*, suggests that tPA and uPA are physiologically distinct and that their

specific biological features determine the extent of matrix degradation and collagen production. The major physiological role of tPA is to convert the inactive zymogen, plasminogen, into plasmin. (Bugge, 1996). However, studies have shown that tPA interferes with platelet aggregation, interrupting thrombotic events that occur following injury (Stricker et al., 1986). Fibrin deposits are rich in platelets, that provide numerous clotting factors, growth factors and mitogens for fibroblasts and inflammatory cells, as well as protease inhibitors, such as PAI-1 (Erickson et al., 1984). Tissue-type-PA deficiency may exert some of its effects on collagen accumulation in these animal models of fibrosis through the accumulation and aggregation of platelets in persistent fibrin deposits, resulting in secretion of potent up-regulators of collagen production, such as TGF-β1. Major fibrin deposits were observed in the skin of tPA-deficient mice injected with fibrin, but not in uPA-deficient mice, indicating that platelet accumulation in these lesions may occur and therefore may play a role in the development of the severe fibrotic skin changes in tPA-deficient mice but not uPA-deficient mice.

Histological examination of fibrin-injected skin in uPA-deficient mice indicated that inflammatory cell infiltrate was more severe than in tPA-deficient and wild-type mice. Image analysis of collagen density in the fascia of uPA-deficient skin demonstrated collagen levels similar to wild-type skin, and suggested that the increase in layer thickness was perhaps due to oedema in the skin rather than collagen deposition. Swaisgood and others (2000) demonstrated similar effects in the lungs of uPA-deficient mice following bleomycin treatment. Histologically, the lungs appeared fibrotic with major structural changes in the alveoli and abundant inflammatory cell infiltrate. However, HPLC analysis of these tissues showed that collagen levels remained unchanged compared with their wild-type counterparts. The group concluded that the absence of uPA in this system may lead to altered levels of activated growth factors and other mediators of collagen synthesis independent of plasmin activity, resulting in reduced collagen levels. Alternatively, evidence exists that uPA-mediated plasmin generation of activated MMP-1 plays a role in the degradation of matrix collagen (Murphy and Gavrilovic, 1999). In a similar manner to fibroblasts *in vitro*, the lack of this interaction in uPA-deficient mice may prevent collagen degradation and thus collagen accumulates around each cell. Fibroblasts surrounded by collagen demonstrate down-

regulation of procollagen gene expression via integrin interactions with the matrix (Riikonen et al., 1995), and may result in reduced collagen deposition compared with tPA-deficient mice.

In contrast to the increased numbers of inflammatory cells, there appeared to be fewer fibroblasts in the fascia of uPA-deficient mice injected with fibrin compared with both tPA-deficient and wild-type mice. These observations suggest that although fibrinolysis is significantly impaired and fibrin persists in these animals, excessive collagen deposition does not occur in the skin because fewer fibroblasts migrate to the site of injury and deposit matrix in the uPA-deficient mice. This theory is supported by the results of several reports which demonstrate impaired cell invasion and fibrinolysis by fibroblasts, smooth muscle cells and inflammatory cells in animals deficient for uPA or plasminogen (Carmeliet et al., 1994; 1997; 1998; Gyetko et al., 1996; Romer et al., 1996). In contrast to the results of Gyetko and colleagues (1996) that showed impaired migration of inflammatory cells in uPA-deficient mice, Carmeliet and others (1994) demonstrated that uPA-deficiency did not affect macrophage migration, even though fibrinolytic activity was significantly impaired. This evidence supports the accumulation of inflammatory cells in the residual fibrin matrix in skin of uPA-deficient mice in this study. Fibrin degradation products may serve as chemoattractants for inflammatory cells (Skogen et al., 1988) which migrate into the fibrin matrix but subsequently degrade fibrin more slowly due to uPA deficiency. It would appear that residual or redundant proteolytic mechanisms are adequate to allow inflammatory cells to infiltrate the provisional fibrin matrix (Eitzman et al., 1996).

There was no evidence of increased angiogenesis in either of the plasminogen activator-deficient mice compared with wild-type mice following injections of fibrin, however, there were more blood vessels in fibrin-injected skin compared with PBS-injected skin in all mouse strains examined. It is well documented that fibrin matrix is associated with angiogenesis (Dvorak et al., 1987; Thompson et al., 1993; Gailit and Clark, 1996). Furthermore, studies have shown that the process of neovascularisation is dependent on plasminogen activator-mediated plasmin activation (Moscatelli and Rifkin, 1988). For angiogenesis to occur, endothelial cells detach from basement membranes of the capillary

plasminogen activator-mediated plasmin activation (Moscatelli and Rifkin, 1988). For angiogenesis to occur, endothelial cells detach from basement membranes of the capillary wall by targeted proteolysis mediated by tPA and uPA. Endothelial cells in the bud of the new vessel migrate through the fibrin matrix, degrading matrix and releasing growth factors, which further promote the angiogenic response (Rogelj et al., 1989). Therefore, it was predicted that there would be fewer blood vessels in fibrin-injected skin of tPA- and uPA-deficient animals compared to wild-type mice due to the potential significance of the fibrinolytic system in this process. However, several reports suggest that there are no changes in blood vessel growth in tPA-, uPA- or plasminogen-deficient mice following injury (Romer et al., 1996, Carmeliet et al., 1997). It is clear that other proteases compensate for the gene deficiency in both tPA- and uPA-deficient mice, potentially, the alternative plasminogen activator in respective deficient strains. In addition, recent evidence suggests that matrix metalloproteinases participate in the process of neovascularisation by acting as pericellular fibrinolysins (Hiraoka et al., 1998). The group demonstrated in tPA-, uPA- and plasminogen-deficient mice that the fibrinolytic system was not required for neovascularisation and that endothelial cell fibrinolytic activity was mediated by membrane type-1 MMP (MT1-MMP), supporting the results of the current study in tPA- and uPA-deficient mice.

In summary, this chapter has demonstrated both *in vitro* and *in vivo* that excessive collagen accumulation is associated with persistent fibrin deposition and/or reduced fibrinolysis *in vitro* and *in vivo*. Collagen production by dermal fibroblasts in the presence of a fibrinolytic inhibitor and tPA-deficient fibroblasts in fibrin gels, was significantly increased. Moreover, deposition of collagen was significantly increased in tPA-deficient mice following subcutaneous injections of fibrinogen and thrombin. Breakdown of fibrin and other incorporated extracellular matrix components by plasmin may be important in limiting collagen accumulation by dismantling the provisional matrix on which fibroblasts invade and secrete collagen. In support of this, reduced fibrinolysis through tPA or plasmin inhibition, and persistent fibrin matrix are associated with excessive collagen accumulation in many fibrotic conditions, including LDS. The mechanisms by which reduced fibrinolysis and fibrin persistence modulate the excessive

deposition of collagen are unknown, however, *in vitro* and *in vivo* evidence suggests both direct fibroblast-fibrin interactions and plasmin-mediated protease activation may play a role. The next chapter will examine the role of persistent fibrin deposition and reduced fibrinolysis on the regulation of collagen gene expression and on MMP-1 activation by dermal fibroblasts in fibrin gels.

Chapter 6
RESULTS AND DISCUSSION

The effect of reduced fibrinolysis and a persistent fibrin matrix on procollagen type I gene expression and proMMP-1 activation by dermal fibroblasts

6.1 Introduction

The previous chapters established that there was a significant increase in collagen production by fibroblasts grown in a fibrin matrix in both *in vitro* and *in vivo* models. Furthermore, reduced fibrinolysis, through inhibition of tPA or plasmin activity, with a persistent fibrin matrix mediated a further accumulation of collagen in these systems. The cellular and molecular mechanisms leading to increased collagen deposition in these systems are not known, but may be due to a number of alternative pathways. There may be an increase in procollagen gene expression by fibroblasts through direct interaction with components of the fibrin matrix. Alternatively, collagen degradation may be decreased through reduced activated and total levels of degrading proteases. Substantial evidence suggests that the fibrinolytic protease, plasmin, plays a major role in the activation of matrix metalloproteinases (MMPs), in particular the major interstitial collagenase, MMP-1 (Rao et al., 1999). The aim of this chapter was to examine the hypothesis that, under conditions of reduced fibrinolysis, collagen accumulation is the result of direct stimulation of procollagen synthesis by persistent fibrin matrix, and reduced levels of active MMP-1, leading to decreased collagen degradation and hence, accumulation.

Procollagen gene expression by human dermal fibroblasts was investigated by Northern analysis, and activation of proMMP-1 was assessed using a specific MMP-1-substrate cleavage assay. Furthermore, the effect of inhibition of MMP activation on collagen accumulation was examined by HPLC analysis of hydroxyproline.

6.2 Results

6.2.1 The effect of reduced fibrinolysis and a persistent fibrin matrix on procollagen type I gene expression

Procollagen type I gene expression was examined by Northern analysis of mRNA isolated from human dermal fibroblasts grown in three-dimensional fibrin gels with or without the fibrinolytic inhibitor, α_2-antiplasmin (1μM) (Figure 6.1A and B). Results from chapter 5 suggested that in the presence of 1μM α_2-antiplasmin, fibroblast-mediated fibrinolysis of the fibrin matrix was reduced by approximately 70% at 24, 48 and 72 hours. However, procollagen type I gene expression was maximal at 24 hours, with no difference in expression in the presence or absence of the inhibitor. Moreover, at 48 and 72 hours, procollagen type I gene expression was increased in the presence of α_2-antiplasmin, with the greatest difference at 48 hours. Levels of procollagen mRNA were higher in fibrin gels with and without inhibitor at 48 hours compared with plastic monolayer cultures. In addition, there was no change in procollagen mRNA levels at 48 hours in the presence of inhibitor, suggesting the increase in procollagen gene expression by fibroblasts in fibrin gels was not due to α_2-antiplasmin stimulation.

The experiment was repeated with triplicate cultures and the increase in procollagen gene expression was assessed by densitometric analysis after 48 hours. Data showed that there was a significant increase in procollagen type I gene expression by dermal fibroblasts in fibrin gels supplemented with α_2-antiplasmin compared with cells in fibrin gels without inhibitor ($p<0.05$, Figure 6.2 A and B). Furthermore, procollagen gene expression was significantly higher when fibroblasts were grown in fibrin gels compared with cells grown on tissue culture plastic, with or without inhibitor ($p<0.05$).

