A COMPARATIVE STUDY OF THE MECHANISMS LEADING TO DERMAL AND LUNG DISEASE IN SYSTEMIC SCLEROSIS

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by

Rama Vancheeswaran

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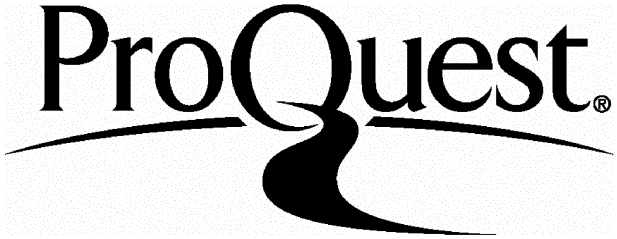
Department of Rheumatology

Royal Free Hospital & School of Medicine

Pond Street

London NW3 2QG



DEDICATION

To my parents and Kevin

ABSTRACT

Systemic sclerosis (SSc) is a multi-system disease of unknown aetiology, characterised by abnormalities in endothelial cell function, immune regulation and connective tissue metabolism. Skin sclerosis is the most apparent and studied feature but lung involvement is the major cause of death. Histo-pathological studies have revealed differences between the skin and lung suggesting distinct organ-specific mechanisms which have been further examined in this project by comparing the *in vivo* expression of Endothelin-1 (ET-1), ET-1 receptors and the major adhesion molecules in the two organs. The characteristics of fibroblasts derived from the two organs were also studied *in vitro*.

Circulating levels of ET-1 were elevated in SSc patients with widepread fibrosis (dcSSc). Lesional biopsies taken from the skin in dcSSc and the lung in fibrosing alveolitis (FASSc) showed a significant increase in the vascular expression of ET-1 and ET-1 receptors. Circulating levels of E-selectin and VCAM-1 were markedly raised in SSc. DcSSc skin biopsies showed a high vascular expression of E-selectin and ICAM-1 while FASSc vessels showed increased levels of VCAM-1 and ICAM-1. A strikingly high expression of ET-1, ET-1 receptors and ICAM-1 was also shown by hyperplastic alveolar epithelial type II cells in FASSc. Substantial differences in cell size and rate of cell proliferation were observed between SSc skin and SSc lung fibroblasts. SSc skin fibroblasts also showed markedly higher levels of matrix production, cell surface antigens including ICAM-1, CDw49d, ectoenzymes, 5A3, 3D3 and 3C4 and T lymphocyte interactions compared with SSc lung cells. However, a sub-population of ICAM-1-high and 3C4-high expressing cells was expanded in both SSc skin and SSc lung fibroblasts compared with normal cells.

This project provides evidence for different cellular abnormalities in the skin and lung which suggest organ-specific disease processes in SSc.

PREFACE

Some of the work reported in this thesis has appeared in the following publications:-

Publications:

1. Higley HR, Persichitte K, Chu S, Waegell W, Vancheeswaran R, Black CM: Immunohistochemical localisation of transforming growth factor beta 1 and type I procollagen in limited (lcSSc), diffuse (dcSSc) systemic sclerosis and morphea. Arthritis Rheum 1994; 37: 278-287

2. Vancheeswaran R, Magoulas T, Efrat G, Olsen IO, Penny RM and Black CM: Circulating endothelin-1 levels suggest different pathogenic mechanisms in systemic sclerosis (SSc) subsets. J Rheum 1994; 21:1838-1844

3. Vancheeswaran R, Azam A, Black CM, Dashwood MR: Localisation of endothelin-1 and it's binding sites in scleroderma skin. J Rheum 1994; 21: 1268-1276

4. Shi-Wen X, Panesar M, Vancheeswaran R, Mason J, Haskard DO, Black CM, Olsen IO, Abraham DJ: Expression and shedding of ICAM-1 and LFA-3 by SSc and normal fibroblasts, Arthritis Rheum 1994; 37: 1689-1697

5. Bou-Gharios G, Osman J, Atherton A, Monaghan P, Vancheeswaran R, Black CM, Olsen I. Expression of ectopeptidases in scleroderma. Ann Rheum Dis 1995; 54: 111-116

6. Bruckdorfer KR, Hillary JB, Bunce T, Vancheeswaran R, Black CM: Increased susceptibility to oxidation of low density lipoproteins isolated from patients with systemic sclerosis. Arthritis Rheum 1995; in press.

Submitted and in preparation

1. Vancheeswaran R, Sinclair H, Kapahi P, Haskard DO, Abraham DJ, Black CM: Adhesion molecules in the scleroderma spectrum disorders. J Invest Dermatol. 1994; submitted

2. R.Vancheeswaran, G. Bougharios, D.J. Abraham, T, Piela-Smith, J.Korn, I.Olsen and C.M. Black: Expression of fibroblast cell surface markers in Systemic sclerosis. Arthritis Rheum. 1995; submitted

3. Vancheeswaran R, Xu SW, DJ Abraham, Korn J, Piela-Smith T, Olsen I, Black CM. Phenotypic and functional characteritics of normal skin and lung fibroblasts. Exp Cell Res. 1995; submitted

4. Shi-Wen X, Vancheeswaran R, Bou-Gharios G, O'Hare M, Olsen I, Abraham D.J, Black C.M. Scleroderma fibroblasts retain abnormal functional and phenotypic characteristics following retroviral infection with a temperature-sensitive SV 40 large T antigen. Exp Cell Res. 1995; submitted

5. Vancheeswaran R, Dashwood MR, Pantelides P, du Bois RM, Black CM. Endothelin-1 immunoreactivity and binding sites in fibrosing alveolitis associated with systemic sclerosis (FASSc). J Clin Invest. 1995; in preparation

6. Vancheeswaran R, Korn J, Piela-Smith T, Olsen I, Black CM. Phenotypic and functional characteristics of scleroderma fibroblasts derived from the skin and lung. J Cell Physiol. 1995; in preparation

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ABBREVIATIONS

SSc	Systemic sclerosis
RP	Raynauds phenomenon
DcSSc	Diffuse cutaneous systemic sclerosis
LcSSc	Limited cutaneous systemic sclerosis
FASSc	Fibrosing alveolitis in systemic sclerosis
PHT	Pulmonary hypertension
ACR	American College of Rheumatology
MHC	Major Histocompatibility Complex
TCR	T cell receptor
BALF	Bronchoalveolar lavage fluid
D_LCO	Carbon monoxide diffusion capacity
ET-1	Endothelin-1
ACE	Angiotensin Converting Enzyme
vWF	von Willebrand Factor
APAAP	Alkaline phosphatase complexed to rabbit polyclonal anti-alkaline phosphatase
ICAM-1	Intercellular adhesion molecule-1
VCAM-1	Vascular cell adhesion molecule-1
LFA	Lymphocyte function antigen
ELISA	Enzyme linked immunosorbent assay
mAb	monoclonal antibody
Tris	Tris (hydroxymethyl) amino methane
PBS	Phosphate buffered saline
TBS	Tris buffered saline
DMEM	Dulbecco's modified Eagle's medium
FCS	Foetal Calf Serum
FCM	Flow cytometric analysis
AFI	Average fluorescent intensity

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Systemic sclerosis: an overview

1.1.1 Definition and history

Systemic sclerosis (Scleroderma; SSc) is a generalised connective tissue disorder characterised by progressive obliterative microvascular lesions, altered immune events and the excessive accumulation of normal extracellular matrix components in the skin and internal organs (Black 1993). The aetiopathogenesis of this disorder is unknown but the final fibrotic response is thought to occur as a result of interactions between endothelial cells, immune cells and fibroblasts (Mauch *et al.*, 1993). Numerous agents are recommended for the treatment of SSc, but as yet no cure is available (Wigley FM., 1992).

Although the designation "scleroderma" was first used by Gintrac in 1847 to describe skin thickening, the existence of this disorder has been documented since the 5th century by Hippocrates (Black *et al.*, 1981). Vascular dysfunction (Raynaud's phenomenon; RP) was first noted by Raynaud in 1862 and the association of SSc with RP was described in 1896 by Hutchison. Pulmonary symptoms in SSc were first recorded by Binz in 1864 (Binz quoted in Wolters 1892). However, it was not until after the careful work by Von Notthaft in 1898 and Matsui in 1924 that both lung parenchymal interstitial change and vascular abnormalities were felt to be directly caused by SSc. The presence of other visceral lesions was described in a detailed review by Goetz in 1945 when he coined the term "progressive systemic sclerosis" for this disorder.

1.1.2 Clinical perspective of SSc

1.1.2.a Incidence and prevalence studies

The incidence of SSc in the UK is 18 cases per million population per year. SSc has a similar incidence in the USA (20 cases per million population per year) (Silman 1991; Steen *et al.*, 1990). The prevalence of SSc patients in the UK at a

particular time has been shown to be 31 per million population (Silman AJ., 1991). It occurs mainly in the 30-60 year age group and rarely in children and the elderly. Females are predominantly affected, four times as often as males with the ratio increasing to 15:1 in females of child bearing age (Silman AJ., *et al.*, 1988). It is more common in black Americans and is more severe in non-Caucasoid individuals (Black 1993). RP occurs as the first symptom in 95% SSc patients and is itself prevalent in 3-10% of the adult population of the UK (Kallenberg 1990). RP is defined as episodic vasospasm characterised by pallor, cyanosis, suffusion and/or pain of the fingers, toes, ears, nose or jaw in response to cold or stress (Black 1993). RP may either be primary or secondary. Secondary RP is associated with an underlying disease (Kallenberg 1990).

1.1.2.b Early detection of disease

As the majority of SSc patients present with RP (Black 1993) , this is the most important stage for identifying patients at risk of developing systemic disease. Prospective studies have found that 1% of patients with RP develop features of connective tissue disease, the most common of which is SSc (Harper *et al.*, 1982; Fitzgerald *et al.*, 1988). The presence of abnormal capillaries is the most significant predictor to date of the subsequent development of SSc. These capillary abnormalities are noted in various sites including the nailfold (90% SSc patients), labial tissue (92% SSc patients) and conjunctival tissue (Maricq *et al.*, 1982; Grassi *et al.*, 1993; Mancel *et al.*, 1993) . Antinuclear antibodies detect 75% of patients with secondary RP (Kallenberg *et al.*, 1980).. Both techniques together detect 95% of RP patients who subsequently develop SSc (Kallenberg. 1990)

1.1.2.c Clinical presentation and course

The most apparent feature of SSc is skin thickening, which is assessed by the skin score, a clinical parameter that measures the extent of skin involvement (Kahaleh *et al.*, 1986). Skin disease occurs in three phases; an early oedematous phase (usually seen within 12 months of onset of disease) followed by a

proliferative phase and then an atrophic phase (Seibold 1994). Systemic disease may occur at any stage in the course of SSc and is usually the major factor affecting disease outcome (Black 1993). The frequency of involvement of the various organs as reported by post-mortem studies is: skin (98%), oesophagus (74%), lung (74%), bowel (39-46%), kidney (49%), cardiovascular disease (41%), skeletal muscle (41%) and the thyroid (24%) (D'Angelo *et al.*, 1969). Now that renal involvement is more successfully managed with vasoactive agents, the major cause of death in SSc is lung disease (Alton *et al.*, 1988; Steigerwald 1988).

Median survival of patients with lung disease is 78 months with a mortality of 60% of patients at 5 years (Altman *et al.*, 1991). The major clinical manifestations of SSc lung disease include fibrosing alveolitis (FASSc) and pulmonary vascular disease (pulmonary hypertension; PHT) (Scully *et al.*, 1989). FASSc is present in 60-100% of SSc patients (Steen *et al.*, 1985). A recent review summarising 11 studies of patients, with FASSc comprising 195 cases, reported different percentages of disease depending on the type of investigation performed. Abnormal pulmonary symptoms were found to identify 54% of patients, radiological evidence was present in 53% and abnormal pulmonary function tests identified 92% of patients (Alton *et al.*, 1988). Additionally, histological evidence of FASSc was present in 87% patients based on a review of 168 post-mortem cases (Alton *et al.*, 1988).

PHT was observed in 50% of SSc patients either as isolated disease or secondary to fibrosis (Scully *et al.*, 1986). PHT is associated with a significantly poorer prognosis than pulmonary fibrosis and tends to occur as isolated disease in patients with lcSSc (Stupi *et al.*, 1986). Other less frequently occurring forms of SSc lung disease include aspiration pneumonia, pleural disease, spontaneous pneumothorax, neoplasms, drug induced pneumonitis and associated pneumoconiosis (Black *et al.*, 1994).

1.1.2.d Disease criteria and classification

The American College of Rheumatology's preliminary criteria for SSc (given below) were defined in a multi-center study of 264 SSc patients and more than 400 patients with other related disorders evaluated within the first two years of diagnosis (1980). These criteria are reported to have a 97% sensitivity and 98% specificity for definite SSc.

American College of Rheumatology (ACR) preliminary criteria for SSc

1. Proximal scleroderma was the single main criterion for diagnosis with a sensitivity of 91% and specificity of more than 90%.

2. Sclerodactyly, digital pitting scars of fingertips, atrophy of the distal finger pad and bibasilar pulmonary fibrosis contributed further as minor criteria in the absence of proximal scleroderma.

3. One major or two minor criteria were found in 97% definite SSc patients, but in only 2% of the comparison patients with systemic lupus erythematosus, polymyositis/dermatomyositis or RP.

The ACR criteria make a clear distinction between SSc and other connective tissue diseases. In addition, it serves to exclude localised scleroderma, characterised by a more benign disease course with no associated visceral involvement (Steen *et al.*, 1990).

Once permanent vascular damage or skin and internal organ changes begin to appear in patients suspected to have SSc, several physical and serological differences separate these patients into distinct groups, each with a separate clinical presentation and a different disease course (Black 1993). Based on a classification system proposed by an international group (see below), SSc can be subdivided into diffuse and limited cutaneous disease (dcSSc and lcSSc, respectively) (LeRoy *et al.*, 1988).

The following criteria are used to distinguish between dcSSc and lcSSc;

A. DcSSc (40% of SSc patients)

(i) Onset of RP within 1 year of skin changes (puffy or hidebound)

(ii) Truncal and acral skin involvement

(iii) Presence of tendon friction rubs

(iv) Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease and/or myocardial involvement

(v) Absence of anti-centromere antibodies

(vi) Nailfold capillary dilatation and capillary destruction

(vii) Anti-topoisomerase I antibodies (Scl-70; 30% patients)

B. LcSSc (60% of SSc patients)

(i) Presence of RP for years (usually decades) before onset of skin changes

(ii) Skin involvement either limited to the hands, face, feet and forearms (acral) or absent skin changes or not present

(iii) A significant late incidence of PHT with or without interstitial lung disease, trigeminal neuralgia, skin calcifications and telengiectasiae

(iv) A high incidence of anti-centromere antibodies (70-80%)

(v) Dilated nailfold capillary loops usually without capillary dropout

1.1.2.e Prognosis and Survival

The prognosis and consequent survival in SSc is dependant upon the extent of organ compromise. LcSSc is associated with a survival rate of 79-84% at 5 years, 47-75% at 10 years and 33-43% at 12 years. In contrast, dcSSc is associated with a survival of 50% at 5 years, 22-26% at 10 years and 17% at 15 years (Barnett *et al.*, 1988). In lcSSc, pulmonary arterial hypertension occurs in 5% of patients (Stupi *et al.*, 1986; Al-Sabbagh *et al.*, 1989). This has a very poor prognosis with a mean duration of survival from detection of 2 years (Stupi *et al.*,

1986). Interstitial lung disease occurs in 70% dcSSc patients and is the main cause of death in this patient subgroup (Steen *et al.*, 1985).

A comprehensive summary of 11 studies comprising 2000 SSc patients showed that adverse factors influencing survival include being older than 40 years, being male or being black (Masi 1988) . Severe RP, a rapid progression of skin involvement plus visceral disease at baseline also portends a decreased likelihood of survival (Altman *et al.*, 1991). Increased mortality is particularly associated with lung disease, (both FASSc and PHT), cardiac failure and hypertensive renal crisis (Black 1994). Recently, in a study of 233 SSc patients, a 2.5 fold higher incidence of malignancies was reported, particularly of the lung (8 fold) (Rosenthal *et al.*, 1993).

1.1.3 Genetic and environmental factors

1.1.3.a Genetic factors

Although SSc is not considered to be a genetic disorder, there is evidence to support a genetic predisposition. This is based on the presence of: (i) familial clustering of SSc, RP and other autoimmune disorders (ii) disease linkage with specific alleles of the Major Histocompatibility Complex (MHC) and (iii) a high frequency of antinuclear antibodies, in blood relatives.

Thirty two cases of familial SSc have been reported so far, in first degree relatives, particularly in affected siblings and in parent and child (McGregor *et al.*, 1988; Briggs *et al.*, 1990). Concordance and discordance of disease in monozygotic twins is also present in the literature (Guseva *et al.*, 1981; Cook *et al.*, 1993). Although these instances are rare, a more common occurrence is the clustering of other connective tissue diseases with SSc (Maddison *et al.*, 1993).

The MHC was the first genetic area to be explored in SSc, based on observations of MHC linked genes with other rheumatic diseases (Welsh 1985; Goldstein *et al.*, 1987). The MHC determines cell surface glycoproteins that present antigen to and interact with T lymphocytes. The presence of such

linkages with disease therefore suggests specific antigen involvement (Schiffenbauer *et al.*, 1987). Increased frequencies of the following genes have been reported in SSc: class I (HLA-A9, A31, B8, B35), II (DR1, DR3, DR11, DRw6, DR52, DQA2, DQB1, DPB1) and III (C4 null alleles) (Briggs *et al.*, 1990; Briggs *et al.*, 1993; Kuwana *et al.*, 1993; Stephens *et al.*, 1993; McHugh *et al.*, 1994). In a recent UK study, the strongest association noted to SSc was the presence of a C4A null allele. An additional susceptibility marker was DQA2 (Briggs *et al.*, 1993).

In SSc, the specific Class II associations differ between populations. For example, DR1 and DR3 are associated with different disease subsets in European and American studies. Furthermore, while DR1, DR3, and DR5 contribute to SSc in Caucasoid patients but in Japanese patients, DR4 is associated with the disease (Briggs *et al.*, 1990; Sasaki *et al.*, 1991). However, both American and Japanese patients show linkage with certain HLA DQB1 alleles (Reveille *et al.*, 1992; Kuwana *et al.*, 1992). These differences may reflect variability in geographic, environmental and racial factors (Briggs *et al.*, 1990). The presence of DR3/DR52a has been noted to be associated with a 16.7 fold excess risk of development of pulmonary fibrosis in patients with either dcSSc or lcSSc (Briggs *et al.*, 1991). This observation suggests the involvement of a specific antigen in SSc lung disease.

There is conflicting evidence for increased autoantibodies in the relatives of SSc patients. While a recent study on 268 blood relatives of 63 patients in the UK showed a higher frequency of antinuclear antibodies in relatives compared with controls (Maddison *et al.*, 1993), no such difference was observed in a study by Barnett *et al.*, 1993.

The MHC linkage associated with many of the autoimmune diseases has prompted interest in defining the antigen specificities of T cell receptors (TCRs). For example, T lymphocytes expressing the TCR Vβ 5.2 gene recognises myelin basic protein in patients with multiple sclerosis (Kotzin *et al.*, 1991). Although no specific association with Vα and β TCR genes are currently known in SSc, an

increased frequency of TCRγ chain polymorphisms were shown in 41% of SSc patients compared with 21% controls (Kratz *et al.*, 1990) . Additionally, increased TCR γδ1$^+$ lymphocytes have been reported in SSc patients, in the peripheral blood, BALF and the gut, but not in skin (Yurovsky *et al.*, 1994). This latter report is particularly interesting in view of the suggestion that TCR γδ1$^+$ cells are involved in a primitive defence mechanism that protects epithelial cells from injury (Janeway 1988; Itohara *et al.*, 1990). The variation in γδ1$^+$ cells in the circulation and lung compared with skin suggests that T lymphocytes populations differ between organs in SSc. This concept is supported by the observation that lymphocytes cloned from skin produce significantly different lymphokines to those seen in the circulation in SSc patients (Ferrarini *et al.*, 1990).

Non-MHC genetic associations have also been reported in SSc. These studies are largely unconfirmed but suggest that mutations in the fibronectin gene, chromosomal instability and the decreased ability of patients to metabolise certain drugs, may contribute to the aetiopathogenesis of SSc-like illnesses.