6.2.2 The effect of reduced fibrinolysis on proMMP-1 activation

The role of reduced fibrinolysis on proMMP-1 activation was examined using a specific MMP-1 substrate cleavage assay, resulting in an increase in fluorescence at 398nm upon cleavage of a fluorescent-quencher molecule, at a site specific for MMP-1. The assay system was initially standardised using serial dilutions of active MMP-1 standard (Figure 6.3). Fluorescence emitted following cleavage of the substrate by MMP-1 was recorded

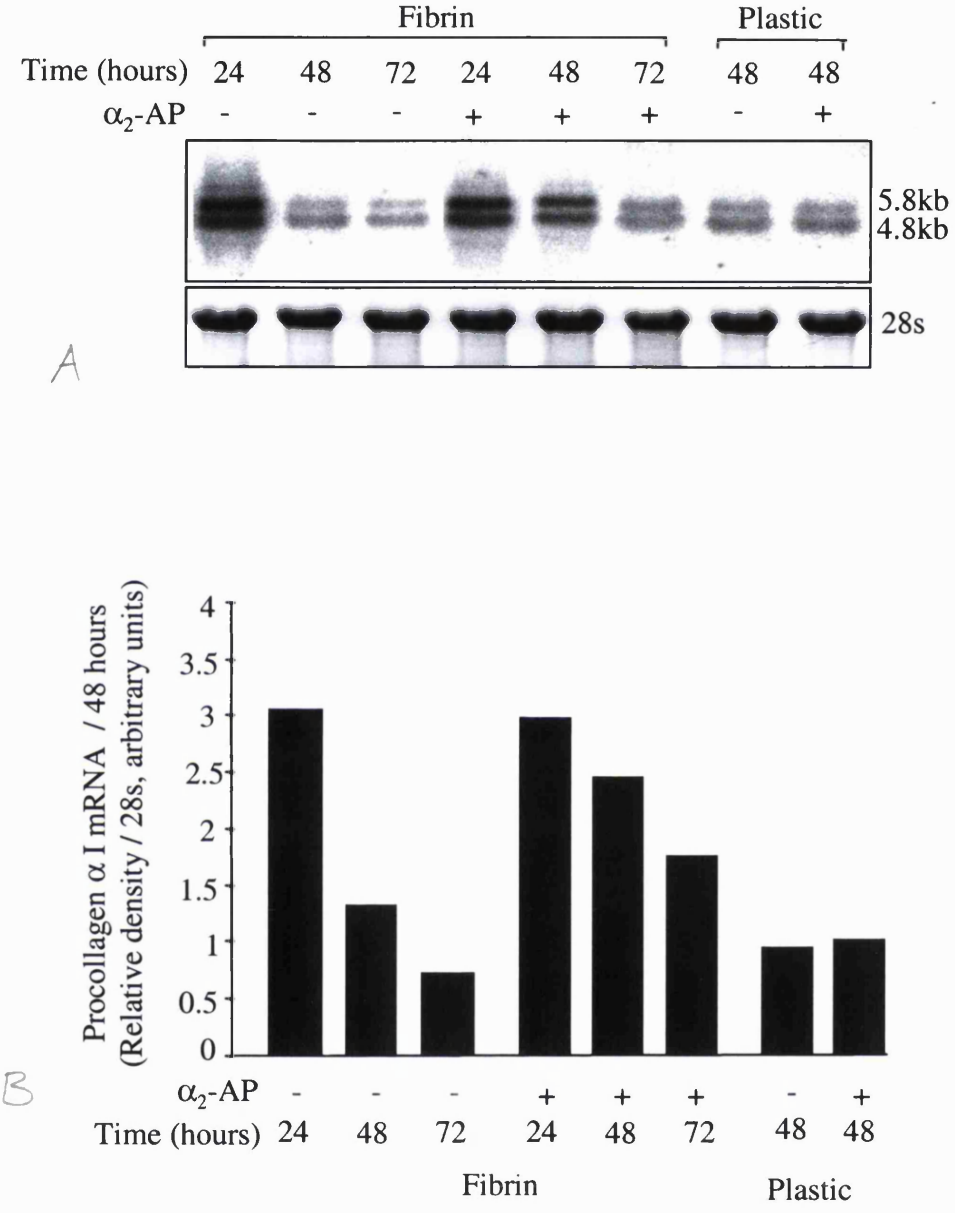


Figure 6.1. The effect of reduced fibrinolysis and persistent fibrin matrix on procollagen α1(I) mRNA expression by dermal fibroblasts grown in three-dimensional fibrin gels. A. Representative Northern blot of procollagen α1(I) in the presence or absence of α_2-antiplasmin at 24, 48 and 72 hours (only 48hrs shown for cells grown on tissue culture plastic). **B**. Densitometric analysis of mRNA transcripts from Figure A.. Values for procollagen α1(I) mRNA are normalised for loading with respect to 28S rRNA band and expressed in arbitrary units. Data are from one sample per condition.

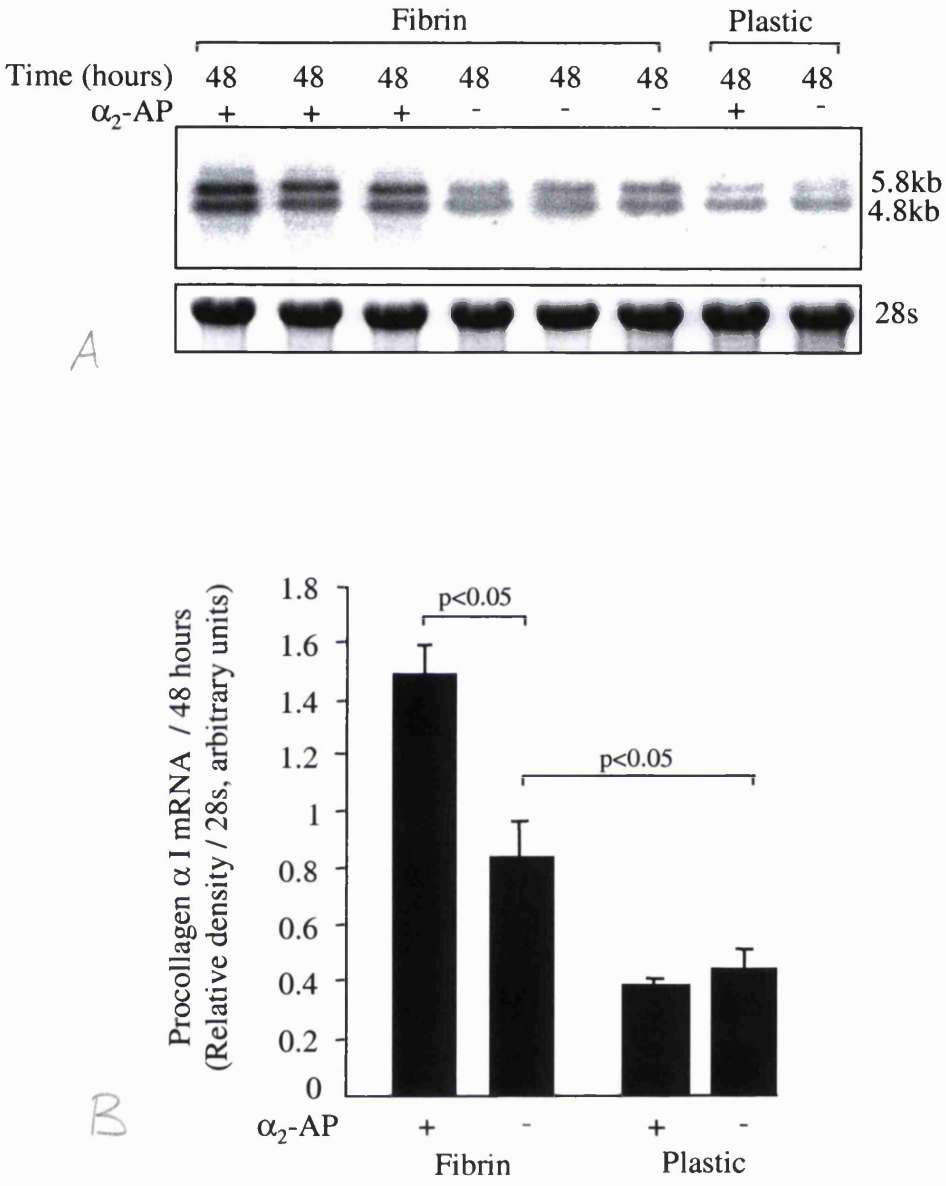


Figure 6.2. The effect of reduced fibrinolysis and persistent fibrin matrix on procollagen α1(I) mRNA expression by dermal fibroblasts grown in three-dimensional fibrin gels. A. Representative Northern blot of procollagen α1(I) in the presence or absence of α_2-antiplasmin at 48 hours (representative lane shown for each condition on tissue culture plastic). **B**. Densitometric analysis of mRNA transcripts from Figure A. Values for procollagen α1(I) mRNA are normalised for loading with respect to 28S rRNA band and expressed in arbitrary units. Results are from three samples for each condition (mean ± standard error, n=6).

at 1 minute intervals until activity had ceased (curve plateau). Using the change in fluorescence, the concentration of active MMP-1 was calculated (Table 6.1) (for equation see Materials and Methods, Section 2.22).

Conditioned media from cultures of dermal fibroblasts in fibrin gels with and without α_2-antiplasmin at 24, 48 and 72 hours was collected, and assessed for MMP-1 activity (Figure 6.4). Data showed a significant decrease in the level of active MMP-1 in the media from cultures of fibroblasts in the presence of α_2-antiplasmin (reduced fibrinolysis) compared to cultures without the inhibitor, at 24, 48 and 72 hours ($p<0.01$). In addition, the levels of active MMP-1 were significantly greater without inhibitor at 48 and 72 hours compared with at 24 hours ($p<0.01$). Moreover, although the amount of active MMP-1 in the reduced fibrinolysis cultures was higher at these time-points, it was not significantly different from the level at 24 hours. In another set of samples, APMA, a proMMP activator, was added prior to assessment of MMP-1 activity. Addition of APMA resulted in complete activation of MMPs and so total MMP-1 levels can be calculated (Figure 6.5). Analysis of total MMP-1 levels following the addition of APMA showed that there were similar levels of total MMP-1 protein produced by fibroblasts in the presence or absence of α_2-antiplasmin at each of the time points examined. A significant increase in total MMP-1 levels was observed at 48 hours compared with 24 hours, however this rate of MMP-1 production appeared to decline at 72 hours.

6.2.3 The effect of MMP-1 inhibition on the accumulation of collagen by dermal fibroblasts in fibrin gels

To examine the effect of reduced MMP-1 activation on the accumulation of collagen, a synthetic MMP-1 inhibitor (MMP-1*i*) was added to the cultures at 1μM. Initially, the efficacy of the inhibitor was examined using the specific fluorescent MMP-1 cleavage assay at 48 hours (Figure 6.6). Data showed that active MMP-1 levels were significantly reduced in the presence of the inhibitor, in cultures with and without α_2-antiplasmin ($p<0.01$). Furthermore, there was no significant difference in the level of total MMP-1 protein produced by fibroblasts in the presence or absence of α_2-antiplasmin and/or

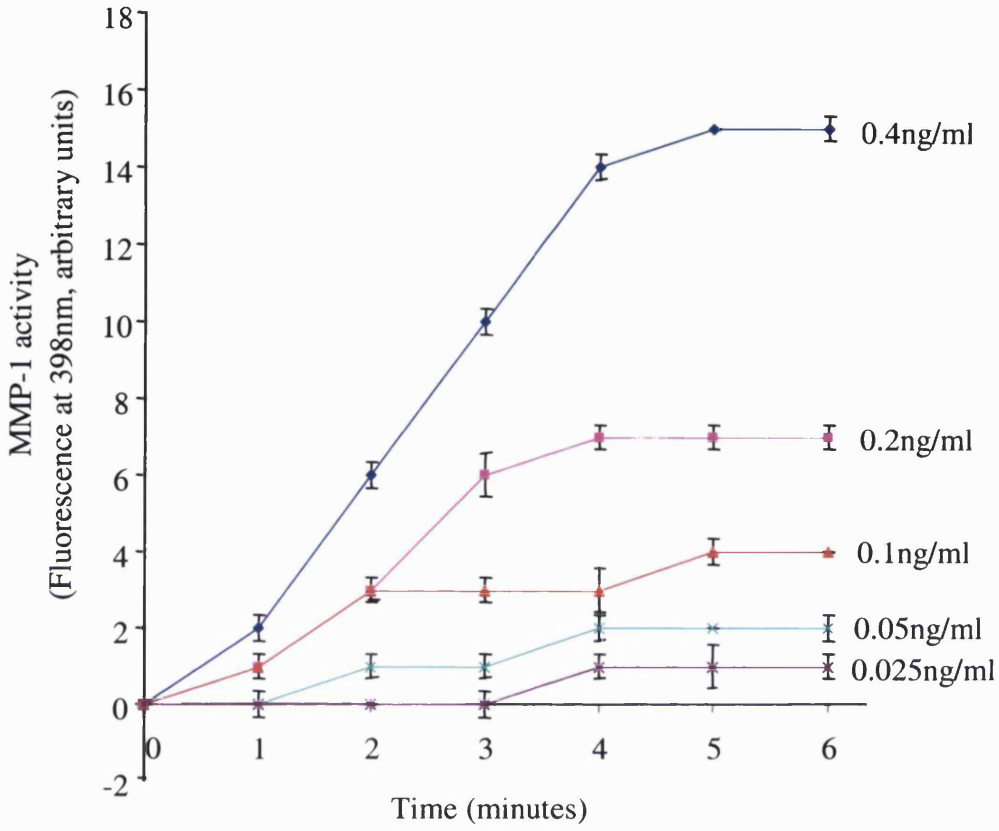


Figure 6.3. Standard curve of MMP-1 activity, using specific MMP-1 substrate cleavage fluorescence assay Data represents n=6 for each enzyme concentration (mean ± standard error).