Fibronectin is an important molecule in many biological processes. The presence of somatic mutations in the fibronectin gene in mononuclear cells and dermal fibroblasts derived from SSc patients was reported by Deguchi *et al.*, 1989 based on a study of Japanese patients. A two nucleotide mutation was noted in the 3' position adjacent to the sequence for the cell binding region of fibronectin and was suggested to provide an abnormality in the feedback regulation of matrix synthesis by fibronectin in SSc fibroblasts. However, similar studies repeated by both Japanese and Dutch groups did not reproduce these results (Verheijen *et al.*, 1990; Shiokawa *et al.*, 1991).

Housset *et al.*, 1969, first described an increased chromosomal breakage rate, acentric fragments and deletions in lymphocytes and fibroblasts in SSc. A high proportion of RP patients, who subsequently develop SSc, were also noted to have a high rate of chromosomal instability relative to healthy individuals (Emerit 1976). Artlett *et al.*, 1993 also revealed an increased level of chromosomal anomalies in SSc patients in both fibroblasts and peripheral lymphocytes. Based

on the above observations and in view of clastogen (bleomycin) induced fibrosis (Finch *et al.*, 1980; Adamson *et al.*, 1990), it appears that chromosomal abnormalities are present in fibrotic lesions but it is not known if such aberrations contribute to the aetiology or form part of the pathology of fibrosis.

Lastly, May *et al.*, 1990, provided evidence that poor metabolisers of both dapsone and mephenytoin have a 10 fold increase in the risk of developing SSc, compared with controls. This observation needs confirmation but is interesting in view of hydralazine induced systemic lupus erythematosus, which is dependant on an interaction between gender, MHC type, therapeutic dose and acetylator phenotype (Batchelor *et al.*, 1980).

The above observations suggest that in SSc, any of these genetic factors may influence the development of disease in different individuals.

1.1.3.b Environmental agents:

A number of environmental agents have been linked with the development of SSc-like features including silica, bleomycin, toxic rapeseed oil (adulterated with aniline), epoxy resins, L-tryptophan and organic solvents such as vinyl chloride (Welsh *et al.*, 1988). Recently, a major focus of research has been the possible association between silicone gel breast implants and SSc but careful retrospective studies of 235 patients with silicone gel breast implants and of 840 female SSc patients found no demonstrable evidence of a statistically significant association (Hochberg *et al.*, 1993; Giltay *et al.*, 1994). However, it is possible that a SSc-like syndrome may occur if any of these agents are imposed on a genetic background which might predispose to SSc.

1.1.4 Other aetiopathogenic agents

The formation of free radicals is implicated in SSc as these agents can induce both vascular activation and chromosomal abnormalities. Free radicals may occur due to hypoxic episodes and because of activated polymorphonuclear cells (Czirjak *et al.*, 1987; Murrel 1993). Evidence for increased oxidation was first demonstrated in stored plasma from SSc patients compared with controls by Blake *et al.*, 1985. Recently, studies in SSc have also revealed deficient levels of the anti-oxidants - selenium and ascorbic acid (Herrick *et al.*, 1994) and increased oxidised lipoproteins (Bruckdorfer *et al.*, 1994) .

Retroviruses: Although a viral aetiology has been suggested in SSc, there is weak data to support this concept. For example, a recent study by Haustein *et al.*, 1993, revealed antibodies to HIV retroviral proteins in 8 out of 29 SSc patients compared with a 3% positivity in 120 normal controls.

1.1.5 Pathology: brief overview

The major hallmarks of SSc are (i) vascular abnormalities characterised by capillary obliteration, endothelial injury and activation, intimal proliferation and medial thinning (ii) tissue infiltration by mononuclear inflammatory cells and (iii) an increased deposition of normal matrix components in the skin and internal organs (Black 1993). The working hypothesis in SSc is that, in the presence of the requisite aetiologic factors and genetic background, endothelial dysfunction and immune abnormalities lead directly or indirectly to the activation or selection of a subpopulation of normal or abnormal fibroblasts (LeRoy *et al.*, 1991; Black 1993; Mauch *et al.*, 1993). Investigators have, however, not yet examined whether all these abnormalities occur in the same individuals or the sequence of occurrence of these abnormalities. Furthermore, it is not clear whether the fibroblast subpopulations that are selectively expanded are normal

or abnormal cells. A detailed examination of each of these pathological features is given in later sections.

1.1.6 Animal models

The availability of animal models of SSc would be invaluable to research in SSc but the existing models display only some features of human disease. A brief account of some of the animal models used in research is given below as they are interesting in view of the presence of end stage fibrosis even in the absence of features considered to be involved in the pathology of human disease.

Chronic graft versus host disease (GVHD): The first animal model (rat) with autoimmune features similar to SSc was described following lymphocyte transfer and bone marrow transplantation into immunologically tolerant recipients (Statsny *et al.*, 1963; Bos *et al.*, 1989). A graft versus host reaction (GVHD) with excessive fibrosis and chronic inflammation was observed but vascular changes in this disease model were absent or vasculitic, in marked contrast to the proliferative, microvascular changes characteristic of human SSc (Bos *et al.*, 1988).

Experimental graft versus host disease (GVHD): is caused by treatment of mice with Tumor Necrosis Factor α (TNFα) (Piguet *et al.*, 1987; Sharpe *et al.*, 1988). Endothelial cell damage with increased levels of Factor VIII and vWF and fibrosis were noted in this model. Thus, an immunological process which damages the endothelium resulted in SSc-like features in this model, implicating pivotal immune and vascular abnormalities in the pathology of fibrosis. This model also suggests that endothelial cell damage occurs secondary to immune activation.

Bleomycin-induced fibrosis in mice: Cutaneous fibrosis resembling that seen in SSc was first described by Finch *et al*, following bleomycin administration (Finch *et al.*, 1980). In susceptible mice, the earliest changes of bleomycin-induced lung damage were endothelial and epithelial changes, similar to that in

human disease, and it was significant that these changes reproducibly preceded the increased production of matrix by fibroblasts (Adamson *et al.*, 1990). However, while marked inflammatory changes were seen in lung disease, skin fibrosis was associated with a notable absence of inflammation and autoantibodies characteristic of human SSc (Mountz *et al.*, 1983).

Spontaneous animal models: These models resemble human SSc more closely since no known aetiologic agents are present and include the University of California Davis L200 chicken model (Gershwin *et al.*, 1981; Van der Water *et al.*, 1989) and two tight skin mouse models; TSK/+ (Bocchieri *et al.*, 1991) and the newly developed TSK/NZB hybrid (Bocchieri *et al.*, 1993). While both the UCD L200 and TSK/+ models exhibit features of dermal fibrosis, differences are present with regard to the profile of autoantibodies in the former and the absence of cellular inflammation and vascular damage in the latter. An early inflammatory component and some SSc autoantibodies have recently been shown to occur in the hybrid TSK/NZB mouse but vascular damage is still notably absent (Bocchieri *et al.*, 1993).

1.2 Pathogenesis I: Vascular hypothesis

Le Roy and Campbell suggested that vascular injury is essential to the pathogenesis of SSc and occurs prior to fibrous deposition (Campbell *et al.*, 1975). Since then, investigators have suggested a likely sequence of events, starting with endothelial damage with an increased adhesion of activated inflammatory cells which then migrate and release cytokines. Subsequent to this, fibroblasts are activated with a resultant increased matrix synthesis and deposition (LeRoy *et al.*, 1991).

Evidence for early endothelial cell damage in SSc was provided by a sequential study of 60 SSc skin biopsies (Prescott *et al.*, 1992), where the first detected histological change was functional and structural endothelial damage with subendothelial oedema. This was followed by platelet aggregation,

lymphocytic migration of both $CD4^+$ and $CD8^+$ T cells, with tissue fibrosis occurring at a later stage after inflammation had subsided. Similarly, studies by Harrison *et al.*, 1991, have shown endothelial and epithelial damage as the first detected ultrastructural change in SSc lung biopsies. Furthermore, as described earlier, studies of bleomycin induced lung fibrosis in mice provide evidence that the earliest change detected in fibrotic lesions are endothelial and epithelial changes (Adamson *et al.*, 1990).

The mechanisms that lead to vascular injury are still unknown but Kahaleh *et al.*, 1979, implicated a non-dialysable heat stable protein, different from immunoglobulins, as responsible for the endothelial cytotoxic activity present in 33-50% SSc sera. This was subsequently thought to be a serine proteinase present in granules of activated T cells (granzyme 1) (Kahaleh *et al.*, 1990). Since then, investigators have suggested many other possible factors including environmental agents, endothelium seeking viruses, free radicals formed by intense vasospasm with resultant reperfusion injury, tumor necrosis factor, protease inhibitors and low molecular weight cytotoxic factors such as complement, histamine, kinins, toxic free radicals, oxidised lipoproteins, thromboxane (Kahaleh 1990).

The immunoglobulin fraction of sera from 33% of SSc patients reveal anti-endothelial cell antibodies which have also been reported in 74% SLE and 33% RA sera (Rosenbaum *et al.*, 1988). If these antibodies were mediating damage *in vivo*, the vascular damage seen in SSc would be similar to other connective tissue disorders rather than the distinct SSc vascular changes observed. These antibodies may therefore occur secondary to endothelial damage. Lastly, the presence of perivascular cell infiltrates may suggest an endothelial cell auto-antigen resulting in increased lymphocyte activation with direct cytolytic interactions mediated by natural killer (NK) and lymphocyte activated killer (LAK) cell interactions (Kantor *et al.*, 1992).

1.2.1 Abnormalities in vascular morphology and function

Vascular changes in SSc are widespread and seen in virtually all organs including the skin, viscera, subcutaneous tissue and muscles (Goetz 1945) and are characterised by (i) vasomotor instability with repeated transient interruptions of tissue perfusion (ii) small vessel structural changes and (iii) intravascular abnormalities manifested by increased platelet activity, decreased red cell deformability and enhanced thrombus formation (Kahaleh 1990).

1.2.1.a Transient disturbances in vascular function

A reduced digital blood flow, pulse volume and abnormal peripheral vasoconstriction in response to cold and stress are consistent findings in RP and SSc patients and precede the development of structural vascular abnormalities (Kallenberg 1990). It is possible that after a limited duration of ischaemia, substantial tissue injury can occur upon reperfusion, perhaps through the production of damaging proteolytic enzymes or free radicals. This is particularly notable as endothelial cells are more susceptible to superoxide injury because of a deficient production of catalase (Shingu *et al.*, 1985). Dysregulation in vascular tone may occur due to an imbalance in factors that induce vasoconstriction compared with vasodilators. In support of these mechanisms, previous studies have shown increased circulating levels of ET-1 (Kahaleh 1991; Yamane *et al.*, 1992), a potent vasoconstrictor agent, with decreased synthesis of nitric oxide (vasodilator) (Kahaleh *et al.*, 1993).

1.2.1.b Structural vascular changes

The structural vascular changes seen in SSc have been examined by nailfold capillaroscopy and histology. By nailfold capillaroscopy, the vascular changes observed include capillary dilatation, distortion and loss resulting in avascular areas (Maricq *et al.*, 1976; Maricq *et al.*, 1982). These abnormalities are also associated with increased globular, eosinophillic deposits of serum protein

in the extracellular spaces in nailfold biopsies (Carpentier *et al.*, 1990). In nutrient microvascular beds, capillaries were decreased, sometimes to 30% of expected density, with a resultant nutritional deficiency and tissue atrophy.

Post-mortem histological examination of the vascular lesions revealed concentric fibrous intimal thickening of small arteries, occasionally with thickening of the media and associated mucoid change in 22% of SSc patients (D'Angelo *et al.*, 1969). Additionally, Rodnan *et al.*, 1980, found a greater than 75% luminal narrowing of the digital arteries in more than 80% of dermal biopsies derived from a large series of SSc patients. These changes were also present in dermal biopsies in RP patients who were destined to evolve into SSc (Birstingl 1971).

Vascular lesions are more pronounced in the microcirculation with a spectrum of ultrastructural changes including gaps between endothelial cells, endothelial cell vacuolisation and swelling, occasional cellular necrosis, reduplication of the basal lamina and perivascular cellular infiltrates consisting predominantly of activated T lymphocytes and macrophages (Fleishmajer *et al.*, 1980).

1.2.2 Endothelial cell factors

Endothelial cell activation is associated with a change in the level of expression of a spectrum of biologically active molecules which allows the cell to manifest new functions (Kahaleh 1990). Table 1.1 shows some of the factors expressed by endothelial cells that may be altered resulting in a microenvironment which favours thrombosis and fibrosis. Determinations of these endothelial cell factors may be useful in assessing both the activity of the disease process and the response to therapeutic agents (Pearson 1993).

Table 1.1 : Molecules synthesised by the vascular endothelium

Vasoactive molecules:	**Growth factors and cytokines**
Endothelin-1 (ET-1)	PDGF,
Endothelium derived relaxing factor-nitric oxide	TGFβ,
	IL-1,
Prostaglandin E2	IL-6,
Prostacyclin (PGI-2)	CSF,
	Platelet Activating Factor
Extracellular matrix	**Anticoagulation factors**
Collagen III, IV and V	Antithrombin III
Sulphated proteoglycans	Thrombomodulin
Fibronectin	Protein S
Thrombospondin	
von Willebrand Factor	
Adhesion proteins	**Enzymes**
E-selectin, P-selectin	ACE
Intercellular adhesion molecule-1 (ICAM-1)	Aminopeptidyl peptidase (CD13)
Vascular cell adhesion molecule (VCAM-1)	Neutral endopeptidase (CD10)
	Dipeptidyl peptidase IV (CD26)

1.2.3 Platelet factors

Platelet activation is also seen in SSc but it is not known if this event occurs prior to, or as a result of endothelial cell damage (Kahaleh *et al.*, 1982; Blann *et al.*, 1993). Platelets can release a number of biologically active peptides including platelet factor 4, β thromboglobulin, thrombospondin, prostacyclin, TGFβ, platelet derived growth factor (PDGF) and serotonin (Kahaleh 1990). Although the role of the coagulation system in the pathophysiology of SSc is unclear, circulating levels of platelet aggregates, β thromboglobulin, vWF and platelet factor 4 are clearly raised in SSc. While two studies showed increased β thromboglobulin in RP (Kallenberg *et al.*, 1982; Blann *et al.*, 1993), Seibold *et al.*, 1985, showed increased levels of β thromboglobulin only in RP patients with minor symptoms of SSc but not in truly primary RP. Furthermore, it was interesting to note that low dose aspirin and dipyridamole therapy did not result in any improvement in SSc or RP, despite reducing β thromboglobulin and platelet factor 4 levels (Beckett *et al.*, 1984).

1.2.4 Endothelial cell -fibroblast interactions

While endothelial cells may cause fibrosis through the increased expression of growth factors, the relationship between these cell types in SSc is unclear. Endothelial cell activation and fibrosis may be two processes that occur separately reflecting different target responses to a common initiating event, or they may be sequential events with the initial target being the endothelial cell with resultant factors acting on the fibroblast. Based on earlier *in vivo* observations in SSc and data from animal models, the latter mechanism appears to be more likely. Factors which are released by endothelial cells that could result in increased fibrosis include TGFβ, PDGF and ET-1 (Kahaleh 1990). TGFβ and PDGF have been extensively investigated in SSc and details of these studies are given in later sections. The role of ET-1, a particularly important candidate in

SSc since it can induce both the vasoconstrictive and fibrotic features characteristic of the disease (Kahaleh 1991) has been examined in detail in this project and is described in Chapters 3 and 4.

1.3 Pathogenesis II: The immune hypothesis

Research on the role of the immune system in general has concentrated on two areas including: the humoral immune system and cellular immunity. There is a growing body of evidence for abnormalities in both of these systems but the critical immunologic event(s) which drives these processes are still unknown.

1.3.1 Autoantibodies

SSc has been considered an autoimmune disease since the 1960's after the introduction of indirect immunofluorescent techniques for the detection of antinuclear antibodies (Beck 1961). Although this abnormality is shared with other autoimmune disorders, the antibodies in SSc are distinct (Tan 1989). There is no evidence for a pathogenic role for these antibodies but they are markers of distinct clinical subsets of SSc and show a relationship with specific Class II polymorphisms. For example, anticentromere antibodies are reported to be associated with DR1, DR4, DR5, DR8, DQ7 and DQB1 alleles and anti-topoisomerase antibodies are associated with DR2, DR5, DR52a and DQB1 (Briggs *et al.*, 1993; Maddison *et al.*, 1993; Reveille *et al.*, 1992). This close relationship suggests a T cell driven process even if the specific T cells are unidentified to date. It is also conceivable that these autoantibodies arise secondary to cell damage or as a result of molecular mimicry, due to homologies between target epitopes and retroviral proteins. For example, epitopes present on topoisomerase-1 show strong sequence similarity to p30gag proteins from the feline sarcoma virus and cytomegalovirus (Maul *et al.*, 1989; Muryoi *et al.*, 1992). Similarly, B cell epitopes on the PM/Scl antigen show homology to the SV40

large T antigen and HIV tat proteins (Alderuccio *et al.*, 1991). An autoimmune phenomenon in SSc patients may therefore be caused by infection with these agents.

The autoantibodies characteristic of SSc are described below:

Anti-scleroderma 70 antibodies are directed against a 70 kD antigen that is a degradation product of DNA Topoisomerase-1 (100kD). Topoisomerase-I catalyses the relaxation of supercoiled DNA resulting in configurations that favour gene expression (Maul *et al.*, 1986; Shero *et al.*, 1986). A survey of data on all complete human genes sequences in 1988 showed that the fibrillar collagen genes have a striking preponderance of binding sites for topoisomerase I (Douvas 1988). Although this observation provides a connection between one of the characteristic antibodies associated with the dcSSc subset and collagen synthesis, the basis for any link is still not known.

Anticentromere antibodies are directed against 3 centromeric proteins: CENP-A, CENP-B and CENP-C of molecular weights 17, 80 and 140 KD (Earnshaw *et al.*, 1987) and are present in patients with the more limited form of SSc (Catoggio *et al.*, 1983). Antibodies to nucleolar antigens are directed against RNA polymerase I,II and III, chromosomal antigens, small nuclear ribonucleoproteins and the PM/Scl antigen. RNA polymerase antibodies are present in 25% of SSc patients with organ involvement and are indicative of a poor prognosis in dcSSc. PM/Scl antibodies are associated with dcSSc patients with muscle involvement and anti 7-2RNP antibodies are directed against mitochondrial antigens associated with the limited subset of SSc (Tan 1989; Reimer 1990).

Other autoantibodies that occur in certain SSc patients include anti-erythrocyte antibodies (6%), antigranulocyte antibodies (18%) and antiplatelet antibodies (24-75%) (Sipos *et al.*, 1988; Girelli *et al.*, 1993) .

1.3.2 T lymphocytes and monocytes

A variety of cellular immunologic abnormalities have been described in SSc. The presence of a SSc-like illness in chronic graft versus host disease strongly implicates a cell mediated immune pathology in SSc. The partial /absent effect of immunosuppressive agents may argue against an immune mediated pathogenesis or simply reflect the late stage at which these agents are used in disease. For example, partial responses have been reported with anti-thymocyte globulin (ATG) and interferon (IFN) (Goronzy *et al.*, 1990; Sinclair *et al.*, 1993; Stevens *et al.*, 1992) and although cyclophophamide has been found to be beneficial in SSc lung disease, this has yet to be tested in controlled trials (Du Bois RM., 1994).

The characteristics of SSc peripheral blood mononuclear cells are described in this section. The local tissue inflammatory cell phenotype in SSc skin and lung is described in detail in later sections. While studies have shown normal absolute lymphocyte counts in SSc (Frieri *et al.*, 1991; Kahan *et al.*, 1991; Kantor *et al.*, 1992), there is increasing evidence for abnormal T lymphocyte subsets, in particular activated helper T cells (Frieri *et al.*, 1991; Fiocco *et al.*, 1993). Decreased $CD8^+$ cells and increased $CD4^+/CD8^+$ T cell ratios (Almendinger *et al.*, 1983; Frieri *et al.*, 1991), a higher proportion of $CD4^+/CD25^+$ and $CD4^+/CD26^+$ T cells (Fiocco *et al.*, 1993), increased double negative ($CD4^-/CD8^-$) TCR α/β (Sakamoto *et al.*, 1992) and increased TCR γ/δ cells (Kratz *et al.*, 1990) have been reported. *In vitro*, SSc lymphocytes have been shown to have depressed non-specific (mitogen driven) T cell responses (Salem *et al.*, 1976; Horowitz *et al.*, 1977), reduced NK cell activity (Kantor *et al.*, 1992) and excess T helper activity (Inoshita *et al.*, 1981). Studies on cultured mononuclear cells have also shown that these cells spontaneously release IL-1 and IL-1 inhibitor and TNF-α (Alcocer-Varela *et al.*, 1985; Westacott *et al.*, 1988; Umehara *et al.*, 1990). A notable correlation between T cell activity (soluble IL-2 receptors) and B cell activation (lamp 2 expression) was recently noted by Holcombe *et al.*, 1993, providing a link between the cellular and humoral immune phenomena.

The presence of activated mononuclear cells and helper T cells is also indicated by raised soluble levels of IL-2 and IL-2 receptors (Kahaleh *et al.*, 1989; Degiannis *et al.*, 1990a; Degiannis *et al.*, 1990b; Kahaleh *et al.*, 1992), increased IL-4 and IL-6, TNF (Needleman *et al.*, 1992), and elevated CD4 but not CD8 (Kahaleh 1991) in the circulation. Although contradictory results are present with regard to soluble IL-2 (Kahaleh *et al.*, 1989; Degiannis *et al.*, 1990a; Needleman *et al.*, 1992), the levels of soluble IL-2 receptors consistently relate with disease progression. The presence of an activated T lymphocyte subset is further supported by the increased expression of proto-oncogenes including c-myc and c-myb by SSc T cells (Kahan *et al.*, 1989). In addition, a nuclear protein which binds specifically with the non-coding sequence of the human c-myc gene correlates with SSc disease activity (Gay *et al.*, 1992). These observations are consistent with the possibility that immune activation determines disease progression. Sfikakis *et al.*, 1994, recently showed an increased T cell mitogenic frequency even in late stage SSc suggesting that abnormal levels of T cell proliferation persist throughout disease.

T cell activation in SSc may occur as a result of stimulation by autoantigens. A number of possible autoantigens have been implicated based on the observations that circulating SSc T cells are reactive to laminin and type I collagen (Huffstutter *et al.*, 1985; Hawrylko *et al.*, 1991). Matrix components such as collagen type IV, fibronectin, laminin and tenascin are known to co-stimulate activation and signal transduction in T cells, which then lead to an enhanced generation of cytotoxic T cells responses and cytokines (Springer 1990). Thus, after the initial immune activation, increased matrix components in SSc may persistently maintain the activation of T cells and the pathological process.

1.3.3 Mast cells

The role of mast cells in the pathogenesis of SSc is unclear. Their involvement is implicated by the presence of a two fold increase in the

numbers of normal and degranulated mast cells in clinically "uninvolved" and fibrotic SSc skin (Seibold *et al.*, 1990). Additionally, recent studies have reported increased levels of dermal nerve growth factor which is known to increase mast cell numbers and stimulate histamine release in SSc dermal biopsies (Tuveri *et al.*, 1993).

1.3.4 Cytokines and growth factors

Recent evidence has suggested that products secreted by activated mononuclear cells may be involved in SSc. Of these, IL-1, IL-2, IL-4, IL-6, IL-8, IFN-γ and growth factors such as TGFβ, PDGF, TNFα, IGF and basic FGF are notably implicated (Takehara *et al.*, 1983; Mauch *et al.*, 1990; Korn 1991; Unemori *et al.*, 1991; Kahari 1993). Except for IFN-γ, increased levels of all the other factors have been described in either the sera or in lesions in SSc patients. It is highly unlikely that one of these factors alone is responsible for the disease. However, the importance of a single cytokine or growth factor may be limited to a disease subset, disease stage or organ. It is also possible that a combination of factors may set the conditions that permit the effector cells to respond to the next sequential signal.

A brief overview of the effects described in the above reviews, which implicate certain cytokines and growth factors in SSc is given in the Table 1.2 below.

Table 1.2: *In vivo* and *in vitro* effects of cytokines implicated in SSc.

Interleukin-1 *In vivo* administration endothelial cell injury and increased vascular permeability sequestration of polymorphonuclear cells in the pulmonary circulation, *In vitro* Inhibition of endothelial cell growth PGE_2 inhibition Increased ICAM-1 and lymphocyte adhesion, Increased IL-1 and PDGF 2.5 fold increase in collagen type I and III mRNA
Tumor necrosis factor α/ Lymphotoxin *In vivo* Severe endothelial cell damage with vascular permeability Acute and chronic inflammation Fibrosis Small bowel necrosis Sole effector of GVHD in animal models *In vitro* Endothelial cell damage with increased levels of Factor VIII and vWF Increased IL-1, PDGF, ICAM-1 synthesis by endothelial cells Increased mRNA for MHC I antigens Increased IL-6 Increased ICAM-1 and lymphocyte adhesion Increased fibroblast growth Increased collagenase (10 fold), increased TIMP mRNA (3 fold)
Interleukin-2 Increased mononuclear cell proliferation
Interleukin-4 *In vitro* Increased B cell stimulation Increased IL-2 mediated T cell proliferation Increased T cell adhesion to endothelial cells by non ICAM/LFA-1 pathways Increased fibroblast chemotaxis Increased collagen and fibronectin synthesis
Interleukin-6 *In vivo* Proliferation and differentiation of T and B lymphocytes, haematopoietic precursors, non-lymphoid cells Increases the acute phase response *In vitro* Potent stimulation of both T and B lymphocyte function Fibrogenesis
Interleukin-8 *In vitro* Potent neutrophil chemotaxis

Transforming growth factor-β 1 *In vivo* Neovascularisation Profound immunosuppression Fibroblast chemoattractant, Increased fibroblast mitogenesis and collagen synthesis Increased PDGF receptor mRNA *In vitro* increased pretranscriptional collagen gene expression Inhibits serine, thio and metalloprotease secretion (collagenase, stromelysin, telopeptidase) Increased TIMP mRNA.
Platelet derived growth factor *In vivo* Directed movement of polymorphs, macrophages, fibroblasts and smooth muscle cells. *In vitro* Increased basic FGF Increased mitogenic proto-oncogene ras expression Increased fibroblast and smooth muscle proliferation Increased collagen and procollagenase synthesis
Interferon γ: *In vivo* Increased lymphocyte binding to endothelial cells Tumoricidal activity, increased phagocytic activity Decreased collagen synthesis at the transcriptional level *In vitro* Increased MHC Class II expression Increased ICAM-1 expression and thus lymphocyte adhesion Inhibits fibroblast proliferation Decreased collagen synthesis
Insulin like growth factor *In vitro* Increased collagen synthesis
Basic Fibroblast growth factor (bFGF) *In vitro* Increased collagen synthesis

It is difficult to determine which of the above factors is most critical to the pathogenesis of SSc because of the variation in the effects of these agents *in vivo* and *in vitro*, the difference in target cell responsiveness to these factors, the presence of cytokines interactions which may modify the effect of given cytokine, and the effect of cytokine inhibitory factors (Needleman 1992).

Difficulties in the assessment of the role of individual cytokines in SSc is exemplified by examining TGFβ. This cytokine is implicated in SSc as it is a potent stimulator of collagen synthesis and endothelial neovascularisation (Ignotz *et al.*, 1986; Roberts *et al.*, 1986). However, actual inspection of SSc biopsies for TGFβ message has yielded inconsistent results. Both no increase in dermal SSc biopsies (Falanga *et al.*, 1990; Peltonen *et al.*, 1990) and increased levels of TGFβ in early dermal SSc lesions (Kulozik *et al.*, 1989; Herrmann *et al.*, 1991; Higley *et al.*, 1993) and pulmonary fibrosis (Broekelmann *et al.*, 1991; Corrin *et al.*, 1994) have been reported. Furthermore, as raised extracellular forms of TGFβ are observed, it has been suggested that increased lesional TGFβ may reflect passive binding to matrix components in SSc (Gabrielli *et al.*, 1993). Lastly, TGFβ is known to cause profound suppression of the cellular immune system and reduce the vascular-immune cell binding *in vitro*, in contrast to the immune hyperactivity and increased endothelial-immune cell adhesion present in SSc (Falanga *et al.*, 1990). Thus, the above results suggest that raised levels of TGFβ occur early and transiently and promote the mechanisms that lead to fibrosis in SSc.

1.4 Pathogenesis III: Abnormal fibroblast hypothesis

The fibroblast is central to the pathogenesis of SSc. The functions of the fibroblast include the deposition, maintenance and degradation of the extracellular matrix. While the deposition of matrix has been well studied, maintenance and degradation are less well understood. The fibrotic response in SSc may result from an imbalance in fibroblast proliferation, increased matrix synthesis or decreased matrix degradation (Mauch *et al.*, 1993). The examination

of fibroblast abnormalities in SSc has been performed predominantly on dermal fibroblasts and is described later in the section on SSc skin.

1.4.1 Fibroblast-matrix interactions

The extracellular matrix is a complex structure composed of different molecules including the collagens, proteoglycans, glycosaminoglycans and elastin. Although the extracellular matrix had been considered to be a passive scaffold for cells, it is now known to exert important biological functions by modulating cell migration, chemotaxis, proliferation and differentiation and lastly the regulation of matrix synthesis (Mauch *et al.*, 1993).

The major cell surface receptors involved in interactions with the extracellular matrix are the integrins which are heterodimeric glycoproteins consisting of two non-covalently associated α (130-200kDa) and β (90-130kDa) chains. Each of these subunits has an extracellular N-terminal domain, a transmembrane segment and a small C-terminal cytoplasmic domain connected to the cytoskeleton. Integrins are implicated in signalling between the extracellular matrix and the intracellular events (Ruoslahti *et al.*, 1987; Hynes 1992). They bind to a number of extracellular matrix proteins including fibronectin, fibrinogen, laminin, collagen, thrombospondin, vitronectin and von Willebrand factor mainly through RGD sequences. This interaction is cation dependant and requires both subunits. Alternatively spliced forms differing in their extracellular domains have been reported and may be important in altering ligand binding affinity (Mauch *et al.*, 1993). The collagen binding α1/β1, α2/β1 and α3/β1 integrins mediate functional interactions between fibroblasts and collagen fibrils (Hynes 1992). The β1 integrins also induce collagenase and stromelysin expression. Abnormalities in either the α or β integrin chains may result in an aberrant regulation of matrix synthesis (Werb *et al.*, 1989).

Other extracellular matrix-cell adhesion proteins have also been identified including anchorin CII, a 47 kDa heat shock protein which binds to denatured

collagen (Mauch *et al.*, 1993) and the ectoenzyme dipeptidylpeptidase IV (DPPIV; CD26) which binds hepatocytes to type I collagen and mediates the initial phase of fibronectin-mediated cell spreading on collagen (Piazza *et al.*, 1989).

1.4.2 Collagen metabolism

The collagen family of molecules include the fibrillar, basement membrane and filamentous collagens which are well characterised. Although these molecules are genetically distinct, they possess a similar basic structure which codes for a repeating $(Gly\text{-}X\text{-}Y)_n$ sequence. The addition of individual amino- and carboxy- propeptide gene sequences, introns followed by significantly different post-translational modifications results in the diverse number of collagens present. Each of the collagen genes are complex and large, with a considerable number of introns which are directly copied by transcription to result in large pre-mRNA molecules (Bailey AJ., *et al.*, 1988, Chu M-L., *et al.*, 1984).

Processing of the pre-mRNA molecules involves approximately 50 excisions and splicing steps. The collagens are then synthesised as pre-pro-collagens with large signal sequences (100 amino acids) which undergo a series of enzymatically controlled post-translational modifications (Krieg *et al.*, 1988). When the newly synthesised pro-alpha chains enter the rough endoplasmic reticulum, proteases remove the signal sequences. Enzymes including prolyl and lysyl hydroxylases convert prolyl and lysyl residues to the respective hydroxylated analogues. These enzymes only work on the substrate when it is present in a non-helical form. Sugar residues are then added, catalysed by galactosyl and glucosyl transferases and intra-chain and inter-chain disulphide bonds are formed. Proα chains are then released from the Golgi complex in polysomes to the exterior of the cell where two specific proteinases present in the extracellular space are responsible for the cleavage of terminal parts of the procollagen I molecule, a prerequisite for the appropriate assembly of type I

collagen into collagen fibres (Prockop *et al.*, 1979; Laurent 1986; Krieg *et al.*, 1988; Mauch *et al.*, 1990).

The pathways of collagen degradation are complex and may occur in both intracellular and extracellular sites. Degradation of mature collagen fibrils in the extracellular space depends on the activity of collagenase, which cleaves the collagen molecule between position 775 and 776 within the triple helix. Once this occurs, the molecule becomes susceptible to various other proteases, or alternatively the denatured chains are taken up by cells during endocytosis and degraded intracellularly. Collagenase is synthesised in an inactive form and controlled by various tissue inhibitory metalloproteases (TIMPs) (Murphy *et al.*, 1988; Birkedal-Hansen *et al.*, 1993).

1.4.3 Regulation of collagen synthesis and other extracellular matrix components

Studies of the molecular mechanisms which control the expression of collagen and extracellular matrix genes may provide a basis for understanding collagen regulation by growth factors and a rationale for therapeutic intervention in fibrotic states (Mauch *et al.*, 1990). A systematic examination of the effects of chimeric reporter constructs of the chloramphenicol acetyl transferase genes and various 5' sequences of the proα1(I) or proα2(I) genes have yielded a wealth of information on collagen regulation. In particular, regulatory elements in the 2000bp and 222bp upstream of the start of transcription site in the α2(I) and α1(I) collagen genes, respectively have been identified. Additionally, proteins that bind to these regulatory sequences have been reported (Hatamochi *et al.*, 1986; Maity *et al.*, 1988; Parker *et al.*, 1990). The presence of increased levels of both α2 (I) and α1(I) collagen chains in almost all physiologic and pathologic situations suggests common regulatory factors for both collagen genes. Three binding sites are common to both genes including a common site for the DNA binding protein, nuclear factor 1 (NF-1) (Oikarinen *et al.*, 1987). TGFβ is generally known to

increase the expression of both genes, and has now been shown to induce the expression of NF-1 (Rossi *et al.*, 1988). Although other cytokines also affect the synthesis of both collagen chains at a transcriptional level, details of the mechanisms involved at the gene level are still unknown. Additionally since individual cytokines upregulate both type I and type III collagen and other extracellular matrix components in SSc, a common pathway for cytokine mediated matrix regulation is indicated. Thus, extracellular matrix genes may be (i) under the control of a master gene and/or (ii) subject to activation by one or more transactivating factors and/or (iii) the modification of one or more suppressor elements (deCrombrugghe *et al.*, 1990).

1.5. Skin involvement in SSc

1.5.1 Histology of normal skin

Microscopic features

Normal human skin is composed of a multilayered cellular epidermis and an underlying dermis which meet at the dermal-epidermal junction. The epidermis normally consists of keratinocytes, immune cells, neuronal cells and melanocytes. It is normally divided into three layers including an uppermost non-cellular cornified layer overlying two cellular layers. The basal cell layer, apposed to the dermis, is immunologically active with the presence of interspersed antigen-presenting cells (Langerhans cells). The dermis contains a variety of elements including vessels, nerves, dermal appendages (hair follicles, arrectores pilorum, sweat and sebaceous glands) and individual cellular components set in a meshwork of extracellular matrix. It is customarily divided into 2 layers: a more superficial papillary dermis and a deeper reticular part (Breathnach 1971; Lever *et al.*, 1983). Normal skin consists of two major types of collagen including type I collagen which is present throughout the dermis and

type III collagen which is more restricted in distribution to type I (Meigel *et al.*, 1977; Lovell *et al.*, 1979).

1.5.2 Pathological features of SSc skin disease

1.5.2.a Histology

SSc skin lesions are characterised by a thickening of the dermal collagen network, varying degrees of arteriolar thickening, a diminution of adnexal and microvascular structures, flattening of the dermal-epidermal junctions and sclerosis of underlying subcutaneous tissue. While inflammatory infiltrates are present early in the development of the lesion, particularly in perivascular sites, they disappear with the deposition of increased fibrous tissue (Fleishmajer *et al.*, 1977a; Rowell 1988)`.

1.5.2.b Inflammatory profile in skin

The cellular infiltrates present in dermal lesions are mainly comprised of activated T lymphocytes, predominantly $CD3^+/CD4^+$, $TCR\alpha^+/\beta^+$, $HLADR^+$ and macrophages (HLA DR^+ and CD 68^+), with few B cells, neutrophils, eosinophils and langerhans cells (Roumm *et al.*, 1984; Gruschwitz *et al.*, 1992; Ishikawa *et al.*, 1992). Dermal mononuclear cells also express the cellular proto-oncogenes c-myc, myb and ras (Gay *et al.*, 1992). In these sites, increased levels of PDGF and PDGF receptors, TGFβ1, IL-1, TNF, IL-6 and IL-8 have been reported (Klareskog *et al.*, 1990; Gay *et al.*, 1992; Higley *et al.*, 1993; Koch *et al.*, 1993). The increased accumulation of inflammatory cells in SSc lesions may occur due to raised levels of immune cell adhesion molecules. Most notably raised levels of β1 and β2 integrins are shown by intradermal leukocytes (Gruschwitz *et al.*, 1992; Sollberg *et al.*, 1992) with increased levels of their counter-receptors (ICAM-1, VCAM-1 , E-selectin and CD44). Some expression of intraepidermal CD44 was shown by Koch *et al.*, 1993, with no epidermal infiltrates.

1.5.2.c Differences in dermal histology between the various subsets of SSc

Although the pathological features seen in SSc are common to the various subsets of disease, the levels of inflammatory activation differ between the SSc spectrum disorders. For example, investigators have shown that localised SSc (morphea) lesions have significantly more macrophages, decreased T lymphocytes and increased TGFβ and $HLADR^+$ expression compared with dcSSc lesions. LcSSc lesions have significantly lesser inflammation (as assessed by the above markers) than both morphea and dcSSc. (Branchet *et al.*, 1992; Ishikawa *et al.*, 1992; Higley *et al.*, 1993)

1.5.2.d Features of SSc fibroblasts *in vivo*

Ultrastructural examination of SSc dermal fibroblasts *in vivo* have revealed cells with a severely dilated rough endoplasmic reticulum and round microvesicles near the cytoplasmic membrane (Fleishmajer *et al.*, 1977b). In situ studies have shown increased levels of the proα(I) collagen mRNA in both the early oedematous and late sclerotic phases of SSc (Scharfetter *et al.*, 1988). In the early phase, increased proα(I) collagen mRNA were associated with cells in the deep dermis, subcutaneous fat and in perivascular sites particularly in the vicinity of surrounding mononuclear infiltrates. In late stage lesions, increased proα(I) collagen mRNA levels were present in the entire dermis and close to the dermal-epidermal junction. Increased levels of total collagen synthesis is also suggested by reports of increased levels of proline hydroxylase (Kawaguchi *et al.*, 1992a; Kawaguchi *et al.*, 1992b) . There are no differences in the distribution and ratio of type I and III collagen in the dermis in SSc (Lovell *et al.*, 1979). SSc fibroblasts are also noted to be heterogeneous in phenotype *in vivo*, expressing different levels of collagen mRNA (Scharfetter *et al.*, 1988), actin and desmin (Sappino *et al.*, 1990) and MHC II antigens (reticular and perivascular cells express higher levels HLA DR) (Branchet *et al.*, 1992).

1.5.3 Features of SSc dermal fibroblasts *in vitro*

In vitro studies on SSc fibroblasts have predominantly examined cells derived from dermal lesions and have highlighted a number of abnormalities, most notably increased levels of matrix synthesis. SSc dermal fibroblasts synthesise 2-4 times higher matrix molecules, particularly type I (α1(I) and α2(I) heterotrimers and rarely α1(I) homotrimers) and type III collagen (α1(III) homotrimers), fibronectin and proteoglycans (both glycosaminoglycans and protein core components) (LeRoy 1974; Uitto *et al.*, 1979; Jimenez *et al.*, 1986; Xu *et al.*, 1991). The regulation of this increased extracellular matrix may occur at a pre-transcriptional stage as there is direct evidence of increased mRNA synthesis (Jimenez *et al.*, 1986; Scharfetter *et al.*, 1988; Vuorio *et al.*, 1991).

In vitro, SSc dermal fibroblasts, unlike normal cells, express high levels of extracellular matrix components and proto-oncogenes for a period of time even after serum deprivation, suggesting an endogenous autocrine ability to maintain this disease phenotype *in vitro* (Jimenez *et al.*, 1986; Trojanowska *et al.*, 1988). In addition, variable levels of cell membrane β1 and reduced levels of α1 integrins have been shown in SSc dermal fibroblasts which may lead to an aberrant control of collagen regulation (Ivarsson *et al.*, 1993). Decreased fibroblast responses to procollagen peptides involved in regulation of collagen synthesis have also been observed (Mauch *et al.*, 1993). Although decreased levels of matrix degrading enzymes may also explain the increased deposition of matrix in SSc, studies have shown stable to increased levels of collagenase (84%-200%) and moderately raised levels of TIMP (167%) in SSc dermal fibroblasts (Chua *et al.*, 1990; Lohmann *et al.*, 1993).