Standard [ng/ml]	δF	Calc. Conc [ng/ml]	Error [ng/ml]
0.4	15	0.417	0.01
0.2	8	0.222	0.008
0.1	4	0.111	0.008
0.05	2	0.056	0.011
0.025	1	0.028	0.01

Table 6.1. Calculation of active MMP-1 levels (ng/ml) from standard equation using change in fluorescence (δF denotes change in fluorescence at curve plateau). Calculated concentrations were similar to standard concentrations added.

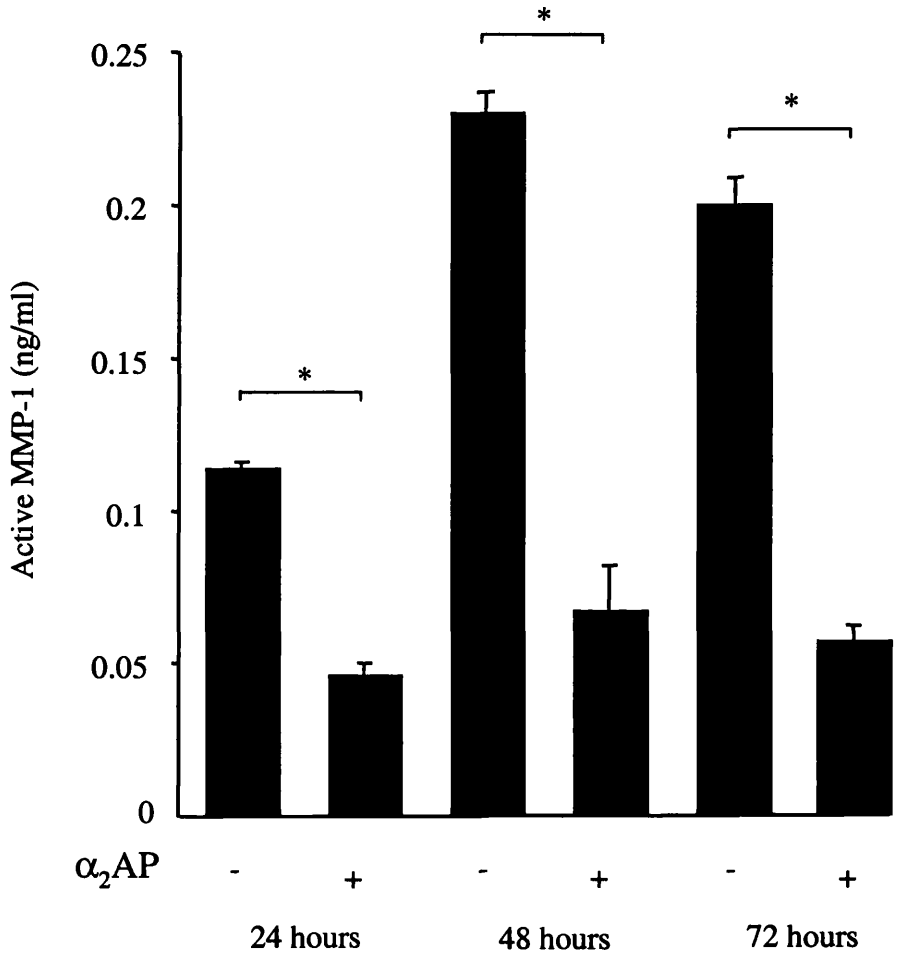


Figure 6.4. Active MMP-1 levels in conditioned media from human dermal fibroblasts in fibrin gels with and without α_2-antiplasmin (α_2-AP). Data represents n=12 for each condition (mean ± standard error) (* = $p<0.01$).

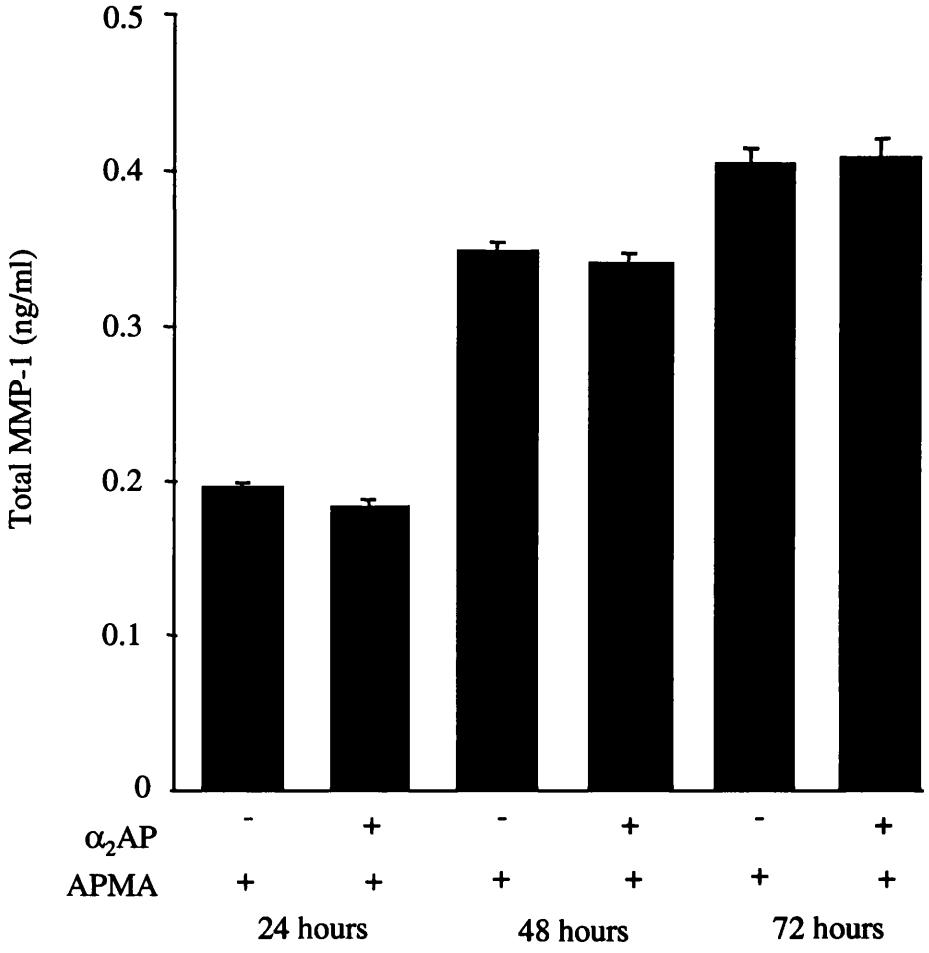


Figure 6.5. Total MMP-1 levels in conditioned media from human dermal fibroblasts in fibrin gels with and without α_2-antiplasmin (α_2-AP). Conditioned media was treated with APMA for total MMP activation. Data represents n=12 for each condition (mean ± standard error).

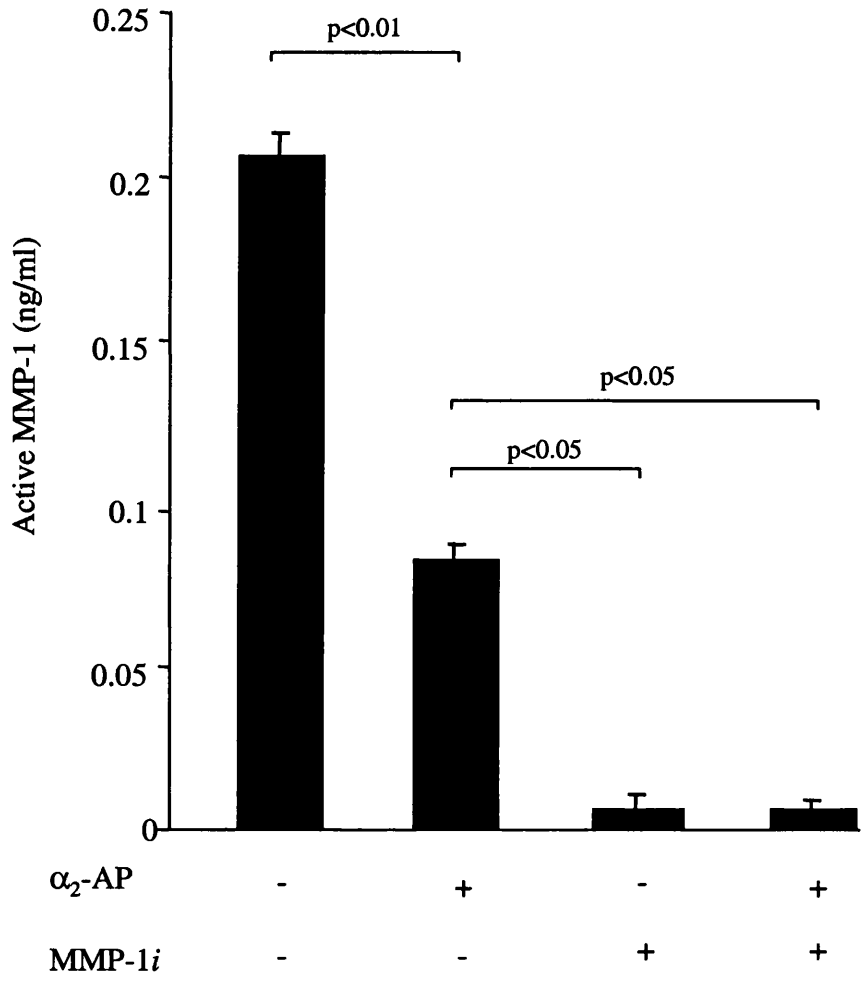


Figure 6.6. Active MMP-1 levels in media from dermal fibroblasts in fibrin gels with and without α_2-antiplasmin (α_2-AP) and/or specific MMP-1 inhibitor (MMP-1*i*). Data represents n=12 for each condition (mean ± standard error).