Apart from the abnormalities of matrix expression, SSc dermal fibroblasts have been found to show other abnormalities including raised levels of ICAM-1, actin, intracellular calmodulin, hsp gene expression (hsp 70) and c-myc expression (Uitto *et al.*, 1979; Deguchi *et al.*, 1990; Sappino *et al.*, 1990; Abraham *et al.*, 1991; Shishima *et al.*, 1992). The observation of expanded sub-populations of

SSc dermal fibroblasts which synthesise high levels of collagen (Needleman *et al.*, 1990b) and ICAM-1 (Needleman 1990a) implicate a clonal expansion of sub-populations of normal cells in disease. SSc dermal fibroblasts also express low levels of dipeptidyl peptidase IV which is an ectoenzyme found to be involved in fibroblast interactions with the extracellular matrix and neuro-peptide metabolism (Bou-Gharios *et al.*, 1995).

SSc dermal fibroblasts are similar to normal fibroblasts with regard to characteristics such as plating efficiency, population doubling times and *in vitro* lifespan (Mauch *et al.*, 1993). These cells are therefore not immortalised or transformed, although increased levels of proto-oncogenes and persistence of their abnormal synthetic phenotype have been reported (Jimenez *et al.*, 1986; Trojanowska *et al.*, 1988). A number of studies have shown differential responses by SSc cells to growth factors such as PDGF, EGF, FGF and TGFβ1 compared with normal cells, indicating variations in the levels of cell surface receptors or in the previous 'priming' of cells by exposure to these agents *in vivo* (Takehara *et al.*, 1991).

1.6 Lung involvement in SSc

1.6.1 Aetiopathogenetic mechanisms of SSc lung disease

1.6.1.a Fibrosing alveolitis (FASSc)

The aetiopathogenesis of FASSc is unknown but injury to the alveolar epithelium and endothelium have been reported as the first event in the disease process (Harrison *et al.*, 1991). The presence of Scl-70 antibodies and/or HLA DR3/DR52a have also been reported to provide a 16.7 fold excess risk factor for pulmonary fibrosis in the presence of SSc. This observation may indicate the involvement of a specific antigen or a specific T cell clonal abnormality in SSc lung disease (Briggs *et al.*, 1991). Although Southcott AM., *et al.*, 1993, did not find any evidence of a clonal expansion of T cells expressing the αβ TCR,

Yurovsky *et al.*, 1994, revealed increased $\gamma\delta1^+$ T cells in BALF. The consistent observation of secondary lymphoid follicles with germinal centres in FASSc similar to those seen in reactive lymph nodes also implicates an immune aetiopathogenesis in the lung (Haslam 1990) .

1.6.1.b Pulmonary hypertension (PHT)

Very little is known of the aetiopathogenesis of PHT in SSc as studies are more difficult to conduct since tools to detect this disease early are not currently available. Barr *et al.*, 1988 and Groen *et al.*, 1989 observed reduced carbon monoxide diffusion (D_LCO) in the steady state and in response to cold challenge in patients with RP and have suggested that pulmonary RP may occur in SSc patients with isolated PHT.

1.6.2 Histology of normal lung

The alveolar wall consists of specialised epithelium and a closely apposed network of capillaries supported by a delicate interstitial matrix. The alveolus is populated by several different cell types that vary in their biosynthetic activities, susceptibility to injury and replicative potential (Crouch 1990).

Epithelial cells form nearly 95% of the alveolar surface area and consist of a single layer of flattened type I epithelial cells linked by tight junctions and supported by a continuous basement membrane. These terminally differentiated cells are vulnerable to a variety of injuries and have little if any replicative or regenerative potential. In contrast, type II epithelial cells, the progenitors of type I epithelial cells, have the capacity to repopulate the alveolar wall after injury. These cells express surfactant, MHC Class II antigens, ICAM-1, growth factors such as TGFβ, PDGF and matrix components including fibronectin, type IV collagen, laminin, proteoglycans and matrix degrading enzymes, cell surface receptors for fibronectin and other matrix components, complement and procoagulant molecules (Crapo *et al.*, 1982).

Endothelial cells line the pulmonary vasculature which constitutes 60% of the total systemic vasculature (Crapo *et al.*, 1982). Fibroblasts are also present in the alveolar wall. In addition to these resident cells, a variety of migratory cells are present in the lung including interstitial and alveolar macrophages, B and T lymphocytes and granulocytes (Kuhn 1988). Although very few granulocytes are seen in the interstitium and airspace normally, the pulmonary microvasculature contains a large marginated pool of granulocytes with a smaller proportion of macrophages (Crouch 1990).

The collagens account for 60% of the total connective tissue and include types I and III , IV, V. VI. Types I and III are ubiquitously present in the lung and co-distributed in a ratio of 2:1, respectively. The other components of normal matrix include elastin, glycoproteins such as fibronectin, glycosaminoglycans and proteoglycans (Hance and Crystal, 1975; Laurent 1986; Crouch 1990).

1.6.3 Pathological features of SSc lung disease

1.6.3.a Fibrosing alveolitis

Gross examination:

The lungs appear stiff and covered with 1-2cm air filled cysts. Getzowa 1945 described a "cystic" morphology where a zone of firm subpleural cysts were present in the lower 2/3 of the lung and "pulmonary sclerotic" changes characterised by diffuse fibrosis, usually maximal at the lung bases with loss of functional lung units.

Histology:

The earliest changes seen in FASSc include a widening of the alveolar walls, capillary congestion and hypercellularity comprising principally of immune cells, particularly mononuclear cells and neutrophils (Rossi *et al.*, 1985; Haslam 1990). These changes are seen in scattered areas with intervening normal tissue and are followed by a pathological process consisting of a combination of inflammatory, destructive and fibrotic changes (Harrison *et al.*, 1989). The

presence of epithelial and endothelial damage was observed as the first detectable event and the predominant feature of FASSc lesions (Harrison *et al.*, 1991). The presence of a marked change in alveolar epithelial cells, including destruction of type I cells and repopulation of the epithelial surface with hyperplastic type II epithelial cells is a consistent abnormality in FASSc. Type II cells were found to express HLA DR and increased TGFβ (Kallenberg *et al.*, 1987; Corrin *et al.*, 1994).

In contrast to the early transient inflammation observed in dermal SSc biopsies, studies performed on post-mortem tissue in FASSc reveal significant inflammation even in late stage lesions (Ashba *et al.*, 1965). A recent study which examined the histological changes in 49 FASSc biopsies from 34 patients found evidence of alveolitis in all biopsies where fibrosis was present (Harrison *et al.*, 1991). Furthermore, unlike the predominantly lymphocytic infiltrates noted in SSc dermal lesions with few plasma cells and granulocytes, FASSc biopsies taken at any stage showed an increased number of macrophages, neutrophils, lymphocytes and eosinophils. Lymphocytic infiltration was present in both the interstitium and the intra-epithelium, preceding the extensive deposition of collagen. Lymphoid follicles comprising of abundant lymphocytes and plasma cells are also present in FASSc but not in SSc dermal lesions.

Recently, FASSc lymphocytes were phenotyped as predominantly antigen primed memory cells; $CD4^+$/ $CD45RO^+$, with a variable number of $CD8^+$ cells (Wells *et al.*, 1993). Mast cells are occasionally present in FASSc, in close contact with fibroblasts. Activated fibroblasts with a resultant increase in connective tissue have been noted early in disease indicating that fibrosis occurs together with inflammation and epithelial/endothelial damage (Harrison *et al.*, 1991).

Bronchoalveolar lavage fluid (BALF):

This technique for sampling the local inflammatory milieu in FASSc has been commonly used for evaluation of lung disease, as it is safe and non-invasive compared with open lung biopsy. A large proportion of the current data regarding SSc lung disease was obtained from studies on BALF.

The main observations noted from examination of BALF in FASSc include an increased cell concentration (3-6 fold) comprising of high numbers of granulocytes (20%; neutrophils and eosinophils) and lymphocytes (20%) and increased immune complexes, immunoglobulin and albumin (Silver *et al.*, 1984; Rossi *et al.*, 1985; Harrison *et al.*, 1990; Wells *et al.*, 1992). Raised levels of epithelial lining fluid glutathione, an indicator of oxidative damage, together with neutrophil degranulation products have also been observed providing evidence for granulocyte mediated tissue damage (Cantin *et al.*, 1987; Sibille *et al.*, 1990; Borok *et al.*, 1991). Increased levels of macrophage derived IL-8 and fibronectin have also been observed in BALF which may account for the increased chemotaxis of neutrophils to FASSc lesions (Kinsella *et al.*, 1989; Carre *et al.*, 1991; Southcott *et al.*, 1993). Du Bois *et al.*, 1992 showed increased soluble levels of ICAM-1 and E-selectin in FASSc patients.

Increased BALF macrophage numbers and raised levels of IGF-1, ET-1, PDGF and TGFβ suggest increased macrophage activation with cytokine release (Wallaert *et al.*, 1986; Martinet *et al.*, 1987; Kinsella *et al.*, 1989; Cambrey *et al.*, 1992; Moreland *et al.*, 1992; Harrison *et al.*, 1994). Reduced levels of IFN-γ have also been reported in BALF (Prior *et al.*, 1992). As each of these agents influence fibroblast proliferation and matrix secretion, they have each been implicated in the pathogenesis of FASSc.

Differential leukocyte counts have been found to be of value in assessing the clinical severity and course of FASSc. For example, increased granulocytes reflect a greater impairment of lung function and a lesser response to treatment while increased lymphocytes reflect early disease and a better response to treatment (Miller *et al.*, 1990; Silver *et al.*, 1990; Harrison *et al.*, 1992; Wells *et al.*,

1992). Although these relationships are clinically useful, serial sampling of BALF has not been found to reflect change in disease, with similar immunological indices in both recent and chronic FASSc (Owens *et al.*, 1986).

1.6.3.b Pulmonary vascular disease

Investigators have reported medial atrophy and concentric intimal proliferation in 29% (D'Angelo *et al.*, 1969) and 57% (Young *et al.*, 1978) of pulmonary arteries in SSc patients. Pulmonary arteries bear the brunt of disease but vessels of all sizes can be involved. Measurements of pulmonary arteries in 58 patients with SSc, including 38 dcSSc and 20 lcSSc patients revealed that the greatest luminal occlusion was seen in lcSSc patients with isolated PHT (Al-Sabbagh *et al.*, 1989). The above studies examined biopsies taken at late stage disease and did not suggest any mechanism for disease pathogenesis.

1.6.4 Features of SSc lung fibroblasts

In comparison with the large number of *in vitro* studies on SSc dermal fibroblasts, very few studies have been performed on FASSc fibroblasts. The main body of evidence for abnormal fibroblast function in FASSc is derived from *in vivo* studies of FASSc biopsies, from fibroblasts cultured from BALF in FASSc patients, from fibroblasts cultured from biopsies taken from patients with other fibrosing lung disorders (cryptogenic fibrosing alveolitis) and animal models of lung fibrosis.

In vivo studies performed on FASSc biopsies revealed similarly dilated endoplasmic reticulum and abundant intracellular collagen in fibroblasts as that seen in dermal lesions (Kuhn *et al.*, 1989). Although increased collagen deposition is histologically evident in biopsies taken from FASSc patients, biochemical studies have not reflected these results. This anomaly may be due to the patchy nature of fibrosis in the lung or the inherent limitations associated with biochemical studies on biopsies. For example, biochemical measurements

of collagen in biopsies require that it is related to other unaltered cellular constituents. So far, levels of DNA have been used which is inappropriate due to the hypercellularity of FASSc biopsies.

In vitro studies of fibroblasts derived from other pulmonary fibrotic lesions have revealed an increased procollagen production in response to increased TGFβ and a faster rate of collagen synthesis compared to controls (Raghu *et al.*, 1989). Studies on rat lung fibroblasts derived from bleomycin-induced lesions also show increased collagen synthesis and fibroblast heterogeneity (Korn *et al.*, 1992). The presence of an abnormal population of fibroblasts in FASSc was suggested by Ludwicka *et al.*, 1992 based on the expression of increased levels of actin and desmin by fibroblasts cultured from BALF.

1.7 Treatment

The current aim of treatment in SSc is to minimise the development of vascular damage and tissue fibrosis by reducing vasospasm, inflammation and/or collagen production. Furthermore, close assessment and management of each of the individual diseased organs is indicated (Torres *et al.*, 1990; Black *et al.*, 1994).

To alleviate vasospasm, vasodilator agents such as calcium channel antagonists (Schmidt *et al.*, 1989), serotonin antagonists (Coffman *et al.*, 1989), prostacyclin analogues (iloprost) (Torley *et al.*, 1991; Wigley *et al.*, 1993) and Angiotensin Converting Enzyme (ACE) inhibitors (Steen *et al.*, 1990; Langevitz *et al.*, 1991) are used. While some benefit may be attained with these drugs in cutaneous RP, vasoactive agents are largely ineffective in PHT (Alpert *et al.*, 1991; Alpert *et al.*, 1992; Baudouin *et al.*, 1993), perhaps due to the development of fixed vascular lesions.

In the early oedematous phase of dcSSc, partial improvement is reported with a number of immunosuppressive agents including methotrexate (van den

Hoogen *et al.*, 1991), anti-thymocyte globulin (Goronzy *et al.*, 1990; Sinclair *et al.*, 1993), cyclosporin A (Gisslinger *et al.*, 1991; Clements *et al.*, 1993) , followed by anti-fibrotic agents such as d-penicillamine (Steen *et al.*, 1985; de Clerk *et al.*, 1987; Ronchetti *et al.*, 1989; Jimenez *et al.*, 1991) and interferon (Gilkeson *et al.*, 1990; Stevens *et al.*, 1992) . Furthermore, initial reports using extracorporeal photophoresis which induces immunosuppression have been promising and are currently being examined in a controlled trial (Rook *et al.*, 1992; DiSpaltro *et al.*, 1993). Although no therapeutic agent is conclusively known to affect SSc lung disease, the combination of corticosteroids with cyclophosphamide has been found to be beneficial . (Harrison *et al.*, 1992; Silver *et al.*, 1993).

Agents that inhibit various stages of the process of collagen synthesis and metabolism have been used with mixed outcomes in SSc (Kahari 1993). Some of these agents have both immune and matrix regulatory effects *in vivo*, including the interferons which reduce collagen synthesis at a transcriptional level, methotrexate which reduces fibroblast proliferation and d-penicillamine which impairs collagen cross-linking with the formation of less stable and easily degraded collagen fibrils. In addition, fibrostatin C, a prolyl-4-hydroxylase inhibitor reduces collagen synthesis *in vitro* (Kawaguchi *et al.*, 1992; Kawaguchi *et al.*, 1992) and 4-hydroxyretinamide is inhibitory in the tsk/+ mouse (Delany *et al.*, 1993). The latter two anti-fibrotic agents are promising but have yet to be tested *in vivo*.

1.8 Aims of this study

Previous studies have predominantly examined SSc skin disease and shown that abnormalities in endothelial function, immune regulation and connective tissue metabolism are the major features of the SSc disease process. The aims of this study were to compare the cellular abnormalities involved in the pathogenesis of SSc in the skin and in the lung in order to determine whether different mechanisms are responsible for the expression of disease in these two organs. In this study, I have therefore examined the following;

1. Serum and tissue levels of Endothelin-1 (ET-1) and ET-1 receptors because this peptide is a potent vasoconstrictor and profibrotic agent;

2. Circulating and tissue levels of E-selectin, VCAM-1 and ICAM-1 because these molecules play a major role in immune cell interactions;

3. The expression of certain fibroblast cell surface antigens and the regulation of extracellular matrix production.

CHAPTER TWO:

GENERAL MATERIALS AND METHODS

2.1 Patient samples and clinical details

2.1.1 Patients

All the SSc patients examined in this project fulfilled the ACR preliminary criteria for disease (1980) and were classified into dcSSc and lcSSc groups, using a modified skin score index (Kahaleh *et al.*, 1986), which ranged from 2 to 10 in patients with lcSSc and higher than 10 in those with dcSSc and internationally accepted criteria (LeRoy *et al.*, 1988). RP was diagnosed when patients presented with episodic vasospasm characterised by pallor, cyanosis, suffusion and/or pain of the fingers, toes, ears, nose or jaw in response to cold or stress (Black 1993), and was subdivided into autoimmune and primary disease based on the presence or absence of anti-nuclear antibodies and abnormal nail fold capillaries, respectively (Kallenberg 1990). The diagnosis of morphea was made on the basis of histopathological confirmation of typical skin lesions (Jablonska *et al.*, 1979).

2.1.2 Clinical parameters

The clinical and laboratory parameters used for the assessment of disease included duration of RP and SSc, skin score, internal organ involvement, grade of severity of Raynaud's disease and serology. The extent of skin involvement in patients with SSc was assessed using a modified skin score index (Kahaleh *et al.*, 1986).

Lung involvement consisted of PHT and FASSc. PHT was diagnosed in SSc patients when the mean arterial pressure was greater than 25/10 mm Hg, either by right heart catheterisation or Doppler echocardiogram (Ungerer *et al.*, 1983; Black CM., *et al.*, 1994). The diagnosis of FASSc was made on the basis of evidence of abnormalities on high resolution computed tomography (CT), a restrictive pulmonary deficit and/or reduced gas transfer measurement and the

exclusion of other known causes of alveolar fibrosis (Harrison *et al.*, 1991). Hypertensive renal disease was diagnosed when patients had evidence of either accelerated hypertension, an abnormal renal biopsy, a fall in creatinine clearance to below 60 ml/min or proteinuria exceeding 500 mg in 24 h in the absence any other cause (Torres *et al.*, 1990).

Detailed records of clinical data including other types of organ involvement, date of collection of blood samples and types of therapy were available on all the patients involved in the study. Records of therapy were available on all patients used in this project and included d-penicillamine, prednisone, α-interferon, cyclosporine-A, azathioprine, anti-thymocyte globulin in the antifibrotic and immunosuppressant group, and nifedipine, ketanserin, diltiazem and iloprost in the vasodilator group.

Anti-nuclear and anti-centromere antibodies were detected by indirect immunofluorescence on Hep-2 cells (1984) and anti-topoisomerase I (anti-Scl-70) antibodies were detected by immunodiffusion performed on agarose gels (Bunn *et al.*, 1994).

2.1.3 Samples

Serum and plasma samples from patients and normal healthy controls were obtained over a period of 5 years. Immediately after bleeding, blood samples were centrifuged at 2500 rpm for 15 min, plasma was separated and stored at -70°C. Serum samples were allowed to clot, centrifuged at 2500 rpm for 15 min, separated and stored at -70°C.

4 mm^2 punch biopsies were taken from the skin of patients and normal healthy volunteers. In the SSc patients, paired skin biopsies were taken from areas that appeared to be clinically "uninvolved" (skin from the back) and from the most recently involved SSc skin to examine disease progression. Lung biopsies were taken from the affected areas of SSc patients with FASSc and

compared with normal lung biopsies taken from the unaffected areas of age matched individuals undergoing lung resection for various malignancies.

The tissue was snap-frozen in liquid nitrogen immediately after biopsy and stored at -70^{o}C until use. Serial cryostat sections (10μm) were cut at -25^{o}C and mounted on the appropriate precoated slides. For immunocytochemistry, the sections were mounted on slides pre-coated with aminopropyl-triethoxysilane (Sigma, UK). For autoradiography, the sections were mounted on gelatinised microscope slides. These sections were then air dried for 45 min, individually wrapped in cling film and frozen at -70°C until use.

2.2 Measurement of circulating levels of Endothelin-1 (ET-1), Angiotensin Converting Enzyme and von Willebrand Factor (vWF)

2.2.1 Circulating ET-1 measurements

Serum samples were thawed at room temperature, diluted with an equal volume of 0.2% Trifluoroacetic acid (TFA) in phosphate buffered saline (PBS) and added to activated SEP-PAK C18 columns (Amersham, UK). Activation of C18 columns was performed by adding 2mls of 100% Ethanol to each of the columns followed by washing in 10mls of 0.1% TFA/H_20. After the addition of serum samples, columns were washed 3 times with 0.1% TFA/H_20, samples were eluted with 2mls of 60% acetonitrile in 0.1% TFA/H_20 and lyophilised until required.

The concentration of circulating ET-1 in these samples was measured after rehydration with 0.5mls of ET-1 assay buffer (0.1% bovine serum albumin in PBS + 1% Tween 20 and 0.01% sodium azide), using a competitive radio immunoassay as previously described (Davenport *et al.*, 1990). In this procedure, the serum samples and known concentrations of unlabelled porcine ET-1 (Novobiochem, Switzerland) were used to block the binding of ^{125}I-ET-1 (Amersham, UK) to rabbit anti-ET-1 antibodies (Peninsula Labs, Belmont, CA). The levels of radiolabelled ^{125}I-ET-1 was measured in the antigen-antibody

complexes by a gamma scintillation counter for 60 min and was found to be inversely proportional to the amount of unlabelled sample ET-1. The concentrations of sample ET-1 were calculated compared with standard levels of peptide.

The sensitivity of the assay was 1.6 pg/ml. Variation between assays of the same sera carried out at different times was approximately 8%, while replicate samples assayed at the same time varied less than 5%. The shelf-life reproducibility of this assay within 6 weeks varied less than 5%. The rabbit anti-ET-1 antibody had maximal reactivity with ET-1 (100%), with minimal reactivity with other isoforms of ET including ET-2 and ET-3 (7%) and proendothelin (17%).

2.2.2 Measurement of serum Angiotensin Converting Enzyme (ACE, EC 3.4.15.1) levels

ACE levels were measured only in selected patient samples where ACE inhibitors were not used in therapy by a spectrophotometric method utilising the synthetic tripeptide substrate N-(3-(2 furyl) acryloyl) L-phenylalanyl-glycylglycine (FAPGG) (Sigma, UK), as described previously (Holmquist *et al.*, 1979) . Briefly, FAPGG was hydrolysed by ACE to furylacryloylphenylalanine (FAP) and glycylglycine (GG) with an associated decrease in absorbance at 340 nm. One unit of ACE activity was defined as the amount of enzyme that was needed to catalyse formation of one micromole of FAP per minute under the conditions of assay. The ACE levels in patient samples were then determined by comparing the sample reaction rate to that obtained with an ACE standard of known activity and concentration. The formula used for this estimation was:

ΔA_{340} per 5 minute for sample = Initial absorbance - final absorbance with test sample

ΔA_{340} per 5 minute for calibrator = Initial absorbance - final absorbance with calibrator

Sample ACE in U/L= $\frac{\Delta A_{340} \text{ per 5 minute for sample}}{\Delta A_{340} \text{ per 5 minute for calibrator}}$ x known activity of calibrator

2.2.3 Measurement of plasma von Willebrand Factor (vWF)

Circulating levels of vWf were measured in plasma using a "capture" enzyme-linked immunosorbent assay (ELISA) as previously described (Gordon *et al.*, 1987). 96 Well plates (Costar) were precoated overnight with a mouse mAb to vWF (provided by Dr J. Van Mourik) at a dilution of 1:2000 in 0.05Mol carbonate buffer, pH 9.6. The plates were then washed in 0.05% Tween 20 in PBS (PBS-T) and standard concentrations of vWF and the plasma samples were added for 1 h at room temperature. After washing the plates thoroughly in PBS-T, ligand capture was detected by the addition of polyclonal sheep anti-human vWF conjugated to alkaline phosphatase (Serotec, UK) for 1 h at 26°C. Binding of this conjugated antibody was detected by incubation with substrate (26.5mg 4-methylumbelli-feryl phosphate (Koch-Light) in 10ml 90% 2-methoxyethanol) in substrate buffer (9.7% dietholamine, 0.01% MgCl, 0.02% NaN_3 at pH 9.3). The fluorescence determination of each well was measured at 25min using a Titertek Fluoroskan II at 460nm. Quantitation of vWF levels in plasma was carried out based on comparison with standard curves.

2.3 Expression of ET-1 and ET-1 binding sites (receptors) in skin and lung biopsies

2.3.1 Immunocytochemistry

2.3.1.a Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

The expression and distribution of various antigens have been examined in frozen sections from normal skin and lung biopsies using the APAAP technique. Prior to the immunocytochemical assay, frozen tissue sections

mounted on glass slides coated with aminopropyl-triethoxysilane (Sigma, UK) were air dried and fixed with an appropriate fixative at 4°C for 5 min. In this project, either acetone: methanol (1:1, v/v) and chloroform: acetone (1:1, v/v) were used. Non-specific IgG binding was then blocked with 20% normal goat serum (NGS) for 20 min and the appropriate primary antibody was added to sections for variable times depending on the type of antibody used. These sections were then washed thoroughly in Tris-buffered saline (TBS) and incubated for a further 30 min with the appropriate complimentary secondary antibody. The sections were again washed in TBS and alkaline phosphatase complexed to anti alkaline phosphatase (same animal species as primary antibody) was added for 30 min. After thorough washing, antigen levels were detected using naphthol AS-MX phosphate coupled to Fast Red TR in N,N-dimethylformamide containing 0.1Mol levamisole (all from Sigma, UK). 0.1Mol levamisole was added to inhibit endogenous alkaline phosphatase. The sections were counterstained with Mayer's haematoxylin.

2.3.1.b Immunocytochemical staining of ET-1

Sections were air-dried for 60 min and fixed with acetone:methanol (1:1) for 5 min at 4°C. Non-specific IgG binding was blocked by incubating the sections with 20% normal goat serum for 20 min at room temperature. Polyclonal rabbit anti-human ET-1 (Cambridge Research Chemicals, UK) was applied at a concentration of 5 μg/ml to the sample sections and incubated overnight at 4°C. Normal rabbit IgG (Serotec, UK), used as a negative control, was also applied to replicate sections at 5 μg/ml. All subsequent manipulations were carried out at room temperature. The sections were washed three times in TBS and the detection of bound antibody to the sections was performed using the APAAP staining procedure.

The histological assessment of the distribution and intensity of staining was made in a blinded fashion by two histopathologists (PD and PJ). ET-1 staining intensity was graded semiquantitatively from (-) to (++) with (-) representing the

absence of any staining and (++) the maximal intensity, corresponding to the intensity of staining with QBEND 10 (anti-endothelial cell marker). The grading was performed by two investigators, without prior knowledge of the clinical diagnosis.

2.3.1.c Identification of cell types in skin and lung

To delineate structures in both skin and lung biopsies, comparable sections from both patient and control biopsies were also stained with mouse monoclonal antibodies that clearly delineate endothelial cells - QBEND 10 (Oxoid, Unipath Labs, UK), smooth muscle (anti-actin; Serotec, UK), macrophages (HLA DR^+ and $CD68^+$) and type II epithelial cells (HLA DR^+ and $CD68^-$) (anti-HLA DR and anti-CD68 were both obtained from Serotec, UK). These mAbs were used at concentrations of 1μg/ml and staining was detected using the APAAP technique.

2.3.2 Autoradiography

2.3.2.a ET-1 binding sites (receptors)

Endogenous peptide levels were reduced by preincubating the mounted sections in 50 mMol Tris HCl buffer, pH 7.4, for 15 min at 22°C, as described previously (Moody *et al.*, 1990; Dashwood *et al.*, 1991; Terenghi *et al.*, 1991; Knock *et al.*, 1993). Slides were then incubated in buffer containing 5 mMol Mg Cl_2, 100 kiu/ml Aprotinin and 1% bovine serum albumin in the presence of 20 to 150 pMol [^{125}I]ET-1 (specific activity 2000 Ci/mmol, Amersham, UK) for 90 min at 22°C. The degree of non-specific binding was established by incubating replicate sections in the presence of 500 nMol (excessive) unlabelled ET-1 (Bachem Fine Chemicals). After incubation, sections were washed in buffer twice for 10 min each, at 4°C, then immersed in cold distilled water and dried in a stream of cold air. Low resolution autoradiography was performed by exposing slide mounted tissue to Hyperfilm 3H (Amersham) in X-ray cassettes for 6 days. [^{125}I]ET-1 binding sites (receptors) were identified in tissue sections, at the microscopic

level, by dipping the sections in nuclear emulsion (LM1, Amersham) at 50°C, and exposing for 3 to 8 days in lightproof boxes at 4°C. The sections were then processed in D19 developer (Kodak), followed by IF23 fixative (Ilford, diluted 1 to 4 in water), each for 5 min at 22°C, after which they were stained with Mayer's haematoxylin and eosin for histological examination. Autoradiographs and stained tissue were examined under dark-field and bright-field illumination on an Olympus Vanox microscope.

2.3.2.b Quantitation of [^{125}I]ET-1 binding sites

The levels of [^{125}I]ET-1 binding to various dermal and pulmonary structures identified by the previously described cellular markers was measured in both patients and controls. The level of [^{125}I]ET-1 binding was assessed by taking exposure meter readings on an Olympus Vanox microscope (at X 200 magnification) of autoradiographic grain accumulations generated from sections incubated with [^{125}I]ET-1 alone (total binding) and from consecutive sections incubated in the presence of excess, unlabelled ET-1 (control non-specific binding). Levels of non-specific binding were subtracted from all the readings to calculate the values for specific [^{125}I]ET-1 binding.

This procedure was performed on an average area of 1900μm^2 (mean of 5 areas) overlying specific structures in dermal sections: papillary and deep dermal vessels, epidermal/dermal border. Vessels in the papillary dermis and within 500μm of the epidermal/dermal border were considered papillary dermal vessels. The levels of [^{125}I]ET-1 binding are expressed as mean +/- sem "arbitrary units" (equivalent to exposure meter reading, in min). In the lung, a similar procedure was performed on an average area of 1900μm^2 (mean of 5 areas) overlying specific structures such as large pulmonary vessels (diameter greater than 20μm), alveolar epithelium, bronchioles and interstitium. The pulmonary interstitium was considered as one group as it was difficult to delineate the vascular, matrix and inflammatory cell components which are in

close proximity to each other. In the case of alveolar epithelium and pulmonary interstitium, these values were further divided by cell number to examine binding sites per cell.

2.3.2.c Competitive binding assays using ET receptor antagonists

The assessment of the levels of each of the different ET receptors in lung biopsies was examined by measuring the inhibition of [^{125}I]ET-1 binding to duplicate 10μm sections by co-incubation with 500nMol unlabelled ET-1, FR 139317 , an ETA selective ligand (2(R)-[2(R)-(2(s)-{[1-(hexahydro-1H-azepinyl)] carbonyl} amino -4-methylpentanoyl)-amino-3-[3-(1-methyl-1H-indolyl)]propionyl]-amino-3-(2-pyridyl)propionic acid, Fujisawa, Japan) , IRL 1620, an ETB selective ligand (Tyr 13-Suc-[Glu9, Ala11, 15]-ET-1(8-21), Du Pont, UK) and Ro 47-0203, a mixed ET receptor ligand (4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamine; Bosentan; generously donated by Dr M Clozel, Hoffman LaRoche, Basel, Switzerland) for 90 min at 22°C. These results were compared with total binding observed in duplicate 10μm sections which were incubated with 100pMol [^{125}I]ET-1 alone for 90 min at 22^{o}C. All the sections were then processed for high and low resolution autoradiography and quantitation of grain density. The effect of each of the ligands are expressed as percentage of total binding.

2.4 Measurement of circulating and tissue expression of E-selectin VCAM-1 and ICAM-1

2.4.1 Monoclonal antibodies

The monoclonal antibodies (mAbs) used to E-selectin were purified mAb 34.27.12 and biotinylated mAb 1.2B6. The mAbs to VCAM-1 were 1E5 and biotinylated 1.4C3 and the mAbs to ICAM-1 were 6.5B5 and 8.4A6 (biotinylated).

All of these mAbs were of the mouse IgG1 class and were generated by Dr D.O. Haskard (Hammersmith Hospital, UK) as previously described (Mason *et al.*, 1993; Wellicome *et al.*, 1993), except for the anti-E-selectin mAb 34.27.12 which was a kind gift from Dr Martyn Robinson (Celltech plc, Slough, UK). The mAbs were purified by affinity chromatography on Protein A Sepharose (Pharmacia, UK).

2.4.2 Enzyme linked immunosorbent assays (ELISAs) for measuring the circulating levels of E-selectin, VCAM-1 and ICAM-1

Two- epitope "sandwich" ELISAs were used to measure the above circulating adhesion molecules in patient and control sera and in known standard sera. This method has been described previously (Mason *et al.*, 1993). Plastic 96 well microtiter plates (Linbro, Flow Laboratories, UK) were precoated overnight with affinity purified monoclonal antibody (mAb) to each of the various adhesion molecules; E-selectin (mAb 34.27.12), VCAM-1 (mAb 1E5) and ICAM-1 (mAb 6.5B5), at 10μg/ml in carbonate buffer (pH9.6) at 37°C. The plates were then blocked with 5% casein /PBS (w/v) for 1 h at room temperature. After washing in 0.25% Tween 20 in PBS (v/v) (PBS-Tween), serum samples (diluted 1:4 with PBS) were added for 2 h at 26°C. After washing the plates in PBS-Tween, ligand capture (circulating adhesion molecules) was detected by the addition of a biotinylated antibody complementary to that of the solid phase (E-selectin; mAb 1.2B6 for detection, VCAM-1; mAb 1.4C3 and ICAM-1; mAb 8.4A6) for detection. Binding of this biotinylated antibody was detected by incubation with a high molecular weight complex of streptavidin-biotin-peroxidase (Dako, UK) followed by 0.5mg/ml o-phenylenediamine in 0.03% hydrogen peroxide in citrate phosphate buffer, pH 5.0. Colour development was stopped with 2Mol sulphuric acid, and the optical density at 491 nm was read with a Titertek ELISA plate reader (Flow Labs, UK).

For quantitation, 3 sera with high levels of circulating E-selectin, ICAM-1 and VCAM-1 were selected as standards and titrated on each assay plate. The results in this study are expressed as ng/ml, based on comparison with these standards. The levels of circulating adhesion molecules in each of the standards has been estimated using recombinant proteins. Regressional analysis of 130 serum samples in this assay indicated high interassay (95-97%) and intra-assay (90-93%) reproducibility for all three adhesion molecules.

2.4.3 In situ expression of E-selectin, VCAM-1 and ICAM-1

2.4.3.a Immunohistochemistry

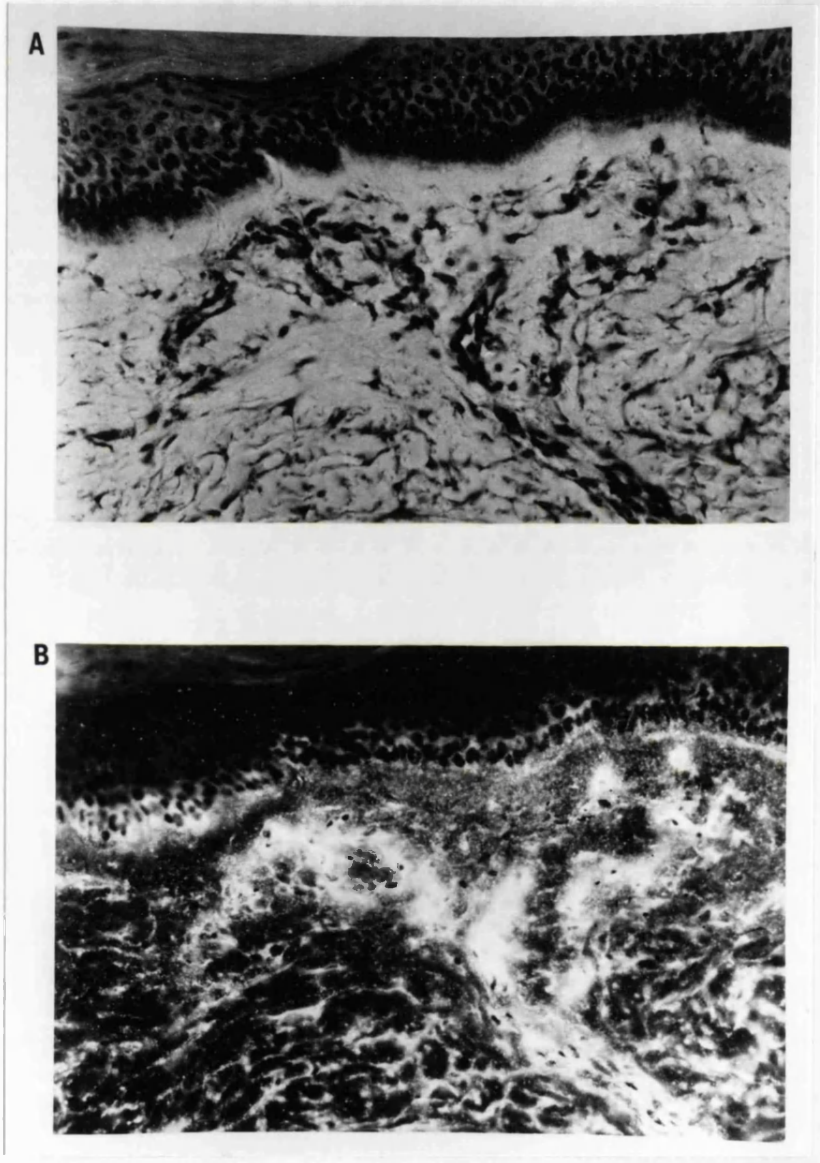
Dermal and lung sections mounted on slides pre-coated with aminopropyl-triethoxysilane were air dried for 60 min, fixed in chloroform : methanol (1:1) for 5 min at 4°C. Non-specific IgG binding was then blocked by incubating the fixed sections with 20% normal goat serum for 20 min at room temperature. All subsequent manipulations were carried out at room temperature. mAbs to E-selectin (1.2B6), ICAM-1 (6.5B5) and VCAM-1 (1E5) were added to replicate sections at a concentration of 0.5-1 μg/ml for 1 h. The concentrations of antibody required for staining were optimised by titration of antibodies on dermal sections from non-SSc inflammatory biopsies (data not shown). A mouse antibody to human endothelial cells (QBEND 10, Unipath Labs, UK) and the relevant isotype matched IgG (Sigma, UK) were also added (1μg/ml) to replicate sections and were used as positive and negative controls, respectively. All the sections were washed three times in TBS and the detection of antibody bound to the sections was performed using the APAAP staining procedure. The histological assessment of the distribution of staining was made in a blinded fashion by two independant histopathologists.

2.4.3.b Quantitation of expression of E-selectin, VCAM-1 and ICAM-1

Quantitation of the intensity of staining was performed by measuring the intensity of fluorescence of the Napthol AS-MX phosphate and Fast red TR combination (Ramshaw *et al.*, 1992; Speel *et al.*, 1992) with an Olympus Vanox AX/2 fluorescence microscope equipped with epi-illumination interfaced with a Peltier cooled CCD video camera with frame store and variable integration control (model VL 350, PCO Optics, Germany) using a KONTRON IPS image analysis system. The instrument settings were set at an excitation wavelength of 568nm and measurements were recorded using a 585nm long path emission filter to optimise fluorescent intensity measurements prior to all analysis. Background levels of fluorescence were then subtracted from the measured levels and are represented as mean fluorescence units (MFU) $\pm$ SEM. Figure 2.1 shows the co-localisation of immunohistochemical and fluorescent ICAM-1 expression in a dcSSc skin section.

The intensity of fluorescence was measured in dermal and pulmonary vessels delineated by the endothelial cell marker (anti-QBEND 10) on an average of 9 vessels per biopsy (at a distance of 100μm in 6 consecutive sections) in both the deep and papillary dermis, large pulmonary (greater than 20μm in diameter) and interstitial vessels. In pulmonary sections, epithelial cell expression ICAM-1 was also analysed by measuring the level of fluorescence associated with alveolar epithelia on an average of 10 alveolar airspaces per biopsy (100μm between each alveolar air space). These measurements were then divided by cell number and the results represent the average alveolar epithelial cell ICAM-1 expression $\pm$ SEM.

Figure 2.1: Co-localisation of immunohistochemical and immunofluorescent staining of Napthol AS-MX phosphate and Fast red TR.



Note A. positive immunolabelling for ICAM-1 in SSc skin section and B. fluorescence associated with immunolabel in same section. Scale bar = 200μm.

2.5 Phenotypic and functional characteristics of fibroblasts *in vitro*

2.5.1 Fibroblast culture

Dermal and lung biopsies were cut into 1-2 mm^3 pieces and cultured in Dulbecco's modification of Eagles Minimal Essential Medium (DMEM), supplemented with L-glutamine (2mMol), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FCS in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air. When the cells became confluent, they were detached using 0.25% trypsin in 20mM EDTA, diluted in half and recultured as described above. These cells were considered to be in passage 2 at confluence.