MMP-1*i* inhibitors following addition of APMA to the samples (data not shown), suggesting that neither inhibitor affects proMMP-1 protein production.

Collagen production by fibroblasts in fibrin gels without α_2-antiplasmin but in the presence of the MMP-1*i* was significantly increased compared with that produced by fibroblasts in the absence of inhibitor at 48 hours ($p<0.01$, Figure 6.7). Collagen production was significantly increased in the presence of α_2-antiplasmin compared with media alone, confirming our previous studies. There was no significant difference in the amount of collagen produced by fibroblasts in the presence of both the MMP inhibitor and α_2-antiplasmin and cultures containing MMP-1*i* or α_2-antiplasmin alone, suggesting that the increased procollagen mRNA levels shown previously may have only a minor effect on the procollagen protein produced. There was no increase in the amount of procollagen produced by dermal fibroblasts on tissue culture plastic in the presence of the MMP inhibitor or α_2-antiplasmin, suggesting that these factors were not directly affecting collagen production by fibroblasts.

In summary, reduced fibrinolysis and a persistent fibrin matrix appeared to enhance procollagen type I gene expression by dermal fibroblasts in fibrin gels. Furthermore, reduced fibrinolysis by α_2-antiplasmin in the same system resulted in a decreased activation of MMP-1. As expected, inhibition of MMP-1 activity resulted in enhanced collagen accumulation by fibroblasts in fibrin gels. Although it is clear that fibrin matrix stimulates procollagen gene expression by dermal fibroblasts, collagen accumulation under conditions of reduced fibrinolysis appears more likely to be due to decreased collagen degradation than increased procollagen type I gene expression.

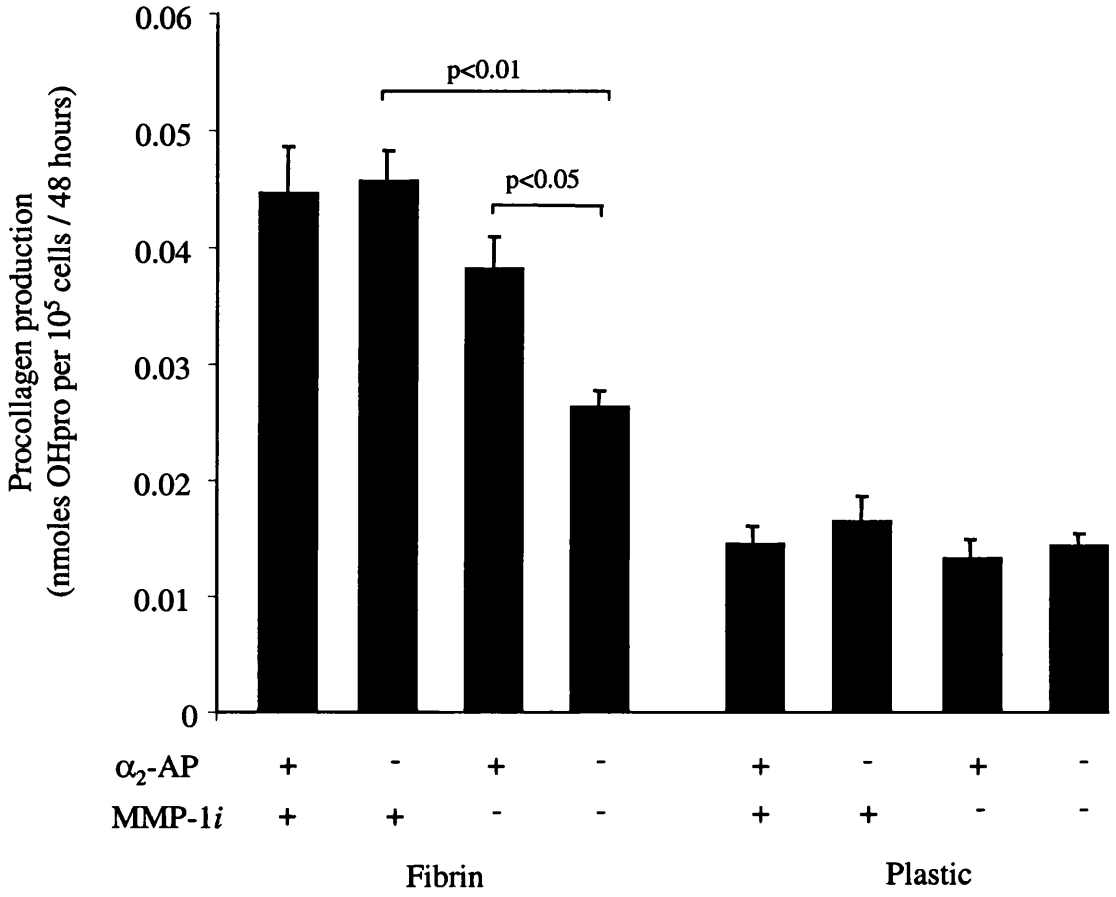


Figure 6.7. Procollagen production by human dermal fibroblasts grown in fibrin gels or on tissue culture plastic, with and without α_2-antiplasmin (α_2-AP) (1μM) and/or a specific MMP-1 inhibitor (MMP-1*i*) (1μM) after 48 hours. Data represents n=6 for each condition (mean ± standard error).

6.3 Discussion

Previous chapters in this thesis have established that collagen production by dermal fibroblasts was significantly increased in response to fibrin matrix in both *in vitro* and *in vivo* models. Furthermore, delayed breakdown of fibrin and its persistence due to reduced fibrinolysis mediated a significant further increase in collagen deposition in these systems. The processes that lead to the deposition of collagen following fibrin accumulation are complex, and presumably involve several cell-cell and cell-ECM interactions, including intricate protease and/or growth factor effects. Excessive collagen accumulation may be the result of increased procollagen gene expression, increased procollagen mRNA stability or a reduction in collagen degradation, principally by interstitial collagenase MMP-1. It is not known if the fibrin matrix directly imparts an effect on fibroblast procollagen production. In contrast, the fibrinolytic protease plasmin, has conclusively been shown to play a major role in the activation of the collagenase MMP-1 (Rao et al., 1999). Therefore, one aim of this chapter was to examine procollagen type I gene expression by dermal fibroblasts in fibrin gels with reduced fibrinolysis, the other was to assess the effect of reduced fibrinolysis on MMP-1 activation by fibroblasts in the same *in vitro* system and the overall effect of this reduced activity on the accumulation of collagen.

Procollagen synthesis is a complex process that involves many regulatory transcriptional and translational steps. The method of collagen measurement used in this study is based on the assay of hydroxyproline by HPLC analysis (Campa et al., 1990), which although accurate, does not distinguish between these two regulatory levels. For example, direct interactions between fibroblasts and fibrin matrix may lead to an increase in collagen production due to increased transcription of the gene, increased levels of post-translational enzymes or a decrease in collagenolytic enzymes. In the present study, fibroblasts grown in fibrin gels with a fibrinolytic inhibitor showed a significant increase in procollagen type I gene expression at 48 hours compared to fibroblasts without inhibitor. In addition, fibroblasts in fibrin gels showed an increase in procollagen gene expression compared with cell on tissue culture plastic after 48 hours. Although it appeared that procollagen mRNA levels declined over the 72 hour time-course, it was

clear that the high levels of mRNA were maintained in cells in fibrin gels with the fibrinolytic inhibitor. As the previous chapter had shown that fibrin gels containing α_2-antiplasmin were degraded slower by fibroblasts compared with gels without the inhibitor, the effect on procollagen gene expression appeared to be due to the presence of persistent fibrin rather than the α_2-antiplasmin peptide.

In support of this data, Gillery and colleagues (1992) demonstrated that dermal fibroblasts in non-retracting fibrin gels, similar to those used in the *in vitro* system of this study, produced increased levels of procollagen type I mRNA compared with fibroblasts grown in monolayer cultures. Moreover, in both systems procollagen gene expression was significantly elevated compared with fibroblasts grown in collagen gels and in retracting fibrin gels. In contrast, Pardes and others (1995) reported a four-fold decrease in procollagen gene expression by fibroblasts grown on fibrin gels compared with monolayer cultures. However, the group did not examine mRNA levels of fibroblasts grown within the three-dimensional fibrin matrix. Addition of the fibrinogen cleavage product, fibrinopeptide B, to fibroblasts grown on plastic, resulted in a significant decrease in procollagen gene expression. They suggested that fibrinogen cleavage products trapped within the polymerised fibrin network may in part be responsible for down-regulation of procollagen gene expression.

In our study, fibrin matrix-fibroblast interactions are likely to occur over the cell surface and so will affect the regulation of collagen gene expression. As stated previously, the mechanisms by which fibroblasts interact with fibrin are not known, although integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ may play a role, directly affecting gene expression through intracellular signalling cascades (Gailit and Clark, 1996). In addition, these integrins have been shown to have strong affinities for other matrix molecules besides fibrin(ogen), including fibronectin and vitronectin. Persistence of the fibrin matrix may result in prolonged or an increased number of cell-matrix interactions and result in enhanced stimulation of collagen gene expression over the course of the study. Several studies have demonstrated that the decrease in procollagen synthesis by fibroblasts in collagen gels was regulated by $\alpha 1\beta 1$ interactions (Langholz et al., 1995) with the collagen matrix. These findings

suggest that procollagen gene expression may be directly regulated by integrin-ECM interactions. Thus, future work would determine the role of candidate integrins αvβ3 and α5β1 in fibroblast procollagen synthesis in fibrin gels, using blocking antibodies or synthetic inhibitor compounds.

Alternatively, a matrix-independent mechanism of collagen accumulation, involving both fibrinolytic and collagenolytic pathways was also investigated. *In vitro*, plasmin catalyzes the intramolecular cleavage that results in the interruption of the cysteine-Zn^{2+} bond. This process gives rise to the so-called 'cysteine switch' mechanism of activation, wherein the switch is closed when the bond is intact and open when the bond is disrupted, allowing proteolysis to occur (Van Wart and Birkedal-Hansen, 1990). Thus, the effect of reduced fibrinolysis on MMP-1 activation by fibroblasts in fibrin gels and the subsequent effect of impaired MMP-1 activity on collagen accumulation was examined. However, quantifying the activity of MMP-1 presents numerous technical obstacles. Techniques used to quantify MMP-1 include the use of radio-labelled collagen, enzyme-linked immunoassays (ELISA) and collagen zymography, but all lack sensitivity and specificity. For example, ELISA does not discriminate between active and inactive forms of the protease, either in its proform or complexed with tissue inhibitor of matrix metalloproteinases (TIMPs). Collagen zymography can distinguish between these forms, however, this method is not sensitive to small changes in MMP-1 activity, partly because fibrillar collagens in the overlay gels are denatured by detergent in the preparation, and also because the technique relies on densitometric analysis, which is semi-quantitative. The development of specific fluorometric oligopeptide substrates developed by Knight and others (1992) allows accurate and sensitive measurement of active MMPs. Furthermore, addition of APMA gives quantitative assay measurement of total enzyme levels. Using this technique, it was discovered that addition of the plasmin inhibitor α_2-antiplasmin to dermal fibroblasts grown in fibrin gels resulted in significantly lower levels of active MMP-1 compared with fibroblasts without the inhibitor. Moreover, as the total levels of MMP-1 (including latent and active) produced by fibroblasts in fibrin gels were similar, irrespective of the presence of the inhibitor, it is likely that plasmin directly affects pro-MMP-1 activation rather than synthesis. Although this latter observation

shows that the MMP-1 levels were similar in both conditions, the assay does not determine active or inactive enzyme complexed with TIMP and so potentially may not be a completely reliable indicator of total MMP levels. Although, the role of TIMPs in this model was not investigated, there is considerable *in vivo* evidence that TIMPs affect the accumulation of collagen in fibrotic disorders through the inhibition of MMP activity. TIMPs are the major physiological regulators of MMP activity in tissue (Nagase and Woessner, 1999) and in fibrotic conditions such as liver fibrosis, their levels have been shown to significantly elevated (Iredale, 1997; Gunther et al., 1999).