2.5.2 Measurement of fibroblast growth

Confluent fibroblasts at passage 2 were seeded at 4×10^3 cells/well into 96 well microtitre plates in complete DMEM and incubated at 37°C in an atmosphere of 5% CO_2 in air. At 24 h intervals, fibroblast proliferation was determined using a colorimetric assay based on the uptake of methylene blue which was equivalent to the number of cells (Oliver *et al.*, 1989). Results are expressed as the percentage increase in fibroblast number above the initial number of cells seeded per well.

2.5.3 Measurement of total cellular matrix expression

2.5.3.a Monoclonal antibodies

MAbs against two intracellular matrix antigens: fibronectin (all isoforms, mouse IgG1) and tenascin (clone BC-4, mouse IgG1), were generously donated by Dr L. Zardi, University of Genoa, Italy. Prolyl hydroxylase, an intracellular enzyme involved in collagen synthesis was obtained from Dako, UK (5B5, mouse IgG1).

2.5.3.b Total cellular matrix expression

Total cellular levels (intracellular and membrane bound) of fibronectin, tenascin and prolyl hydroxylase were examined by FCM analysis as described later after permeabilization of the cell membrane with the detergent 0.1% saponin in PBS, prior to the addition of the primary antibodies. This procedure has been previously described (Abraham *et al.*, 1988). The primary antibodies used in these experiments were all used at a concentrations of 0.5μg/ml, which were found to produce optimal levels of FCM staining.

2.5.4 Measurement of type I collagen secretion

The secretion of type I collagen by cultured fibroblasts was measured using an inhibition ELISA, as previously described (Rennard *et al.*, 1979). Briefly, DMEM supplemented with 5% FCS and 50μg/ml of L-ascorbate (Sigma, UK) was added to confluent fibroblast monolayers in 25cm^2 tissue culture dishes for 24 h. After this period, the medium was removed and replenished with DMEM and ascorbate for a further 24 h. Throughout these incubations, the cells were kept at 37°C. After this period, the medium were collected and frozen at -70°C until required, and the number of viable fibroblasts were measured after trypan blue staining and direct counting in a haemocytometer, after detachment of cells from the monolayer using 0.25% trypsin. Prior to the assay, plastic 96 well microtiter plates (Falcon, UK) were precoated overnight with 200ng/well of purified human collagen (Southern Biotechnology, UK) in 0.05Mol sodium bicarbonate buffer (pH9.6) at 4°C (Plate 1). Standard or serum were also pre-incubated overnight at 4°C in separate 96 well plates with 1.0 μg/ml goat anti-human type I collagen antisera (Southern Biotechnology, UK) (Plate 2). Plate 1 was then thoroughly washed and the contents of plate 2 were transferred to plate 1 and incubated for 1 h at 37°C. The amount of free antibody bound to the Plate 1 was quantified using a polyclonal anti-goat IgG conjugated to alkaline

phosphatase. The absorbance of the reaction on addition of p-phenol phosphate (Sigma 104) was measured at 410nm on a Titertek ELISA plate reader. Finally, the amount of collagen in the test samples was quantified based on the results obtained form standard curves. These results are expressed as ng/ml/million cells.

2.5.5 Monoclonal antibodies used for measurement of fibroblast surface antigens

The following monoclonal antibodies (mAbs) were used to measure fibroblast membrane antigens; (i) MHC I (clone PA2.6, mouse IgG2a, donated by J.Grundy, Royal Free Hospital), and MHC II (clone L234, mouse IgG2a, Becton Dickinson, UK). (ii) the adhesion molecules: ICAM-1 (CD54; clone 15.2, mouse IgG1), CD29, integrin β1 (clone B-D15, mouse IgG1,) and CDw49d, VLA 4 α-chain (clone HP2/L, mouse IgG1) all obtained from Serotec, UK and LFA-3 (CD58; clone TS 2/9, mouse IgG1, American Type Culture Collection, U.S.A.). (iii) the ectoenzymes: CD10, neutral endopeptidase (clone B-E3, mouse IgG2a), CD13, aminopeptidase N (clones B-F10, mouse IgG1) and CD26, dipeptidyl peptidase IV (clone BA5, mouse IgG2a), also purchased from Serotec, UK, and (iv) fibroblast surface antigens: 5A3 (a proteoglycan molecule, mouse IgG1), 3D3 (mouse IgG1), 3C4 (mouse IgG1) and 7D3 (mouse IgM), provided during a collaborative study with Professor JH Korn and generated as described below.

2.5.6 Preparation of novel monoclonal antibodies against fibroblast surface markers (performed by Professor J.H. Korn's group, Boston University School of Medicine, USA).

Hybridoma cells were obtained in a standard manner as described previously (Shulman *et al.*, 1978). Balb/c female mice were immunized with 10^6 early passage foreskin fibroblasts (line SB) and spleen cells fused with the Sp20/Ag14 myeloma cell line using polyethylene glycol. Resultant hybridomas

were initially screened for antibody against fibroblast membrane antigen by a cell-ELISA as previously described (Piela-Smith *et al.*, 1991). Determination of immunoglobulin type was performed by agar gel immunodiffusion (ICN Immunobiologicals, USA). Selected hybridomas were cloned by limiting dilution and propagated as ascites tumours. Two of these mAbs have been characterised and are described in chapters 6 and 7.

2.5.7 Immunostaining of fibroblasts for flow cytometry analysis (FCM)

The expression of cell surface molecules on fibroblasts was analysed and quantified using flow cytometry (FCM) as previously described (Abraham *et al.*, 1990). Cells were grown to confluence in replicate 25 cm^2 tissue culture flasks and detached from the monolayers using EDTA (20mMol) for 20 min at 4°C. They were centrifuged at 400xg for 10 min and the pellet resuspended repeatedly to produce a single cell suspension. Aliquots containing approximately $2x10^4$ cells were used to examine the level of expression of each of the surface antigens. The cells were washed in buffer containing 2% FCS in PBS, centrifuged as described above and the desired monoclonal antibodies were added at their optimal concentrations (given below) for 60 min at 20°C. 50µg/ml of secondary anti-mouse antibody conjugated to FITC (anti-mouse IgG for all the mAbs except 7D3 where anti-mouse IgM was used) was applied for 30 min at 20°C. The cells were then washed in 2% PBS/FCS and fixed in freshly-prepared 2% paraformaldehyde in PBS, for 10 min at 20°C. The cells were then centrifuged and resuspended in 100µl of 2% PBS/FCS and stored at 4°C. The fluorescence intensities of the stained cells was measured by FCM using a FACScan (Becton Dickinson, Twickenham, UK.), after the cells were gated on the basis of their size and granularity. Antigen expression is expressed as the average fluorescence intensity (AFI) of 5,000 individual cells. An isotype-matched irrelevant primary monoclonal antibody was used as a control for non-specific binding.

2.5.8 Optimal concentrations for the various mAbs used in this study

The optimal staining concentrations for all the mAbs used in this study were established by FCM after titration assays were performed on normal dermal fibroblasts. The concentrations that were used to achieve maximal staining for each of the mAbs are shown below:

Mabs	Concentrations used
MHC I (clone PA2.6)	0.5 μg/ml
MHC II (clone L234)	1.0 μg/ml
ICAM-1 (clone 15.2)	1.0 μg/ml
CD29 (clone B-D15)	0.5 μg/ml
CDw49d (clone HP2/L)	1.0 μg/ml
LFA-3 (clone TS 2/9)	2.0 μg/ml
CD10 (clone B-E3)	1.0 μg/ml
CD13 (clones B-F10)	1.0 μg/ml
CD26 (clone BA5)	1.0 μg/ml
5A3	1.0 μg/ml
3D3	0.2 μg/ml
3C4	2.0 μg/ml
7D3	2.0 μg/ml

2.5.9 Immunocytochemical staining for light microscopy

The expression and distribution of antigens which react with the mAbs 5A3, 3D3, 3C4 and 7D3 were examined in frozen sections of normal skin and lung biopsies using the APAAP procedure described above. 10μm sections mounted on glass slides coated with aminopropyl-triethoxysilane (Sigma, UK) were fixed in chloroform: acetone (1:1) at 4°C for 5 min. Non-specific IgG binding was blocked with 20% normal goat serum for 20 min and the mouse anti-human mAbs 5A3, 3D3,

3C4 and 7D3 were added to replicate sections at 0.5 - 1μg/ml, for 60 min. The following antibodies were also used to delineate dermal and lung structures. They included mouse monoclonal antibodies to (i) fibroblasts (5B5; prolyl hydroxylase,) (ii) macrophages (CD68) (iii) keratinocytes (anti-keratin), (v) smooth muscle (anti-actin), all purchased from Dako, UK and (vi) human endothelial cells (QBEND 10, Unipath Labs, UK) which were used at concentrations of 1μg/ml.

2.5.10 Measurement of interactions between fibroblasts and activated lymphocytes

2.5.10.a Cellular components:

Fibroblasts: Fibroblasts were plated at 10^4 cells per well of a flat bottomed 96 well plate (Falcon, UK) and cultured for 24 h in complete DMEM at 37°C in an atmosphere of 5% CO_2 in air.

Lymphocytes: Peripheral blood was obtained from normal individuals and the mononuclear cells separated on Ficoll (Histopaque 1077, Sigma, UK). The cells were washed with RPMI 1640 medium and adherent mononuclear cells, comprising mainly of monocytes and some B cells, were depleted from the cell suspensions by binding to plastic tissue culture dishes for 2 h at 37°C. Activated T lymphocytes were obtained by culturing the enriched suspension of T cells at a density of 1-2x10^6 cells/ml in RPMI 1640 medium (Gibco, UK) containing 100u/ml penicillin, 100μg/ml streptomycin, 100μg/ml glutamine, 10% heat inactivated FCS, 5x10^{-5}Mol 2-mercaptoethanol and 4μg/ml of concanavalin A for 5 to 7 days. The proportion of lymphoblasts accounted for 75-85% of the total activated cell population, as determined by microscopic examination while T-cells were greater than 90% when measured by FCM using the mAb anti-CD3 (Dako, UK).

2.5.10.b Lymphocyte adhesion assays

The adhesion of activated T lymphocytes to skin and lung fibroblasts was measured using a radioisotopic assay with [^{3}H] leucine-labelled lymphocytes, as described previously (Ager *et al.*, 1990). Briefly, activated T cells were radiolabelled for 60 min at 37°C in leucine-free MEM containing 5% dialysed FCS and 10 μCi/ml of [4,5-^{3}H] leucine (60 Ci/mmol, Amersham, UK). After extensive washing to remove excess [4,5-^{3}H] leucine, lymphocytes were resuspended in HEPES-buffered RPMI 1640 plus 1% heat inactivated FCS and $5x10^5$ cells were incubated on fibroblast monolayers in the 96 well plates for 60 min at 37°C (lymphocyte: fibroblast ratio of 50:1). The wells were then washed five times with prewarmed (37°C) Hanks buffered saline plus 1% FCS. The presence of an intact fibroblast monolayer with adherent lymphocytes was examined by phase contrast microscopy before the monolayers were solubilised in 25μl 90% formic acid. Samples were resuspended in 5mls of "Cytoscint", (LBK, UK), a scintillant fluid and counted on a Beckman β counter for 60 secs. The number of lymphocytes bound to the fibroblasts was determined by comparison with a standard curve of known numbers of labelled lymphocytes. The results are expressed as mean number of lymphocytes bound per fibroblast ± standard error.

2.5.10.c Inhibition of adhesion using mAbs

Monoclonal antibodies

Various mAbs were used to assess the role of specific adhesion molecules in lymphocyte interactions with fibroblasts. These included 4 antibodies to different epitopes of ICAM-1 including: (i) clone 15.2, (mouse IgG1, Biogen, UK) (ii) BBA4 (British Biotechnology, UK) and (iii) 6.5B5 and 8.4A6 (generously donated by Dr D.O.Haskard, Hammersmith Hospital, UK). Two antibodies were used against LFA-3; including clone TS 2/9 (mouse IgG1, American Type Culture Collection, U.S.A.) and BRIC 5 (Serotec, UK). MAbs to MHC I (clone

PA2.6, mouse IgG2a, described previously) and MHC II (Becton Dickinson, UK) and normal mouse IgG (Serotec, UK) were also used.

The fibroblast monolayers were first pre-treated for 60 min with different concentrations of each these antibodies before the addition of labelled lymphocytes and continuation of the binding assay described previously. These experiments were performed to establish the saturating concentrations of the various antibodies (data not shown). The effects of each of the four ICAM-1 antibodies and 2 LFA-3 antibodies known to react with different target antigen epitopes were also examined on 3 different fibroblast cultures in triplicate (2 normal lung fibroblast cultures and 1 normal skin fibroblast culture) in order to select the antibody which mediated optimal fibroblast-lymphocyte inhibition (data not shown).

Based on the results of the above experiments, saturating concentrations of the mAbs to ICAM-1(clone 15.2), LFA-3 (clone TS2/9), MHCI and MHC II and an isotype matched IgG were selected to examine the molecules involved in lymphocyte interactions with fibroblasts. These results are expressed as percentage inhibition of binding compared with the maximal binding observed in wells pre-incubated with the isotype matched IgG.

CHAPTER THREE: CIRCULATING LEVELS OF ENDOTHELIN-1 IN SSc

3.1 INTRODUCTION

Endothelin-1 (ET-1) is the major isoform of the endothelin family of peptides which includes ET-1, ET-2 and ET-3. ET-1 is implicated in the pathogenesis of SSc as it is a potent vasoconstrictor and has fibrogenic properties (Kahaleh 1991). ET-1 is considered to be involved in cutaneous vasoregulation on the grounds that it can induce profound vasoconstriction and skin pallor after intradermal injection (Brain *et al.*, 1989). ET-1 also is a potent mitogen for smooth muscle cells and fibroblasts with a notable effect on increasing collagen synthesis by fibroblasts (Komuro *et al.*, 1988; Kusuhara *et al.*, 1989; Kahaleh 1991).

ET-1 mRNA has been demonstrated in endothelial cells in the vasculature of the skin, heart, lung, pancreas, spleen, kidney, posterior pituitary and cerebral neurones (Haynes *et al.*, 1993). Other cells also known to synthesise ET-1 include macrophages, polymorphonuclear cells, alveolar and bronchial epithelial cells, mast cells, smooth muscle cells and keratinocytes (Ehrenreich *et al.*, 1990; Sessa *et al.*, 1991; Haynes *et al.*, 1993). ET-1 is generated from a 212 amino acid precursor form, preproendothelin-1 which then forms an intermediate compound, proendothelin-1, by the action of intracellular endoproteases (Haynes *et al.*, 1993). The cellular secretion of proendothelin-1 occurs before conversion to the mature peptide by an enzymic cleavage at the trp 21-val 22 site by "endothelin converting enzymes". The exact enzymes involved in this activity are still unknown but a recent study implicated neutral endopeptidase (NEP; EC 24.11; CD10) in porcine lung membranes (Abassi *et al.*, 1992; Gardiner *et al.*, 1992; Murphy *et al.*, 1993).

ET-1 levels are raised in response to a range of stimuli including hypoxia, sheer stress, cold temperatures, vasoactive hormones, procoagulant factors and cytokines such as TGFβ1 and IL-1 (Kurihara *et al.*, 1989; Kourembanas *et al.*, 1991; Haynes *et al.*, 1993). The induction of ET-1 by such varied stimuli may occur through several regulatory DNA sequences in the 5' promoter region of the gene for preproendothelin-1. These sequences also include binding sites for NF 1,

acute-phase regulatory elements and a sequence at position 109 to 102 that is identical to the well characterised AP-1 site which binds c-fos and c-jun (Inoue *et al.*, 1989a; Lee *et al.*, 1990; Lee *et al.*, 1991).

ET-1 is known to mediate varied biological effects through at least two different ET receptors corresponding to two different ETA and ETB receptor genes. The ET_A receptor has a higher affinity for ET-1 than ET-2 and ET-3, predominates on vascular smooth muscle and mediates vasoconstriction via phospholipase C and intracellular calcium ion mobilisation (Martin *et al.*, 1990; Lin *et al.*, 1991). In contrast, the ET_B subtype receptor is non-selective for the different ET isopeptides and when present on vascular endothelium mediates vasodilatation (Takayanagi *et al.*, 1991). Clozel *et al.*, 1994, have suggested that ETB receptors can be further divided into two subtypes: ETB1 and ETB2 on the basis that ETB receptors can mediate both vasodilatation and vasoconstriction. Lastly, the existence of a third ET receptor, ETC, which has a higher affinity for ET-3 compared with ET-1 has also been proposed, but not yet confirmed (Emori *et al.*, 1990).

Circulating levels of ET-1 have been reported to be elevated in a number of disorders, including inflammatory arthritides (Miyasaka N., *et al.*, 1992), bowel disease (Murch SH., *et al.*, 1992), primary PHT (Stewart DJ., *et al.*, 1991, Lippton HL., *et al.*, 1989), hypertensive renal failure (Schichiri M., *et al.*, 1990, Stockenhuber F., *et al.*, 1992), Raynaud's phenomenon (Zamorra *et al.*, 1990) and SSc (Kahaleh 1991; Yamane *et al.*, 1992). In order to determine the significance of ET-1 in SSc, circulating levels of ET-1 were measured in SSc patients with different subsets of disease (dcSSc and lcSSc) with and without lung disease (PHT and FASSc). The relationship of circulating levels of ET-1 with various clinical indices and previously documented parameters of vascular damage (vWF and ACE) (Pearson, 1993) was also assessed.

3.2 PATIENTS AND STATISTICS

3.2.1 Patients samples

Serum and plasma samples were taken from 111 patients with SSc (91 females, 20 males) including 64 patients with dcSSc and 47 with lcSSc, 17 patients with primary RP (12 females, 5 males) and 22 normal individuals (12 females, 10 males). Venesection and collection of blood samples was always performed approximately 4 hours after patients had been in the Rheumatology Out Patients Department. This time period was necessary to minimise any effects of ambient temperatures on circulating ET-1 levels. The SSc patients were further sub-divided into groups based on clinical and laboratory evidence of pulmonary vascular disease (PHT) or hypertensive renal disease (25, 36 and 12 patients, respectively).

3.2.2 Statistical analysis

The results in this study are expressed as the arithmetic mean of circulating ET-1 levels ± the standard error (SE). Comparisons between levels in the different patient groups was made by the Mann Whitney U test for non-parametric variables, in which p values less than 0.05 were considered significant. The relationship between clinical parameters and circulating ET-1 levels was assessed using linear regression analysis where p values less than 0.05 were considered significant.

3.3 RESULTS

3.3.1 Circulating ET-1 levels and SSc and RP

In order to examine the association of ET-1 with vascular disease and fibrosis, circulating levels of ET-1 were measured in patients with predominantly vascular disease (primary RP), limited fibrosis and no evidence of hypertension (lcSSc with no PHT or hypertensive renal disease) and widespread fibrosis with no evidence of hypertension (dcSSc with no PHT or hypertensive

renal disease). As shown in Table 3.1, primary RP patients had significantly higher levels of circulating ET-1 than normal healthy volunteers (7.6 $\pm$ 0.5 versus 5.8 $\pm$ 0.2, p=0.001). Patients with limited skin involvement and no PHT or hypertensive renal disease had the lowest circulating ET-1 levels in the study population (4.8 $\pm$ 0.4). Patients with widespread skin fibrosis (dcSSc) with no PHT or hypertensive renal disease also had significantly elevated levels of circulating ET-1 compared with controls (7.9 $\pm$ 0.5 versus 5.8 $\pm$ 0.2, p<0.01).

3.3.2 Circulating ET-1 Levels and organ involvement

To examine the association of circulating ET-1 levels with fibrosis, dcSSc patients were compared with lcSSc with similar organ involvement. DcSSc patients with widespread skin fibrosis and no clinical evidence of lung or kidney involvement had significantly higher levels of circulating ET-1 than similar lcSSc patients (7.3 pg/ml versus 5.0 pg/ml, p=0.009) (Table 3.1). A similar result was also observed when dcSSc and lcSSc patients with no PHT and hypertensive kidney disease were compared (7.9 pg/ml versus with 4.8 pg/ml, p<0.0001, respectively).

The presence of PHT and hypertensive kidney involvement in dcSSc patients was not associated with an increase in levels of circulating ET-1 (6.7 compared with 7.3 pg/ml). This was in contrast to patients with lcSSc, where the presence of PHT was associated with a marked increase in the level of circulating ET-1 relative to lcSSc patients with no evidence of lung and kidney involvement (6.7 versus 5.0 pg/ml, p=0.009).

3.3.3 Circulating ET-1 Levels and parameters of vascular damage

To examine the presence of vascular activation/injury in patients with RP and SSc, levels of ACE and vWF were measured. As shown in Table 3.2, the average serum ACE levels in units/l were significantly lower in the patients with

dcSSc, lcSSc and primary RP compared with normal individuals (51.7, 37.4 and 34.4 U/l versus 87.8 U/l, respectively). This was in contrast to the raised average plasma vWF levels seen in the dcSSc and lcSSc groups but not in RP patients compared with normal individuals. There was no significant difference between any of the disease groups for levels of ACE and vWF. Furthermore, although ACE levels did not relate with circulating ET-1 (fig 3.1A), the levels of vWF were found to correlate significantly with circulating ET-1 ($r=0.4$, $p=0.02$, fig 3.1B).

3.3.4 Circulating ET-1 Levels and clinical parameters

The association of circulating ET-1 levels with various clinical parameters was also examined. In SSc patients, circulating ET-1 levels were found to relate significantly with the extent of skin fibrosis (skin score), as shown in Figure 3.2A ($r=0.20$, $p=0.027$) but not with parameters of lung or kidney disease (D_LCO, Figure 3.2B, and creatinine clearance, Figure 3.2C). In addition, circulating ET-1 levels were found to have an inverse relationship with the duration of SSc and RP in months ($r=-0.2$, $p=0.008$ and $r=-0.2$, $p=0.007$, respectively) (Figures 3.3A and 3.3B). Other clinical parameters documented in this study for all patients including the presence and type of autoantibodies (ANAs, Scl-70 and anticentromere antibodies) and the use different clinical therapies (α-interferon, cyclosporine A, azathioprine, anti-thymocyte globulin) were not found to correlate with circulating ET-1 levels (data not shown). Lastly, smoking was not found to relate to circulating ET-1 levels in this study.

Table 3.1. Circulating ET-1 levels.

Patient group	n•	Mean circulating ET-1 levels (pg/ml)		
(i) Normal controls	22	5.8 ± 0.2		
(ii) Primary RP	17	**7.6 ± 0.5^{+}**		
SSc patients	**n•**	**dcSSc**	**n•**	**lcSSc**
(iii) Without lung or kidney disease	16	7.3 ± 0.4*	22	5.0 ± 0.5*
(iv) Without PHT or hypertensive renal disease	39	**7.9 ± 0.5^{+*}**	35	4.8 ± 0.4*
(v) PHT	15	6.7 ± 0.6	10	**6.7 ± 0.6‡**
(vi) Hypertensive renal disease	10	6.6 ± 0.6	2	7.0 ± 0.5
(vii) PHT and hypertensive renal disease	25	6.7 ± 0.6	12	6.8 ± 0.6

The results are shown as the arithmetic means of the circulating ET-1 concentrations in pg/ml ± the standard error (SE). n• represents the number of patients in each group.

$^{+}$p Values were <0.05 on comparison of the average patient circulating ET-1 levels in each disease subset with the circulating ET-1 level in normal individuals.

* p Values were <0.05 on comparison of the average circulating ET-1 levels between the dcSSc and lcSSc subsets.

There were only 2 patients in the lcSSc group with hypertensive renal disease, thus no statistical comparisons were made.

‡ patients with lcSSc and PHT had significantly higher circulating ET-1 levels (p values were <0.05) than the lcSSc patients without hypertensive organ disease.

Table 3.2 Serum ACE and plasma vWF Levels.

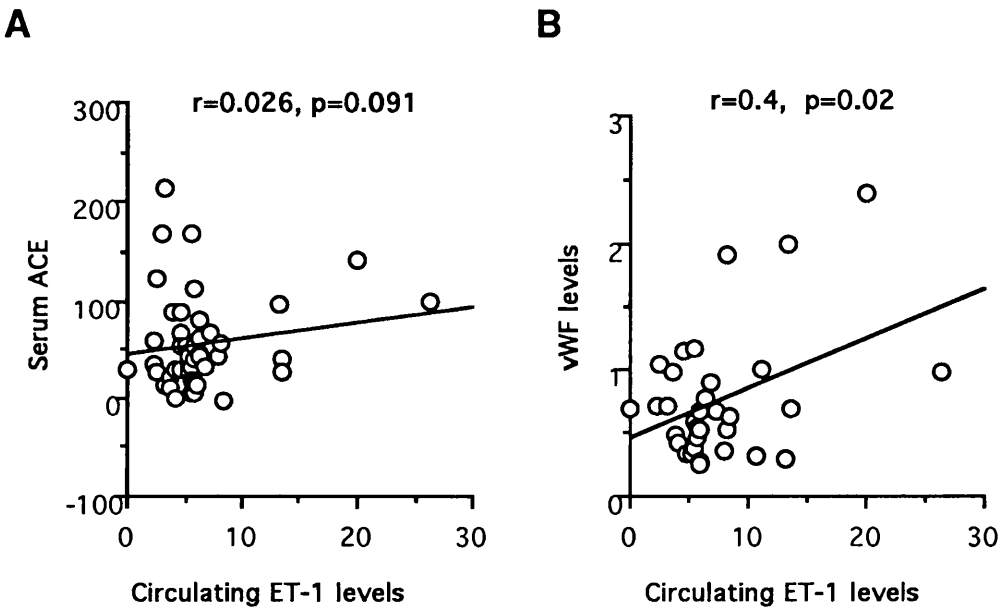
Disease group	n+	Mean ACE levels in U/l	n+	Mean plasma vWF levels in U/ml
Normal controls	13	87.8 ± 4.7	6	0.62 ± 0.16
Primary RP	5	**34.4‡** ± 4.5	5	0.53 ± 0.18
DcSSc	42	**51.7‡** ± 8.3	42	**0.75** ± 0.05
(i) Without lung or kidney disease	10	61.8 ± 6.2	11	0.81± 0.18
(ii) Without PHT or hypertensive renal disease	11	48.4‡ ± 5.0	11	0.61 ± 0.11
(iii) PHT	4	38.7‡ ± 3.7	4	0.53 ± 0.09
(iv) PHT and hypertensive renal disease	3	81.4 ± 5.8	4	1.01 ± 0.08
LcSSc	27	**37.4‡** ± 4.1	26	**0.94** ± 0.06
(i) Without lung or kidney disease	11	48.7‡ ± 4.5	8	0.74 ± 0.08
(ii) Without PHT or hypertensive renal disease	7	36.8‡ ± 3.4	10	0.86 ± 0.11
(iii) PHT	5	38.3‡ ± 3.1	5	1.02 ± 0.3
(iv)PHT and hypertensive renal disease	0	ND	0	ND

The mean levels of ACE* and vWF+ are shown for the various categories of patients.

n+ is the number of patients in each of the subgroups.

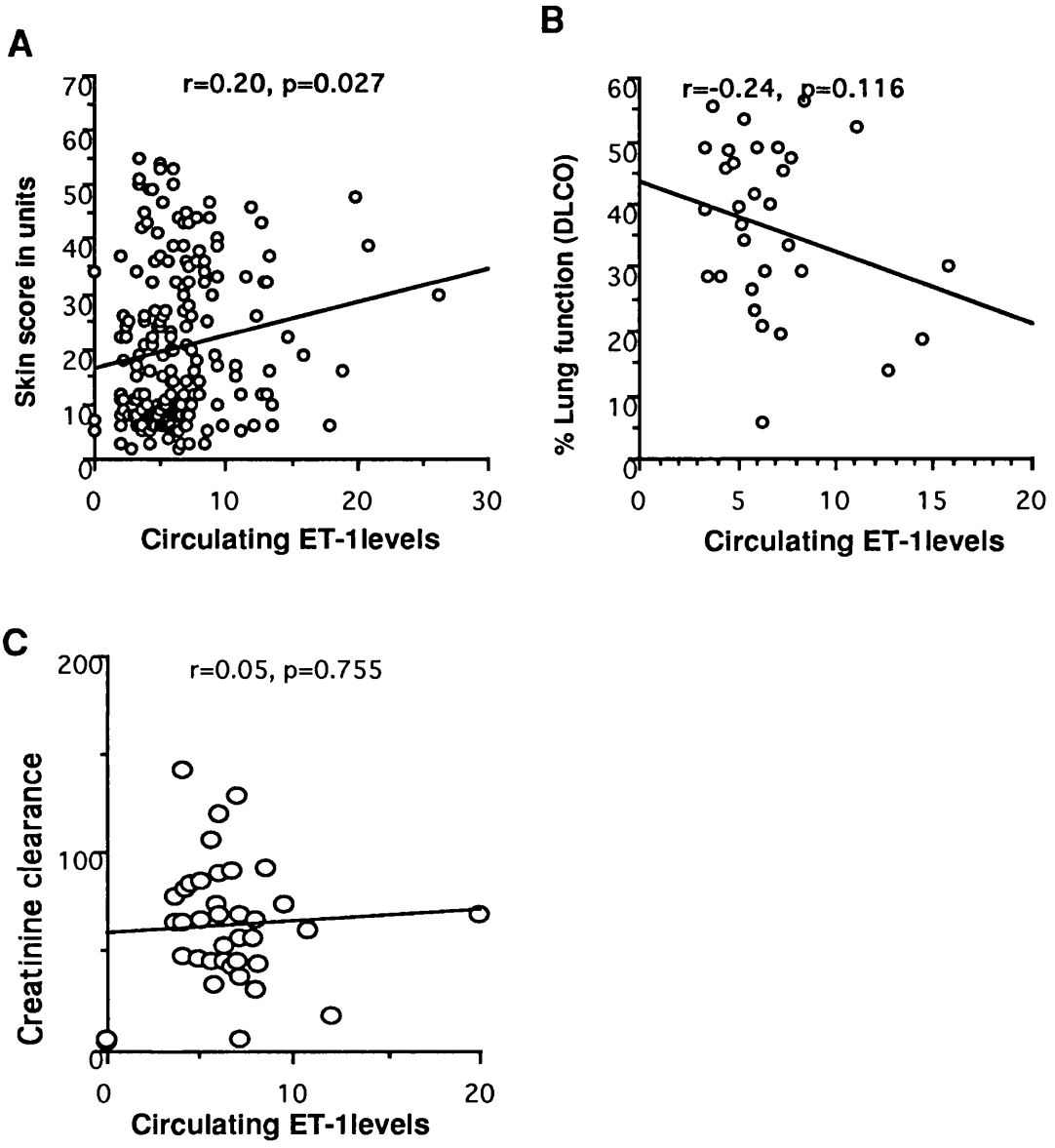
‡ p values were <0.05 on comparison of the average ACE and vWF levels in the dcSSc, lcSSc and RP patients compared with levels in normal controls. ND - not determined

Figure 3.1: Relationship between circulating ET-1 levels and other vascular parameters



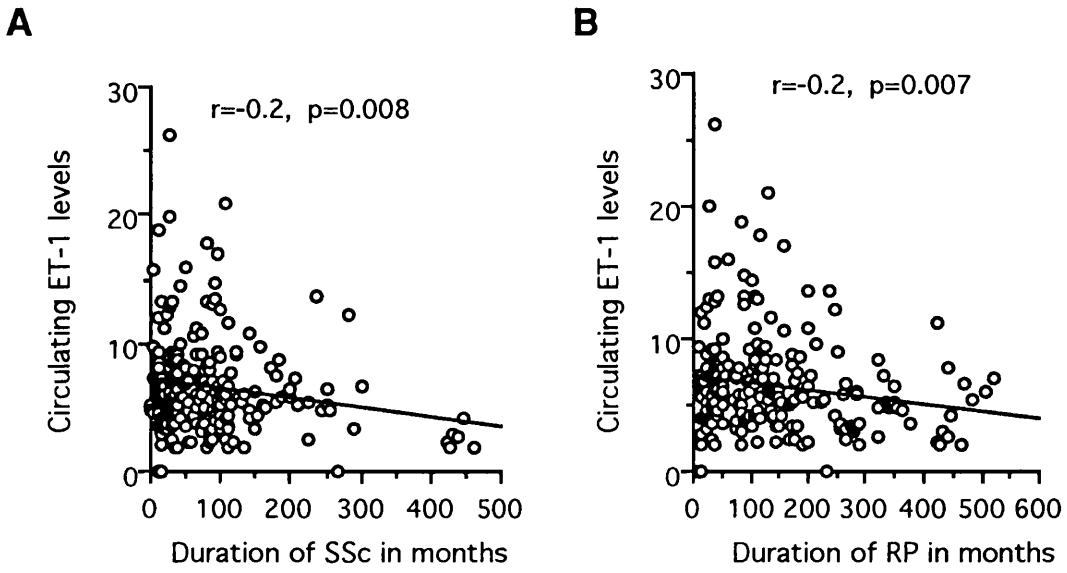
The graph represents the association of circulating ET-1 levels (pg/ml) with **A.** serum ACE levels in U/l **B.** plasma vWF levels in U/ml.

Figure 3.2: Relationship between circulating ET-1 levels and skin score, D_LCO and creatinine clearance



Relation between circulating ET-1 levels (pg/ml) and : **A.** skin score in 111 SSc patients as assessed using a modified skin score index ; **B.** carbon monoxide diffusion capacity (D_LCO) in SSc patients with lung involvement (PHT or lung fibrosis) (15 dcSSc, 10 lcSSc) and; **C.** creatinine clearance in SSc patients with hypertensive renal disease.

Figure 3.3: Relationship between circulating ET-1 levels and disease duration



A. Relationship between circulatingET-1 levels in pg/ml and the time duration in months from the date of diagnosis of SSc to the date of collection of the sample. **B.** Relationship between circulating ET-1 levels in pg/ml and the time duration in months from the date of onset of RP to the date of collection of the sample.

3.4 DISCUSSION

In vitro studies have shown that ET-1 is not only a vasoconstrictor (Brain *et al.*, 1988; Yanagisawa *et al.*, 1989), but also has fibrogenic effects (Kusuhara *et al.*, 1989; Takuwa *et al.*, 1989; Kahaleh 1991). It is therefore possible that this peptide could have an important role in vascular dysfunction or in the over-production of collagen seen in SSc (Kahaleh 1991). The results of this study revealed that raised levels of circulating ET-1 were present in patients with RP and SSc which concurred with previous reports (Zamorra *et al.*, 1990; Kahaleh 1991; Yamane *et al.*, 1992). However, circulating ET-1 levels did not distinguish RP patients from those with SSc.

Examination of circulating ET-1 levels in patients with SSc revealed differences between the levels of the peptide in patients with dcSSc compared with lcSSc. In dcSSc which is characterised by extensive fibrosis of the skin and the viscera, particularly the lung (LeRoy *et al.*, 1988; Steen *et al.*, 1985a), significantly high levels of circulating ET-1 were present compared with patients with limited skin fibrosis. Thus, circulating ET-1 appeared to be directly related to fibrosis which is supported by the relationship of circulating ET-1 levels to skin score observed in this study.

In addition, there also appeared to be an association between raised levels of circulating ET-1 and vascular disease, since circulating ET-1 levels were found to be elevated in patients with RP and in lcSSc patients with PHT. In dcSSc, the presence of PHT and hypertensive renal disease was not associated with a further increase in circulating ET-1 levels relative to dcSSc patients without hypertensive disease. However, the presence of PHT in lcSSc patients was associated with a significant elevation in circulating ET-1 levels relative to lcSSc patients with no pulmonary vascular disease suggesting that this peptide could provide a potentially useful, non-invasive early diagnostic tool for recognising PHT which should be investigated in further longitudinal studies. This observation is particularly significant as PHT is the major cause of mortality in

lcSSc patients, perhaps due to detection and treatment of this complication at a late stage of disease (Stupi *et al.*, 1986; Black *et al.*, 1994). Other markers of PHT such as reduced levels of D_LCO, arterial pressure measurement by right heart catheterisation or Doppler echocardiogram are not sensitive markers of asymptomatic PHT. Anti Scl-70 and anticentromere antibodies are also not useful in delineating patients at risk of development of isolated PHT in SSc.

Levels of ACE and vWF have been previously used as parameters of vascular activation/injury (Pearson 1993). The levels of ACE and vWF were similar in all the patient groups in this study. The presence of reduced levels of circulating ACE and raised levels of plasma vWF in SSc and reduced levels of ACE in RP are similar to earlier studies of these markers in both diseases (Gordon *et al.*, 1987; Woolf *et al.*, 1987; Matucci-Cerenic *et al.*, 1990a; Matucci-Cerenic *et al.*, 1990b; Blann *et al.*, 1993). The relationship observed between circulating ET-1 levels and vWF, but not circulating ET-1 and ACE further suggest that these vascular markers do not reflect the same biological processes. This lack of correlation between ACE and vWF has been reported by earlier investigators and may be because plasma vWF reflects varied biological processes such as endothelial injury and platelet activation while reduced levels of serum ACE indicates endothelial injury only (Matucci-Cerenic *et al.*, 1990b; Pearson 1993). In this study, the levels of these markers suggest that although endothelial cell damage in the two subsets of SSc is similar, circulating ET-1 is significantly different in dcSSc compared with lcSSc.

The significantly lower levels of circulating ET-1 in lcSSc compared with primary RP was interesting particularly in view of the presence of vascular disease in both conditions. This observation may be explained by an increased "mopping up" of the peptide in the skin in lcSSc compared with primary RP skin which concurs with results shown by Terenghi *et al.*, 1991 where ET-1 binding sites where significantly higher in SSc than primary RP skin.

SSc patients with extensive fibrosis and lcSSc patients with PHT both have elevated levels of circulating ET-1. The reason that similar levels of circulating

ET-1 have selective effects ie. fibrosis or vascular dysfunction in patients is not yet known. It is conceivable that particular responses may be mediated by an altered expression of each of the ET receptors. An increased level of ETA receptors (vasoconstrictor) or a decreased level of ETB (vasodilator) may result in vasoconstriction. Such a mechanism has been shown by Yorikane *et al.*, 1993, where PHT occurred in rats when increased levels of ET-1 were present with decreased ETB receptors. ET-1 is also known to stimulate fibroblast collagen synthesis equally through both ETA and ETB receptors but collagenase through ETA receptors only (Katwa *et al.*, 1993; Guarda *et al.*, 1993). Thus, it is conceivable that a decreased level of ETA receptors and an increased level of ETB receptors may be associated with a net increase in collagen accumulation with no vasoconstriction.

The pathophysiological function of increased levels of circulating ET-1 is not clear as the concentrations of the peptide seen in the circulation of patients are 10 times lower than those needed to induce biological effects *in vivo* in experimental animals and *in vitro* (Haynes *et al.*, 1993). Thus circulating ET-1 levels may reflect an increased local expression of the peptide in tissues and spillover into the circulation. Circulating autocrine hormones tend to be released luminally but the study by Yoshimoto *et al.*, 1991 who showed that cultured endothelial cells were found to release substantially more ET-1 to the basement membrane side, rather than luminally, provides support for a local rather than a circulating autocrine role for ET-1. ET-1 has also been observed in lymphatic fluid and BALF suggesting that these compartments also reflect local tissue production (Morel *et al.*, 1989; Cambrey *et al.*, 1993)

The data from this study provides evidence for the involvement of ET-1 in fibrosis in patients with dcSSc and vascular disease in patients with RP and lcSSc. It also suggests that further examination of the localisation and expression of ET-1 and ET-1 receptors in dermal and pulmonary biopsies may be helpful in delineating the precise role of ET-1 in the expression of disease in the skin and lung in SSc.

CHAPTER FOUR:

LOCALISATION OF ENDOTHELIN-1 AND ET-1 RECEPTORS IN SSc SKIN AND LUNG

4.1 INTRODUCTION

The previous chapter showed that circulating levels of ET-1 were related to the extent of fibrosis and vascular disease in SSc. In order to examine the localisation and level of expression of ET-1 in the two most affected organs in SSc, immunocytochemical studies were performed on biopsies taken from the skin and lung and compared with normal tissue. In addition, the localisation and level of expression of ET-1 receptors were examined in replicate sections from the same biopsies.

The studies performed on SSc skin were predominantly performed on biopsies taken from patients with dcSSc while those on SSc lung were carried out on biopsies obtained from patients with FASSc lesions. The expression of ET-1 and ET-1 receptors were not examined in PHT lesions as it is not current clinical practise to biopsy patients with PHT. Furthermore, the expression of the different ET receptors (both ETA and ETB) were also examined in FASSc biopsies and compared with normal healthy lung in order to investigate if an altered expression of either ET receptors is associated with disease.