Measurement of MMP-1 activity in the media isolated from fibroblasts in fibrin gels in the presence of the α_2-antiplasmin strongly suggests that the fibrinolytic pathway plays a major role in the activation of MMP-1. This led to the investigation of the effect of reduced MMP-1 activity on collagen accumulation in the fibrin gel system, using a specific MMP-1 inhibitor. The synthetic inhibitor inhibited interstitial collagenase MMP-1 at relatively low concentrations (1nM) but may also inhibit gelatinases and stromelysins at high concentrations (Odake et al., 1994). The process of fibrillar collagen degradation is dependent on the specific cleavage by MMP-1 (Liu et al., 1995), thus the inhibitor provides a means to examine the effects of MMP-1 inhibition on collagen accumulation. HPLC analysis showed that collagen accumulation by fibroblasts in the presence of the inhibitor was significantly increased compared with fibroblasts in the absence of the MMP-1 inhibitor after 48 hours, confirming that MMP-1 activation plays a major role in the amount of collagen deposited in the fibrin matrix. In support of these findings, other *in vitro* studies have shown that interstitial collagenases degrade matrix collagens only after plasmin-dependent removal of the surrounding glycoproteins (Montgomery et al., 1993). The lack of ECM glycoprotein degradation would prevent fibroblast-derived collagenases from remodelling deposited collagen fibrils, and may contribute to the accumulation of collagen in our system.

The effects of MMP-1 inhibition on collagen accumulation *in vivo* have been demonstrated by the development of mice with a targeted mutation in the collagenase cleavage site of a collagen molecule (Liu et al., 1995). These mice showed a number of

alterations in tissue remodeling, reflecting the inability to degrade type I collagen, including thickening of the dermis and fascia, hair loss and spontaneous ulcer formation. Histological analysis of the skin revealed extensive accumulation of collagen which the group proposed was indicative of normal high rates of collagen type I degradation required to maintain tissue integrity. These findings and other recent data suggest pathogenic scenarios develop if MMP-1 activation is reduced. Haraguchi and others (2001) showed that rats with experimental glomerulonephritis treated with recombinant tPA, resulted in a significant increase in plasmin generation. Plasmin activation resulted not only in reduced fibrin deposition but a significant increase in MMP activation and a concomitant reduction of collagen accumulation in the glomeruli.

In conclusion, this chapter has demonstrated that reduced fibrinolysis and a persistent fibrin matrix caused a significant increase in procollagen gene expression by dermal fibroblasts with significantly decreased MMP-1 activity. The interaction between fibrinolytic and collagenolytic pathways has significant biological relevance since MMP-1 inhibition resulted in excessive accumulation of collagen by fibroblasts in fibrin gels. Numerous other studies have shown that decreased MMP-1 activity, either through reduced active protease levels or increases in their inhibitors, leads to collagen accumulation. Therefore, the stimulation of procollagen gene expression by persistent fibrin matrix and the decreased activation of MMP-1 by the impaired fibrinolytic pathway may play major pathophysiological roles in the processes of tissue repair and in the subsequent development of skin fibrosis. Under conditions of persistent fibrin deposition and reduced fibrinolysis, collagen accumulation is likely to result through a combination of both these pathways.

Chapter 7

SUMMARY AND OVERALL DISCUSSION

The role of fibrin and its persistence in skin fibrosis

7.1 Summary of results

Lipodermatosclerosis refers to the progressive replacement of subcutaneous fat tissue by a collagen-rich fibrous matrix following prolonged periods of venous hypertension in the lower leg. The cellular and molecular mechanisms by which venous hypertension causes the development of LDS and how this leads to ulceration and delayed healing, remain unknown. Findings in Chapter 3 showed that procollagen synthesis was increased in LDS skin and was associated with skin thickening and fibrotic changes. It was also established that fibroblast-like cells residing in the subcutaneous fat septae synthesise procollagen. This is the first evidence to fully support the theory that the subcutaneous fat layer is progressively replaced by collagen in LDS. There was little inflammatory cell infiltrate in LDS skin samples, suggesting that the development of fibrosis was not necessarily perpetuated by the presence of inflammatory cell mediators. However, fibrin accumulation in the dermis was a prominent feature of LDS skin and this may elicit a continued tissue repair response, ultimately leading to excessive ECM deposition and, in particular, collagen.

Fibrin is deposited in the interstitium of LDS skin following extravasation of plasma fibrinogen due to the effects of venous hypertension (Browse, 1982; Leach and Browse, 1986; Margolis et al., 1996). In addition, patients with LDS show reduced fibrinolytic activity in blood and tissues, with decreased levels of tissue plasminogen activator (tPA) and enhanced levels of plasminogen activator inhibitors, ultimately leading to chronic and persistent fibrin accumulation in the skin (Margolis et al., 1996; Rogers et al., 1999). During normal tissue repair, fibrin provides a provisional wound matrix that promotes inward migration of inflammatory cells, endothelial cells and fibroblasts, and acts as a scaffold which is replaced by granulation tissue, rich in collagen (Dvorak, 1986). Indeed, the deposition of collagen by fibroblasts is thought to be intimately associated with the pre-existing fibrin matrix (Dvorak, 1986).

However, persistent fibrin deposition in organs and tissues is also a major characteristic of fibrotic disease (Brown et al., 1989). This thesis addressed the hypothesis that persistent fibrin deposition and reduced fibrinolysis are associated with excessive accumulation of collagen, resulting in the development of skin fibrosis, as in LDS. Novel strategies were used to investigate the role of fibrin and its persistence in collagen deposition both *in vitro* and *in vivo*, and to elucidate potential cellular and molecular mechanisms that regulate this fundamental matrix-remodelling event.

In Chapter 4, the effect of a fibrin matrix on collagen deposition by dermal fibroblasts was examined using both *in vitro* and *in vivo* model systems. Collagen production by human dermal fibroblasts was increased in three-dimensional fibrin gels compared with that produced by fibroblasts grown on fibrin monolayers or on tissue culture plastic. *In vivo*, repeated subcutaneous injections of fibrinogen and thrombin in Balb-c mice resulted in a significant increase in collagen deposition with a inflammatory response. This led to the development of a novel, standardised murine skin fibrosis model. In Chapter 5, under conditions of reduced fibrinolysis, dermal fibroblasts were shown to produce significantly increased amounts of collagen in three-dimensional fibrin gels compared with cultures in the absence of the plasmin inhibitor, α_2-antiplasmin. In addition, dermal fibroblasts isolated from tPA-deficient mice produced significantly greater amounts of collagen in fibrin gels compared with wild-type control cultures. The effect of reduced fibrinolysis on excessive collagen accumulation was confirmed *in vivo*, as repeated subcutaneous injections of fibrin resulted in a significant increase in collagen deposition in the skin of tPA-deficient mice compared with wild-type controls. These results demonstrate that excessive collagen accumulation was associated with reduced fibrinolysis and a persistent fibrin matrix both *in vitro* and *in vivo*.

Two potential mechanisms that may explain excessive collagen synthesis in systems with reduced fibrinolysis and persistent fibrin matrix were investigated in Chapter 6 (Figure 7.1). Firstly, results indicated that an increase in fibroblast procollagen gene

expression contributed to collagen accumulation in fibrin gels in the presence of a fibrinolytic inhibitor. Secondly, that the reduced activation of the collagen-degrading protease, MMP-1, through inhibition of plasmin, resulted in enhanced collagen accumulation in the same system. The combined effect of increased fibroblast procollagen gene expression and reduced MMP-1 activation resulted in the excessive accumulation of collagen in conditions of reduced fibrinolysis and persistent fibrin.

7.2 Discussion

This thesis has demonstrated that fibrin matrix is associated with increased collagen production by dermal fibroblasts, and that reduced fibrinolysis with persistence of fibrin accentuates this effect resulting in a further significant increase in collagen accumulation. It appears that collagen accumulation in these systems occurs through both enhanced procollagen gene expression and decreased collagenase activity (Figure 7.1). Increased procollagen type I gene expression by dermal fibroblasts in fibrin gels under conditions of reduced fibrinolysis supports one of the preliminary observations of this thesis, the presence of significantly increased numbers of procollagen-producing fibroblasts in the skin of patients with LDS, shown by *in situ* hybridisation. Furthermore, repeated subcutaneous injections of fibrin in fibrinolytically-deficient mice resulted in excessive collagen accumulation and similar histological fibrotic features to LDS skin. In particular, parallel collagen bundles with numerous fibroblasts in the dermal and fascial layers and progressive replacement of the subcutaneous fat layer with collagen-rich tissue mimicked the histological features observed in LDS. It is also likely that fibroblast procollagen gene expression would be increased in these fibrin-injected skin samples, although this was not examined. These findings indicate that chronic leakage of fibrinogen and the deposition of persistent fibrin matrix in the skin are directly associated with excessive accumulation of collagen. This process may substantially contribute to the development of fibrotic disease.

In addition to the effects of reduced fibrinolysis and fibrin persistence on elevated procollagen gene expression and excessive collagen synthesis, results from Chapter

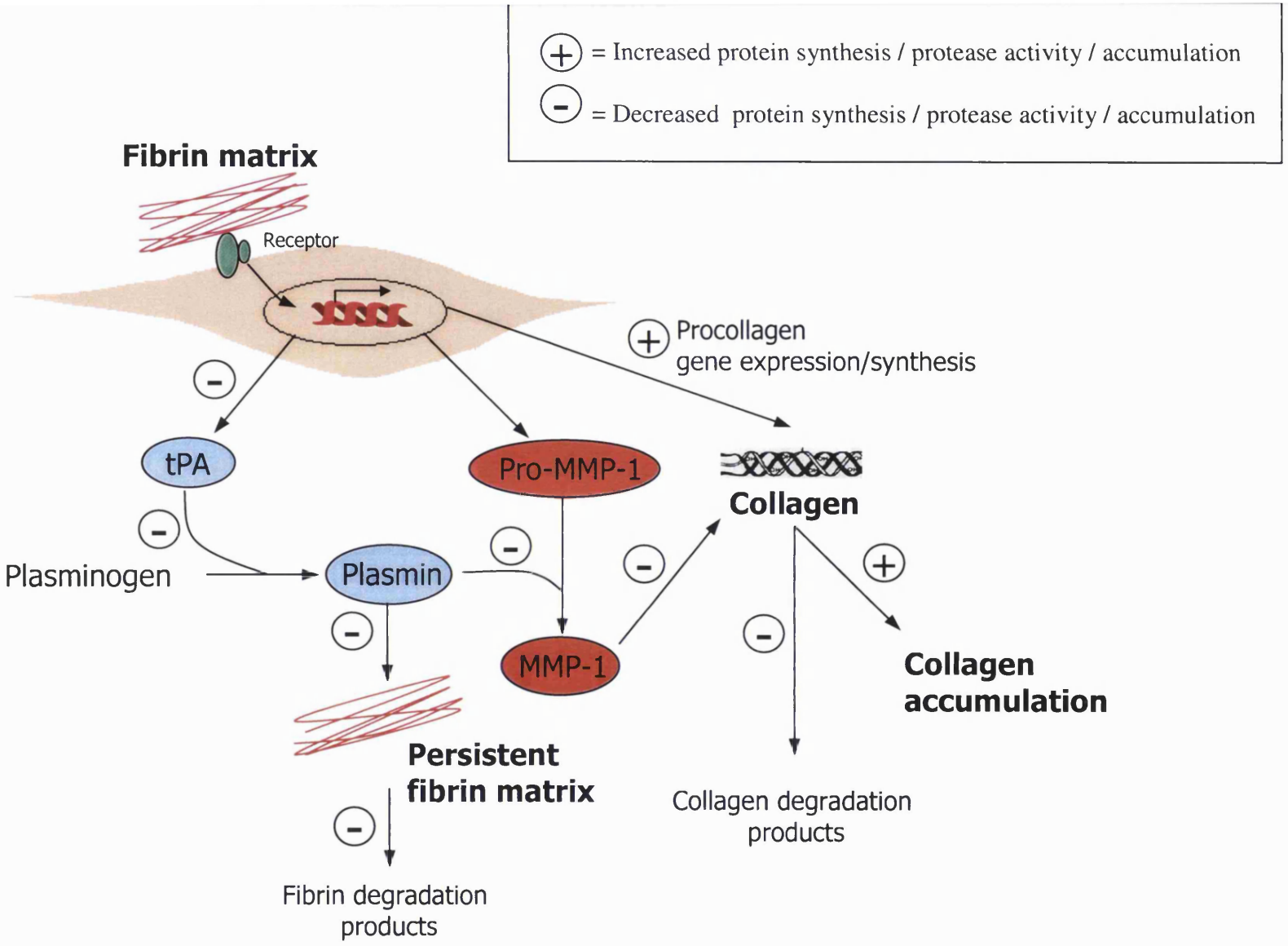


Figure 7.1. The mechanisms of collagen accumulation by fibroblasts under conditions of reduced fibrinolysis in a persistent fibrin matrix. This thesis has demonstrated that fibrin matrix is associated with increased procollagen production. Furthermore, persistent fibrin matrix, due to reduced levels of the fibrinolytic proteases, tPA and plasmin, mediates a further increase in collagen accumulation by fibroblasts. This accumulation is due to both enhanced procollagen gene expression and decreased levels of active, but not total, interstitial collagenase (MMP-1).

6 demonstrated that collagen accumulation may also be the result of reduced collagenase activity (Figure 7.1). MMP-1 activation by dermal fibroblasts grown in fibrin gels was significantly reduced in the presence of a plasmin inhibitor, even though the total level of MMP-1 protein was not affected. These *in vitro* findings may have a significant pathological relevance in conditions such as LDS. Although MMP levels were not examined in LDS skin in this study, others demonstrated significantly increased levels of MMP inhibitors, TIMP-1 and TIMP-2, in LDS skin and wound fluid isolated from chronic venous ulcers (Herouy et al., 1998). This corresponded to an overall decrease in MMP-1 activity. Herouy and colleagues (1998) proposed that the inhibition of MMPs by elevated TIMP levels in chronic wounds resulted in impaired cellular responses and reduced extracellular matrix remodelling, leading to the accumulation of collagen and delayed wound healing. Results from the present study indicate that the reduced fibrinolysis previously observed in LDS skin, may lead to decreased MMP activation. Such a decrease in active protease levels may perpetuate the accumulation of collagen. Further evidence for this concept comes from additional studies assessing MMP-1 activity in other fibrotic skin conditions, such as hypertrophic scars and scleroderma. There was almost a 75% decrease in MMP-1 activity levels compared with controls (Arakawa et al., 1996; Kikuchi et al., 1997) and this was directly associated with an enhanced accumulation of collagen. The effects of MMP-1 inhibition on collagen accumulation *in vivo* have been demonstrated by the development of mice with a targeted mutation in the collagenase cleavage site in the collagen molecule (Liu et al., 1995). These mice showed a number of alterations in tissue remodelling, reflecting the inability to degrade type I collagen, including thickening of the dermis and fascia, and extensive accumulation of collagen, which the group proposed was indicative of normal high rates of collagen type I degradation required to maintain tissue integrity. Interestingly, Brinckmann and others (1999) proposed that alterations in the collagen type I fibril diameter and cross-linking shown in LDS skin, may result in impaired collagen remodelling and thus accumulation. These effects may be similar to those observed by Liu and colleagues in the collagenase-resistant transgenic mouse. Future studies to establish the biological and pathological

significance of this mode of collagen accumulation, could be performed using the skin fibrosis model in plasminogen activator-deficient mice. The level of active murine interstitial collagenase, which cleaves undenatured fibrillar collagens within the triple helical domain similar to human MMP-1 (Krane et al., 1996), as well as other MMPs and their inhibitors, would need to be analysed in tissue samples. These studies would confirm that decreased MMP activation through diminished plasmin levels leads to excessive collagen accumulation in the skin *in vivo*.

Various targets in the collagen synthesis pathway have been proposed that may prevent the excessive deposition of collagen. Fibrillar collagens are synthesised in a precursor form that contain procollagen peptides which are enzymatically cleaved in the extracellular space by procollagen-C- and procollagen N- proteinases (reviewed by Chambers and Laurent, 1997). Intracellularly, several post-translational modification steps, involving hydroxylation of lysine and proline residues (lysyl oxidase and prolyl-4-hydroxylase respectively), are required for secretion and therefore determine the amount of protein synthesised. In conditions where collagen synthesis is induced, so too are the levels of these enzymes. Inhibition of these enzymes offers potential therapeutic intervention (Prockop and Kivirikko, 1995). Although several inhibitors of prolyl hydroxylase exist, a clinically effective inhibitor has not yet emerged (Franklin, 1997). There is considerable concern about potential effects of impaired collagen regulation on tissue integrity which may arise long-term in the treatment of chronic fibrotic disorders. Moreover, the inhibition of protein-C-proteinase is considered an attractive alternative to collagen deposition inhibition, but drug development is still at a relatively early phase (Prockop and Kivirikko, 1995). Alternatively, fibroblast-matrix interactions may be targeted in an attempt to prevent, for example, fibrin matrix-mediated collagen synthesis. Drugs aimed at interfering with such interactions, for example integrin $\alpha v\beta 3$ and $\alpha 5\beta 1$ blocking peptides, may have therapeutic potential in fibrotic disorders. Indeed, this concept is supported by studies which demonstrate that inhibition of these integrins with specific antibodies resulted in a significant decrease in procollagen production

by fibroblasts following application of mechanical load (Reynolds, PhD thesis, 2000).

Therapies could be introduced targeting rapid degradation of fibrin in patients with fibrotic disorders in which chronic and persistent fibrin is a feature. Treatment of patients with LDS and chronic venous ulcers with pro-fibrinolytic agents such as stanozolol, pentoxyfilline or iloprost has proved encouraging but not significantly effective (Coleridge-Smith, 1996). Development of efficacious fibrinolytic agents for treatment of skin disorders is lacking, although several profibrinolysins exist for treatment of other fibrotic disorders, such as post-operative adhesions. Post-operative adhesions have been treated with fibrinolytic agents since 1909 (Hellebrekers et al., 2000), and since then, pepsin, trypsin, plasmin, streptokinase, uPA and tPA have all been used with positive effect, in particular the third-generation mutants of tPA. Progress in the development of new thrombolytic agents such as tPA may lead to even better results when these agents are used in the prevention of LDS and venous ulcer development. Furthermore, the concept of profibrinolysis in the prevention of collagen deposition may be examined in the animal model of skin fibrosis presented by this study.

In conclusion, this study has shown that reduced fibrinolysis and persistent fibrin matrix leads to excessive accumulation of collagen, through increased procollagen gene expression and reduced levels of active interstitial collagenase. As discussed in earlier chapters, the mechanisms by which fibroblasts interact with fibrin matrix are unclear. Moreover, reduced levels of fibrinolytic proteases lead to decreased collagenase activation, thus perpetuating the accumulation of collagen. This thesis proposes that these events occur in the development of LDS and other fibrotic diseases where persistent fibrin deposition is a feature, including scleroderma, pulmonary fibrosis, atherosclerosis and tumour formation. These studies may provide potential therapeutic targets aimed at preventing excessive collagen accumulation in these disorders since there is continuing need for the development of new treatments of fibrotic disease. If the development of LDS were to be halted,

ulcer formation may be prevented, significantly reducing patient morbidity as well as the subsequent social and economic effects of its development.

7.3 Future studies

The mechanisms by which fibroblasts interact with fibrin matrix remain unclear. Future studies will focus on isolating the pathways by which fibrin, and other components of the provisional fibrin matrix, stimulate procollagen synthesis by fibroblasts. Integrin-fibrin interactions appear the most likely and may be examined both *in vitro* and *in vivo*, using specific blocking antibodies targeted against integrins such as $\alpha 3\beta 1$ and $\alpha 5\beta 1$. Blocking such integrins may result in decreased procollagen synthesis by dermal fibroblasts in fibrin gels, when examined by HPLC analysis of hydroxyproline. Furthermore, transgenic animals deficient in specific integrins/integrin sub-units, will provide useful tools in combination with the animal model of skin fibrosis, in which fibrin is delivered directly subcutaneously. Reduced collagen deposition in the skin of these animals compared with wild-type controls would provide substantial evidence for the role of integrins in the interaction between fibroblasts and the fibrin matrix.

Although this thesis has demonstrated that persistent fibrin deposition and reduced fibrinolysis were associated with further accumulation of collagen *in vitro,* through enhanced procollagen gene expression and impaired collagenase activation, these pathways are yet to be demonstrated *in vivo*. Firstly, procollagen gene expression will be examined in plasminogen activator-deficient animals following repeated administration of fibrin by *in situ* hybridisation analysis, similar to that demonstrated in Chapter 3 of this thesis. Impaired interstitial collagenase activity is more difficult to demonstrate *in vivo*, since isolation and maintenance of active and inactive proteases from tissues samples is complex, and often results in activation of all proteases present. Nonetheless, immunohistochemistry for collagenase with and without its pro-peptide may demonstrate relative levels of the active and inactive proteins. Alternatively, *in situ* zymography may highlight the regions of the fibrin-injected wild-type skin where MMP activity is high, compared with relatively low levels of activity in plasminogen activator-deficient skin, in particular, in the tPA knock-out. Studies will subsequently proceed to samples taken from the legs of patients with LDS, demonstrating reduced MMP-1 activity, suggesting that reduced

fibrinolysis leads to collagen accumulation in human fibrosis through an alternative mechanism, other than increased procollagen gene expression induced by persistent fibrin deposition.

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APPENDIX

Appendix A

Patient Classification Data

Patient number	Sex	Age	Duration of CVI (years)
1	Female	68	0
2	Male	63	0
3	Male	48	0
4	Male	59	0
5	Male	65	0
6	Male	69	0
7	Male	54	0
8	Male	61	0
9	Male	69	0
10	Male	54	0
11	Female	49	0
12	Female	65	0
	Mean	60.33	0
	SEM	2.17	0

Table A1. CEAP Class 0 - Normal control patient group (n=12)

Patient number	Sex	Age	Duration of CVI (years)
1	Female	57	32
2	Female	34	5
3	Female	60	5
4	Female	48	13
5	Male	39	15
6	Female	35	10
7	Male	45	10
8	Female	35	7
9	Male	44	5
10	Male	51	8
11	Male	38	11
12	Female	42	4
	Mean	44.00	10.42
	SEM	2.49	2.20

Table A2. CEAP Class 3 - Chronic venous insufficiency without skin changes patient group (n=12)

Patient number	Sex	Age	Duration of CVI (years)
1	Male	43	11
2	Female	34	10
3	Male	60	10
4	Male	43	10
5	Male	32	5
6	Female	28	8
7	Female	67	45
8	Female	43	5
9	Male	32	10
10	Female	28	7
11	Female	44	22
12	Male	46	11
	Mean	41.67	12.83
	SEM	3.51	3.18

Table A3. CEAP Class 4 Lipodermatosclerosis patient group (n=12)

Appendix B

B1 General molecular biology procedures

These procedures are all based on established methods (Sambrook and Maniatis, 1989), with modifications being noted. All molecular biology procedures described were routinely used in the preparation and analysis of nucleic samples.

B1.1 Materials

Precautions to minimise extrinsic RNase contamination were followed, based on previously published recommendations (Sambrook and Maniatis, 1989). Diethylpyrocarbonate (DEPC)-treated water was prepared by adding 0.5ml DEPC (Sigma, Poole, UK) to 500ml of deionised distilled water. The water was vigorously shaken and incubated overnight at 37°C. The water was then subsequently autoclaved and stored at room temperature.

B1.2 Gene cloning and bacterial transformation

The procollagen type I cDNA (Hf677) and the expression vector pGEM7zf+ were digested with ECORI restriction enzyme (Promega, Southampton, UK) prior to incorporation of the insert into the vector. Briefly, 5μg of insert and vector cDNA was incubated for 2 hours at 37°C with 1μl of ECORI (10units per μl) restriction enzyme with supplied enzyme buffer (according to manufacturer's instructions, Promega, Southampton, UK). The resultant DNA was purified by phenol:choloroform extraction (see Appendix B1.6). The cohesive termini of the vector and insert were ligated in equimolar quantities following heating of the DNA mixture to 45°C, melting reannealed cohesive ends. The mixture was cooled to 0°C and 0.1unit of bacteriophage T4 DNA ligase (Promega, Southampton, UK) was added, together with the supplied DNA ligase buffer and 1μl of 5mM ATP. The reaction was incubated for 4 hours at 16°C, and transferred directly to bacterial transformation without purification.

E.coli bacteria were transformed with the pGEM7zf+ plasmid vector containing the procollagen type I α-chain (COL1A1) cDNA sequence in order to amplify the copy number of the plasmid. E.coli DH5α were supplied in a competent state by Gibco

Laboratories (Paisley, UK), and were kept on dry ice before thawing very briefly. Lyophilised plasmids were thawed slowly and reconstituted in double distilled water to a final concentration of 1μg/ml. To a sterile 10ml polypropylene tube, 100μl of the thawed E.coli suspension was pipetted into fresh microfuge tube and kept on ice, before 10μl (10ng) of plasmid solution was added. The samples were mixed and the tubes incubated at 4°C for 30 minutes. The cells were then heat-shocked by incubating the tubes at 42°C for 90 seconds before placing them at 4°C for 2 minutes to allow transformation of the plasmid DNA into the E.coli cells.

Under sterile conditions 0.9ml of S.O.C. growth medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$, 10mM $MgCl_2$, 20mM glucose) was added to each cell suspension and the samples were shaken at 37°C for 1 hour. Meanwhile, the agar bacterial plates were prepared. Lennox agar (32g) was mixed with 1 litre of double-distilled water in a conical flask. The flask was sealed with a bung and autoclaved. A sterile field was a established with a bunsen burner, and ampicillin (Gibco, Paisley, Scotland) was added to the agar to a final concentration of 100μg/ml. Into a sterile petri-dish, 10ml of the agar solution was poured and the plates were dried uspside down at 4°C for 20 minutes.

With a sterile pipette tip, 100μl of each sample was plated and spread with a wire loop. The plates were allowed to dry for 45 minutes and then incubated upside down overnight at 37°C. The following day, two to six individual colonies were removed from the plate with a sterile loop and were used to inoculate 5ml of autoclaved LB broth (Sigma, Poole, UK) supplemented with 100μg/ml ampicillin. The cultures were grown for 16 hours at 37°C with constant shaking and were then stored at 4°C for up to 1 month.

B1.3 Small-scale preparation of plasmid DNA

Small-scale preparation of DNA was performed to isolate the amplified COL1A1/plasmid DNA from the cells. A 1.5ml aliquot of a single bacterial colony grown in 5ml of LB broth medium containing ampicillin was transferred to a sterile microfuge tube. The remaining 3.5ml was stored at 4°C indefinitely. The cell suspension

was centrifuged at 12000rpm for 5 minutes at 4°C and the medium was removed by aspiration, leaving the bacterial pellet. The protocol of alkaline lysis was used as recommended by Sambrook and Maniatis, (1989) to break down the cell membranes.

Briefly, the bacterial pellet was resuspended in 100µl of ice-cold solution containing 50mM glucose, 25mM Tris-HCl, pH7.6 and 10mM EDTA, by vigorous vortexing. A solution containing 0.2mM NaOH, 1% SDS was prepared and chilled on ice, and 200µl was added to each bacterial sample and the contents were mixed by inverting the tube rapidly five times. The samples were kept on ice and 150µl of ice-cold solution 3 (60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml H_2O) was added. The tubes were vortexed gently to disperse solution 3 through the bacterial lysate. The tubes were stored on ice for 3-5 minutes, followed by centrifugation at 12000rpm for 5 minutes at 4°C. The supernatant was transferred to a sterile microfuge tube and an equal volume of phenol:chloroform (Sigma, Poole, UK) was added. After vigorous vortexing for at least 1 minute, the samples were centrifuged for 5 minutes at 12000 rpm. The aqueous top layer was removed and placed into a fresh microfuge and the lower proteinaceous layer was discarded. The DNA was precipitated by the addition of two volumes of 100% ethanol with 50µl of 3M sodium acetate, and the samples were stored for 2 hours at -80°C. The tubes were subsequently centrifuged at 12000rpm for 15 minutes at 4°C, the supernatant was removed and discarded. The DNA pellet was washed with 250µl of 75% ethanol and was centrifuged for 5 minutes at 12000rpm. The ethanol was removed and the pellet was dried for 10 minutes. The nucleic acid pellet was dissolved in 50µl of sterile TE (Tris-EDTA) buffer (pH8) or sterile DEPC-treated water and stored at -40°C until *in vitro* transcription was performed.

B1.4 Spectrophotometric evaluation of nucleic acids

Quantitation of purified nucleic acid samples was performed by spectrophotometry. Nucleic acids absorb light maximally at 260nm. At this wavelength 50µg/ml of DNA or 40µg/ml of RNA have an absorbance of 1.0. The presence of contaminating protein in the sample is measured at 280nm. Calculation of the ratio of absorbance at 260nm and 280nm (A_{260}/A_{280}) was performed to estimate the purity of the preparation. A ratio between 1.7 and 2.1 was judged to be pure.

B1.5 Agarose gel electrophoresis of nucleic acids

A 1% solution of electrophoresis grade agarose (Sigma, Poole, UK) in 100mls of 1 x TBE buffer, diluted from a stock solution of 5 x TBE buffer (0.45M Tris-borate, 0.01M EDTA) was prepared. The solution was heated to dissolve the agarose in a microwave oven. The gel was cooled to approximately 60°C and 2µl of 10mg/ml ethidium bromide solution (Sigma, Poole, UK) was added. The agarose solution was poured into a casting tray (Bio-Rad Ltd, Hemel Hampstead, UK) to a thickness of approximately 5mm, and allowed to set at room temperature for 30 minutes.

Each DNA sample, containing up to 5 µg of DNA was brought up to a volume of 20µl with the addition of 2µl of gel loading dye (Sigma, Poole, UK) and double distilled water. The gel was submerged in 500ml of 1 x TBE in a gel electrophoresis tank (Bio-Rad, Hemel Hampstead, UK,) and the samples were loaded on to the gel, with a lamda DNA/Hind III fragment ladder (0.5µg/ml) or 1kbp DNA ladder (0.5µg/µl) (Gibco, UK). A voltage of 70V was applied to the gel from a Bio-Rad Powerpac 300 (Bio-Rad, Hemel Hampstead, UK) for 45 minutes. The gel was visualised by FLA-3000G imager (Fujifilm, Japan) and AIDA v.2.31 (Raytek Ltd, UK) imaging software.

B1.6 Phenol-chloroform purification of DNA samples

DNA can be concentrated and purified into a pellet from solution that may contain protein using the phenol:chloroform purification technique. Removal of contaminating proteins from DNA samples were performed after DNA isolation and purification when the OD260/280 ration is less than 1.6, and following plasmid linearisation prior to *in vitro* transcription. Phenol:cloroform (500µl) (Sigma, Poole, UK) reagent was added to the DNA samples in a microfuge tube. The mixture was vortexed thoroughly and centrifuged at 12,000rpm for 15 minutes at 4°C. The top aqueous layer was removed carefully and put into a sterile microfuge tube. The lower phenol:chloroform layer was discarded and 1ml of ice-cold 100% ethanol and 50µl of 3M sodium acetate was added to each sample and was stored at -80°C for 30 minutes. The samples were centrifuged at 12000rpm for 15 minutes, the supernatant discarded and the pellet was washed with 75% ethanol before

recentrifugation for 5 minutes at 4°C. The ethanol was removed and the pellet dried before redissolving in sterile water.

B1.7 RNA electrophoresis

RNA electrophoresis was used in conjunction with Northern analysis to determine the specificity of binding of the labelled riboprobe to procollagen type I α-chain DNA. Total RNA isolated from dermal fibroblasts was loaded onto the agarose gel and ran as follows.

A 20x solution of running buffer containing 0.4M 3-N-(morpholino) propanesulphonic acid (MOPS) pH7.0, 0.1M sodium acetate and 0.02M EDTA was prepared in a foil-wrapped bottle and autoclaved. In a pyrex conical flask, 2g of electrophoresis grade agarose (Sigma, Poole, UK) was dissolved in 162ml double-distilled water by autoclaving. The agarose was cooled and 10ml of the 20 x running buffer, 10ml of water and 18ml of 37% formaldehyde (Sigma, Poole, UK) were added and mixed to produce a 1% agarose solution in 1 x MOPS buffer and 1.1M formaldehyde. The gel solution was poured into an RNase-free casting tray.

In a sterile microfuge on ice, 5μg of RNA (varying volumes depending on concentration of each sample) was mixed with an equal volume of RNA loading buffer (Sigma, Poole, UK). A 3μl aliquot of RNA ladder (Promega) was also prepared. The total RNA samples were denatured by heating at 65°C for 15 minutes. The gel was submerged in approximately 500ml of 1 x MOPS, the comb was removed and the gel was pre-run at 80V for 5 minutes. Denatured RNA samples were placed briefly on ice prior to loading. The gel was run at 80V for approximately 2 hours and was visualised by UV illumination of ethidium bromide by FLA-3000G imager (Fujifilm, Japan) and AIDA v.2.31 (Raytek Ltd, UK) imaging software.

B1.8 Northern analysis of procollagen type I riboprobe specificity

Procollagen type I mRNA levels of human dermal fibroblasts *in vitro* were examined by hybridisation with the newly synthesised procollagen riboprobes following isolation of RNA by the TRIzol method according to the manufacturer's instructions (Gibco, Paisley, UK). RNA was isolated and transferred to a nylon membrane as described in Section

2.21.1. The membrane was then pre-hybridised in a solution of 5 x Denhardt's solution, 5 x SSC, 0.1% SDS containing 100mg/ml denatured salmon sperm DNA (Gibco, Paisley, UK) for 45 minutes at 65°C. The digoxigenin-lebelled riboprobe was heated to 65°C for 15 minutes to denature the secondary RNA structure, and was added to the hybridisation solution (containing 15ml DEPC-treated water, 6.25ml 20x SSC, 2.5ml Denhardt's solution, 1.25ml 10% SDS and 100µl of denatured salmon sperm DNA). This solution was poured onto the membrane and was incubated in a rotating oven for 16hours at 65°C. The membrane was removed and washed in 2 x SSC, 0.1% SDS for 30 minutes at room temperature followed by a high stringency wash in 0.2 x SCC, 0.1% SDS for 1 hour at 65°C.

The membrane was wrapped in Saran Wrap and exposed to Kodak X-OMAT film (Sigma, Poole, UK) until a suitable signal was visible. The fluorescent signal was then semi-quantitated using the FLA-3000G imager (Fujifilm, Japan) and AIDA v.2.31 (Raytek Ltd, UK) imaging software. Absorbance values of the signals representing hybridised procollagen probes were normalised relative to the loading of total RNA (ribosomal 28S RNA band) in the same sample. The riboprobe showed a strong signal for the characteristic two collagen transcripts, represented by 4.8kb and 5.8kb (4.8kb band and 1kb poly-adenosine tail) bands on the blot.

Appendix C

C1.1 Routine tissue culture techniques

Cells were routinely grown in 75cm^2 tissue culture flasks in Dulbecco's Eagle Modified Medium (DMEM)/10% (Foetal Calf Serum) FCS containing 100μg/ml penicillin (Gibco, Paisley, Scotland) and 100μg/ml streptomysin (Gibco, Paisley, Scotland). The cultures were incubated in a humidified atmosphere of 10% CO_2 in air at 37°C. Each cell line was tested every 3 months for mycoplasma contamination using a commercially available mycoplasma detection kit (Roche Diagnostics, UK). Culture medium was changed every three days and were passaged on reaching confluence.

Cell sub-culturing was performed by removing the culture medium, washing twice with 10ml PBS (pH7.4), before adding 2ml trypsin/EDTA solution (trypsin 0.05% w/v, EDTA 0.02% w/v, Gibco Life Technologies Ltd, Paisley, Scotland) to the cell layer. The cells were then incubated at 37°C until detachment from the tissue culture plastic-ware occurred as observed using inverted light microscopy (Axioscop 20, Carl Zeiss Ltd, Welwyn Garden City, UK). To the trypsinised cell suspension, 8ml of 10% FCS/DMEM media was added to inactivate the trypsin. The cell suspension was centrifuged at 1000rpm for 5 minutes. The supernatant was then discarded and the cell pellet was re-suspended in 9ml 10% FCS/DMEM. From this cell suspension, 3ml were added to three tissue culture flasks containing 9mls 10% FCS/DMEM (1:3 split).

C2.1 Storage of primary cell lines

C2.1.1 Freezing of cells

Primary cultures were frozen for storage and thawed as needed. Cells for storage were trypsinised and the cell suspension centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in freezing mix containing 16ml 20% FCS and 10% dimethylsulphoxide (DMSO) (Sigma, Poole, UK) in DMEM which was added drop-wise to the cells. A 1ml aliquot of this mixture, representing one confluent flask was added to a labelled Nunc cryotube and frozen overnight at -70°C. Frozen cell aliquots were then transferred to liquid nitrogen.

C2.1.2 Thawing of cells

A single cryotube of cells was removed from the liquid nitrogen and thawed rapidly at 37°C in a water bath. The cell suspension was transferred to 10ml of DMEM/10% FCS and was centrifuged for 5 minutes at 1000rpm. The media was discarded and the cell pellet reconstituted in 10ml DMEM/10%FCS and transferred to a $75ml^2$ tissue culture flask. Cells were incubated in a humidified atmosphere of 10% CO_2 in air at 37°C.

Appendix D.

D1.1 Genotype confirmation of tissue and urokinase plasminogen activator deficient mice by polymerase chain reaction (PCR)

Genomic DNA was obtained from tail-tips of plasminogen activator deficient mice prior to dermal fibroblast isolation or subcutaneous injection of fibrinogen and thrombin, to confirm gene inactivation. Briefly, tips were cut into small pieces with a sterile scalpel and lysed in a solution containing 100mM EDTA, 200mM NaCl, 50mM Tris/HCl, 1% SDS and 50mg/ml proteinase K (Sigma, Poole, UK) overnight. The DNA was isolated in isopropanol, washed in 70% ethanol and redissolved in sterile water to a final concentration of 100ng/μl. Primer sets for tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) were purchased from MWG Biotech-AG, (Ebersberg, Germany), with the following sequence:

tPA840: cgc tgt act ctt gcc cat gag gac
tPA740: gga aag gtg tga ctt acc gtg gca ccc ac
uPAG240: cag agg gga gag act gcc cgg ctt ggg
uPAG689: ggt act ccc cgt ggc ttc ttc ctc cct tg
pGKpA: aat gtg tca gtt tca tag cc

Primers were used in the PCR reaction to amplify regions of modified genomic DNA that results in plasminogen activator gene deficiency (Figure D1). PCR reaction buffer was made up in the following ratios for each set of tPA or uPA primers:

dH_2O	68%
10x dNTP	11.1%
10x KCl	11.1%
25mM $MgCl_2$	6.66%
Primer 5' (20μM)	0.88%
Primer 3' (20μM)	0.88%
Common (20μM)	0.88%
Taq polymerase	0.44%

Each reaction contained 5' and 3' primers (for tPA or uPA respectively) and common primers as described in Figure D1. Each PCR tube contained 2μl genomic DNA (100ng/μl), and 18μl of reaction buffer. Tubes were loaded onto the PCR machine (PCR9700, Applied Biosystems, California, USA) and the machine was programmed as follows to enable optimum amplification of the tPA and uPA genes; 95°C for 5 minutes; 95°C, 60°C, 72°C for 1 minute each for 35 cycles; 72°C for 10 minutes, and then the PCR product was stored at 4°C. The PCR products were removed from the machine and 2μl of gel loading dye was added to each PCR reaction. The PCR products were then loaded onto a 2% agarose gel in 1x TBE buffer, and run at 85V for 20 minutes. The gel was imaged using the FLA-3000G imager (Fujifilm, Japan) and AIDA v.2.31 (Raytek Ltd, UK) imaging software for ethidium bromide. The genotype of each mouse was determined by the band pattern of the PCR products for each plaminogen activator (Figure D1).

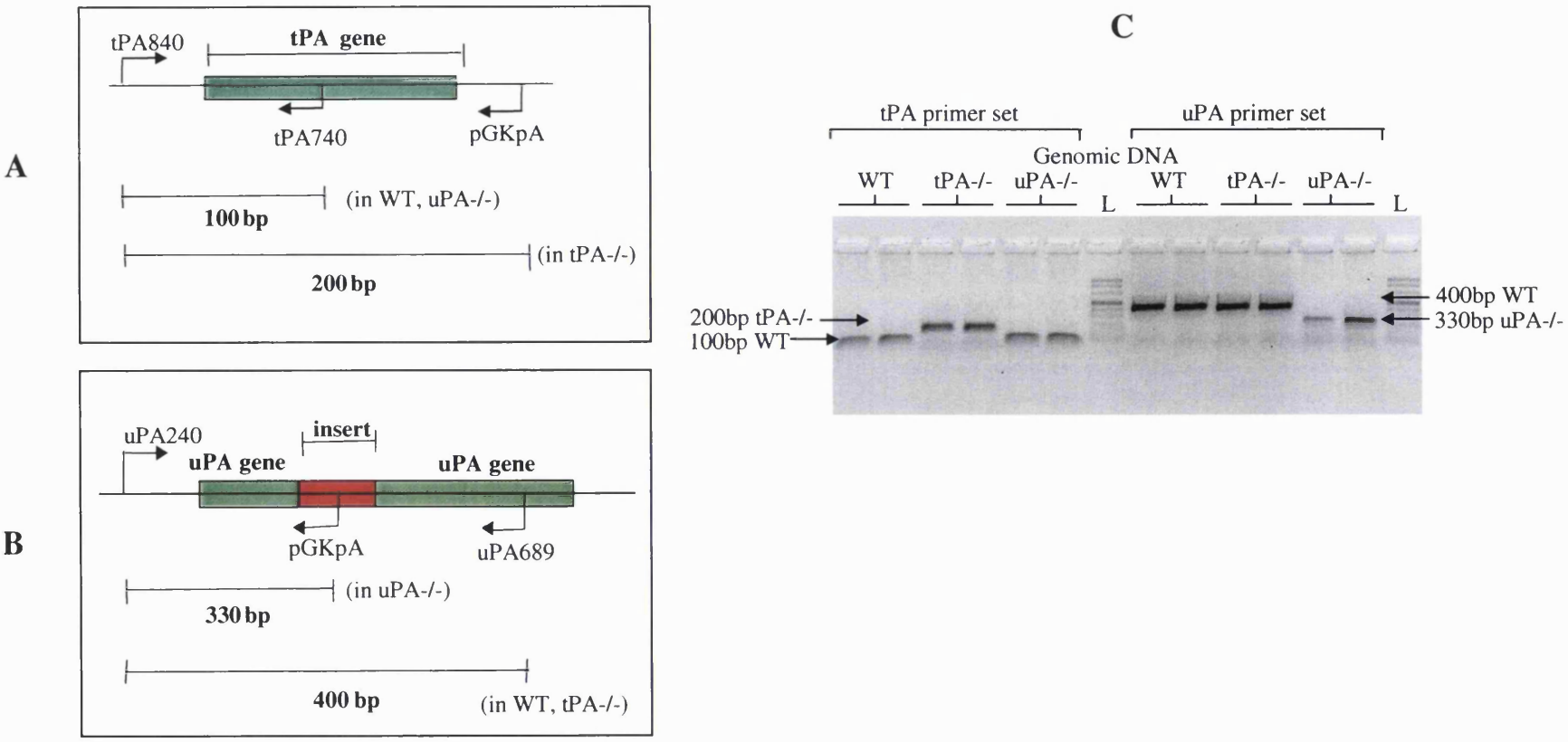

Figure D1. Confirmation of plasminogen activator gene deficiency by polymerase chain reaction. Hybridisation sites for **A.** tPA primers and **B.** uPA primers on the respective genomic DNA gene sequences. **C.** Agarose gel with PCR products demonstrating mouse genotype according to band size and migration pattern. tPA deficiency was shown by the larger 200bp PCR product due deletion of tPA 740 primer recognition sites. uPA-deficiency was demonstrated by the smaller 300bp PCR product due to the insertion of the common primer, pGKpA, recognition sites. (L = ladder)