4.2 PATIENTS AND STATISTICS

4.2.1 Patients

Dermal biopsies were taken from 9 patients with dcSSc (3 males, 6 females; aged 26-55 years, average age 43 years) and 5 normal volunteers (1 male, 4 females; aged 24-70 years, average 37 years). Paired skin biopsies were taken from clinically "uninvolved" and from the most recently involved SSc skin of patients to examine disease progression. Lung biopsies performed for clinical staging were taken from an affected area of the lung in seven patients with FASSc (all female, aged 37-56 years, average age 44 years) and compared with normal lung biopsies taken from the periphery of unaffected regions of

lobectomy or pneumonectomy samples from four age matched individuals undergoing thoracotomy for tumor resection (3 males, 1 female, average age 48 years).

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4.2.2 Statistical analysis

Comparisons between the levels of [^{125}I]ET-1 binding to specific structures in the skin and lung from patients and controls were made using the Student's unpaired t-test. P-values lesser than 0.05 are considered significant.

4.3 RESULTS

4.3.1 Skin

4.3.1.a Immunocytochemistry.

The localisation and level of ET-1 immunostaining was examined in biopsies taken from paired clinically "uninvolved" and involved skin obtained from dcSSc patients and from normal healthy volunteers. ET-1 staining was detected in association with the dermal vasculature identified by the endothelial cell marker - QBEND 10 which included the papillary (PV, see figs 4.1B and 4.1C) and deep dermal vessels (DV) as well as the microvessels around hair follicles, sweat glands and other dermal appendages (AV). This pattern of staining was not seen in normal control sections incubated with the anti-ET-1 antibody (Fig 4.1A). The ET-1 staining was localised as a diffuse band around both the endothelium and smooth muscle layer of the papillary microvessels in the immunoreactive sections (Fig 4.1B).

An increased level of ET-1 staining was noted in association with papillary vessels (PV) in 6/8 clinically uninvolved skin biopsies and in 5/8 involved skin biopsies from the dcSSc patients (Table 4.1) in contrast to the barely detectable

level of staining noted in papillary dermal vessels in 2/5 biopsies and in deep vessels in 1/5 biopsies obtained from normal individuals (Table 4.1). An increased level of ET-1 staining was observed in all but one of the sections obtained from early active SSc lesions (involved skin biopsies), either in association with papillary or deep dermal vessels. Histological examination of the skin biopsy with barely detectable ET-1 staining showed features of markedly increased collagen deposition and the loss of most of the normal dermal structures such as vessels, sweat glands, hair follicles and a flattening of the epidermis. (Fig 4.1D). These findings of increased collagen deposition and a relatively "featureless dermis" were suggestive of established late stage fibrotic lesions (Fleishmajer *et al.*, 1977a).

The ET-1 staining pattern seen in SSc sections was not seen in comparable sections incubated with normal rabbit anti-human IgG sera (Figs 4.1E and 4.1F). The vascular structures associated with skin appendages such as hair follicles, sweat glands and adipose tissue also exhibited an increase in ET-1 staining in the sections from the dcSSc biopsies but not in those from normal individuals (Table 4.1). As shown in Table 4.1, in a number of the SSc biopsies (see Fig 4.1 G) but not in controls (not shown), ET-1 staining was also localised to matrix cells that were probably fibroblasts or dermal macrophages. There was no ET-1 staining associated with the epidermal keratinocyte layer (KC) in both the SSc and control biopsies (Table 4.1).

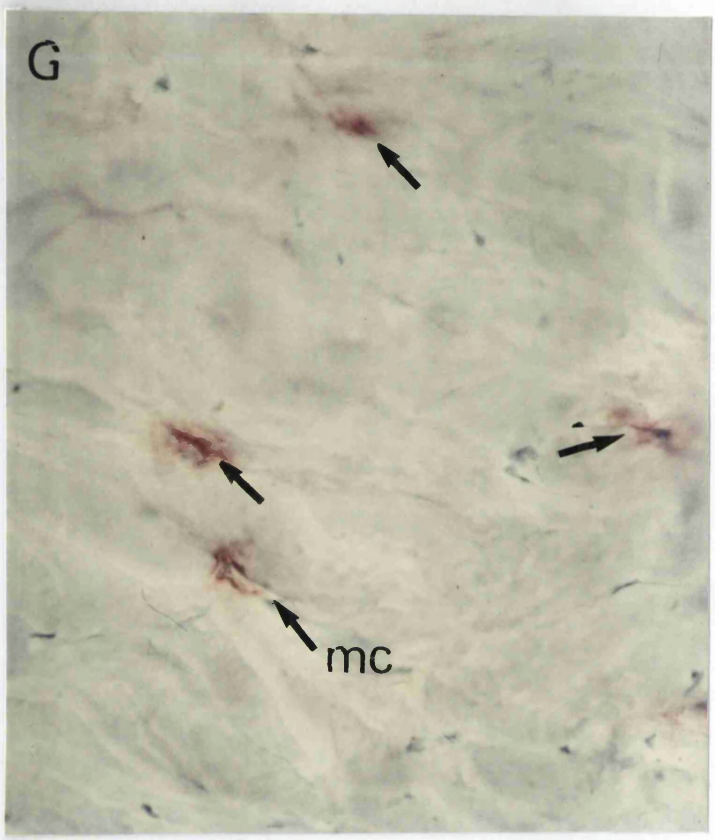
Table 4.1. Endothelin-1 (ET-1) staining in sections of SSc compared with normal skin biopsies

SSc patients	Clinically "uninvolved" skin					Clinically involved skin				
	KC	PV	DV	AV	MC	KC	PV	DV	AV	MC
1. SS	-	++	++	+	-	±	+	+	±	+
2. AJ	ND	+	+	++	++	-	++	++	++	++
3. HDC	-	++	+	±	+	±	++	++	+	-
4. CH	-	-	+	+	+	-	-	-	-	-
5. IC	-	-	±	±	-	-	+	+	-	-
6. AV	±	++	+	±	-	±	++	-	±	-
7. WOB	-	+	±	±	±	-	+	-	+	+
8. MM	-	ND	±	±	+	+	+	+	±	-
9.AS	-	++	±	+	-	-	-	-	++	-
Controls	KC	PV	DV	AV	MC					
1. KH	-	±	±	-	-					
2. CS	-	-	-	-	-					
3. BL	-	±	-	-	-					
4. CG	-	-	-	-	-					
5. SR	-	-	-	-	-					

KC-keratinocytes, PV- papillary vessels, DV-deep dermal vessels, AV- microvasculature associated with dermal appendages such as hair follicles, sweat glands, MC-matrix cell (fibroblasts or macrophages)

-= no staining, ±= minimal staining, +=moderate staining, ++= marked staining.

ND- not determined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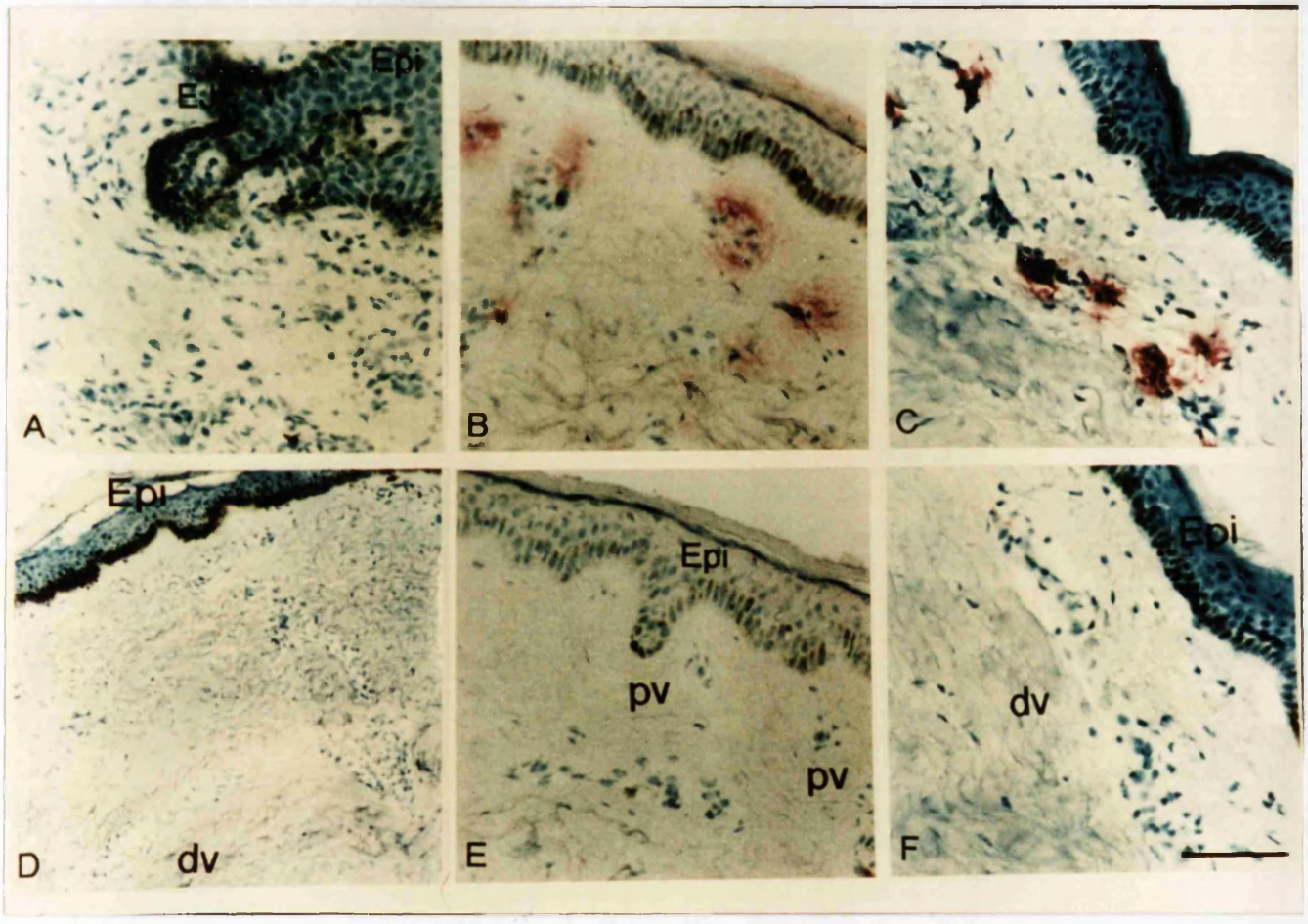
mc

Figure 4.1: ET-1 staining in sections from normal individuals and SSc patients



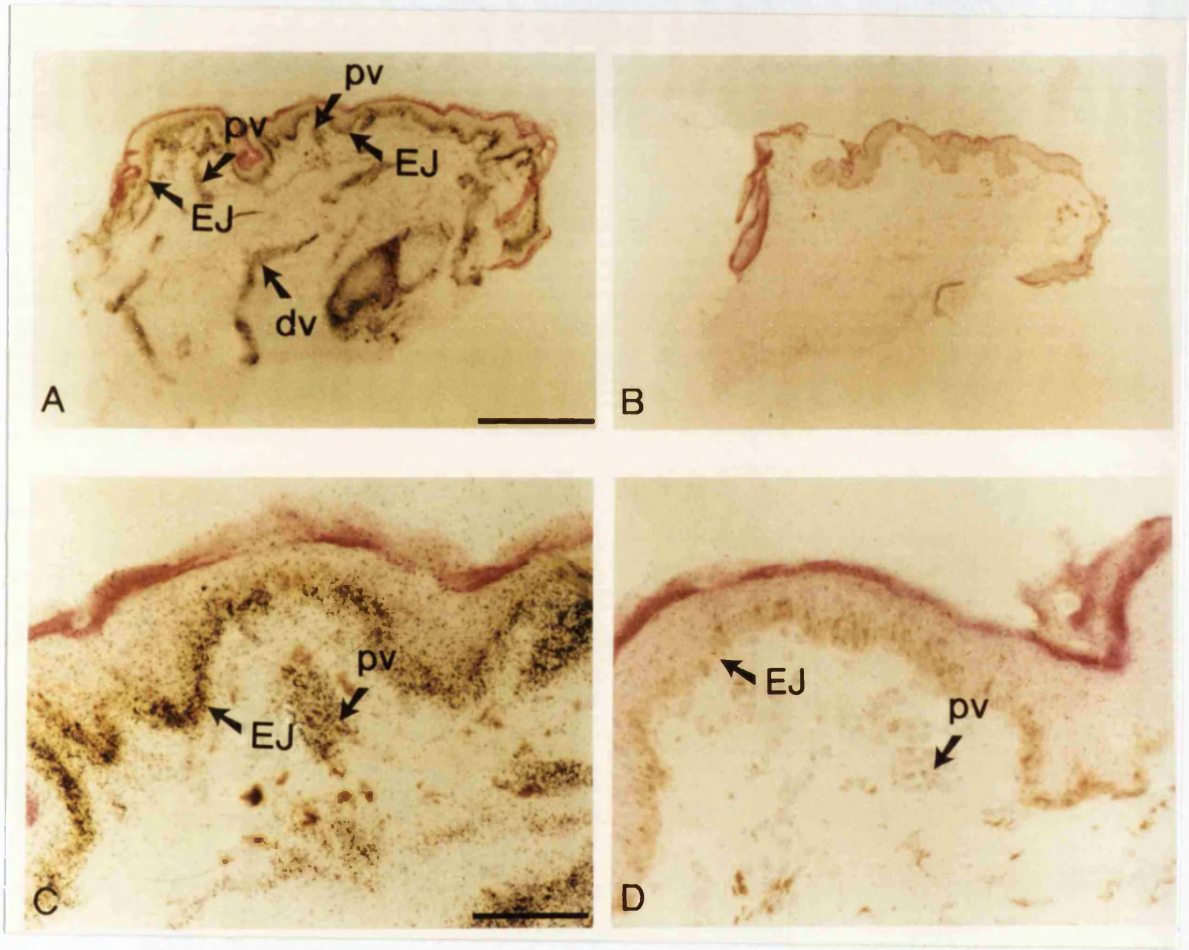
A. Cryostat section from normal control biopsy. Note the lack of staining to the dermal vasculature and epidermis. B. Positive ET-1 staining of papillary vessels (PV) in clinically uninvolved skin section from an SSc patient. C. Marked ET-1 staining of papillary vessels in early involved SSc skin section. D. Negative ET-1 staining in late stage fibrotic lesion. Note marked collagen deposition in the dermis. E and F. Replicate negative control sections to B and C, respectively, incubated with normal rabbit anti-human IgG . Scale bar = 200μm except for fig D where bar is 400μ. G. Positive ET-1 staining of matrix cells (MC) in a representative SSc biopsy on opposite page.

4.3.1.b Autoradiography.

To evaluate the localisation of ET-1 receptors in normal and dcSSc skin, consecutive sections were incubated with [^{125}I] ET-1 and processed for high resolution autoradiography. Extensive [^{125}I]ET-1 binding was observed in the control (Fig 4.2A), SSc clinically "uninvolved" and involved skin sections (Figs 4.3A and 4.4A, respectively) which was markedly reduced, by approximately 80%, in the presence of excess unlabelled ET-1 (Figs 4.2B, 4.3B and 4.4B), indicating that these sites were "specific" ET-1 binding sites (receptors). ET-1 receptors were particularly evident on papillary and deep dermal vessels in both normal and SSc skin (Figs 4.2, 4.3, 4.4). ET-1 receptors were also associated with the vascular supply of hair follicles and sweat glands, erector pili muscles and the dermal/epidermal junction. ET-1 receptors were not detectable in matrix cells.

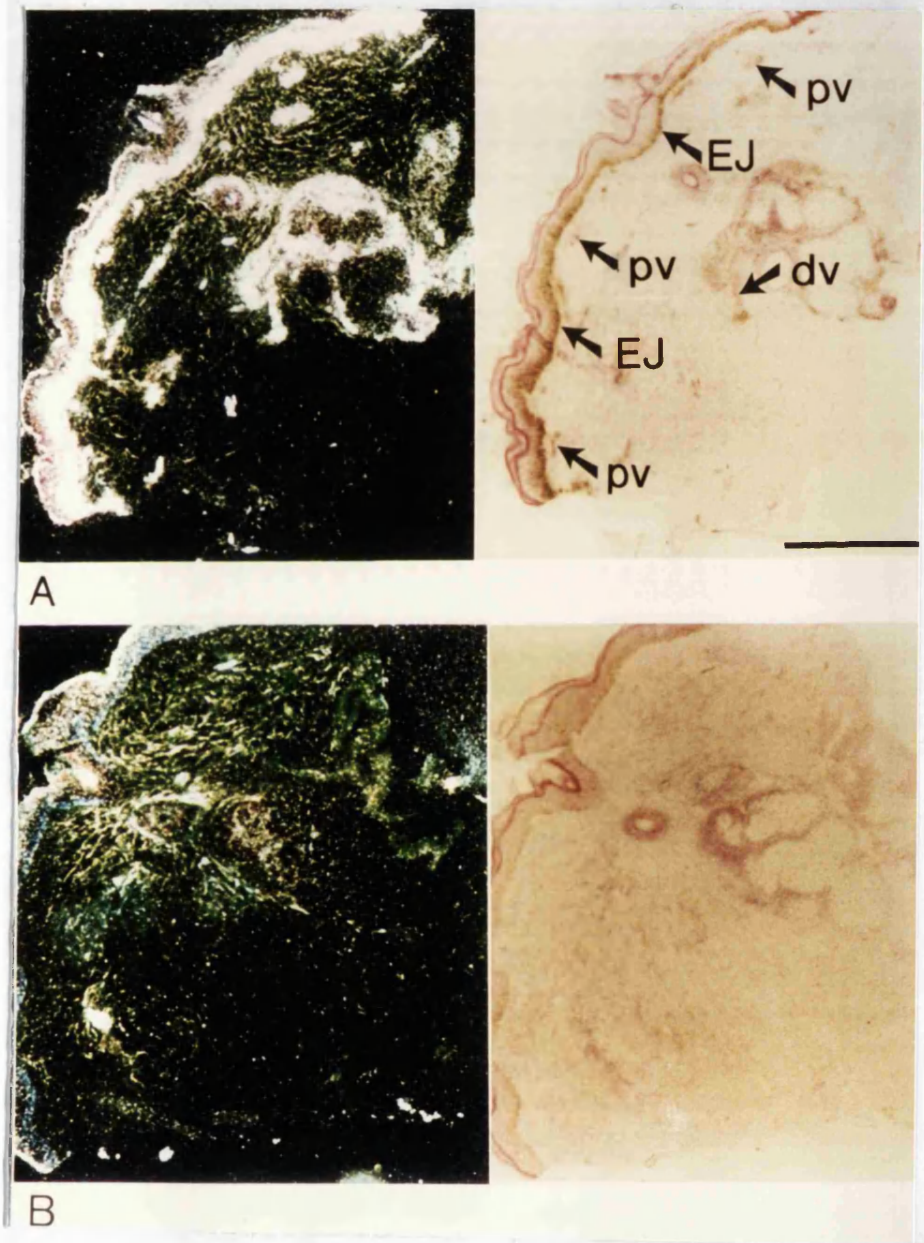
The quantitative assessment of autoradiographs indicated that there was a very significant increase in ET-1 receptors in papillary microvessels in both the clinically "uninvolved" and involved tissue (0.60 $\pm$ 0.03 units; $p<0.0005$ and 0.40 $\pm$ 0.01 units; $p<0.0008$ respectively) compared with similar normal vessels (0.30 $\pm$ 0.02 units). Additionally, there was a significant decrease in ET-1 receptors with progression of disease (Fig 4.5) i.e. from clinically "uninvolved" to involved skin. This increase in ET-1 receptors associated with papillary vessels in disease compared to normals was not seen on examination of the deep dermal vessels (0.20 $\pm$ 0.01 and 0.20 $\pm$ 0.04) in the "uninvolved" and involved skin, respectively, versus 0.20 $\pm$ 0.022 in similar control vessels. Interestingly, the papillary dermal vessels in SSc skin, displayed an increase in ET-1 staining (Fig 4.1B and 4.1C), implying that there was an increase in both ET-1 and ET-1 receptors in these vessels. There was also an increase in ET-1 receptors associated with the dermal/ epidermal border (basal keratinocyte layer) in the clinically "uninvolved" and involved SSc skin (0.50 $\pm$ 0.021, units; $p<0.0001$ and 0.3 $\pm$ 0.04 units; $p<0.001$, respectively) compared with normal skin (0.20 $\pm$ 0.008 units).

Figure 4.2: ET-1 receptors in a normal skin biopsy



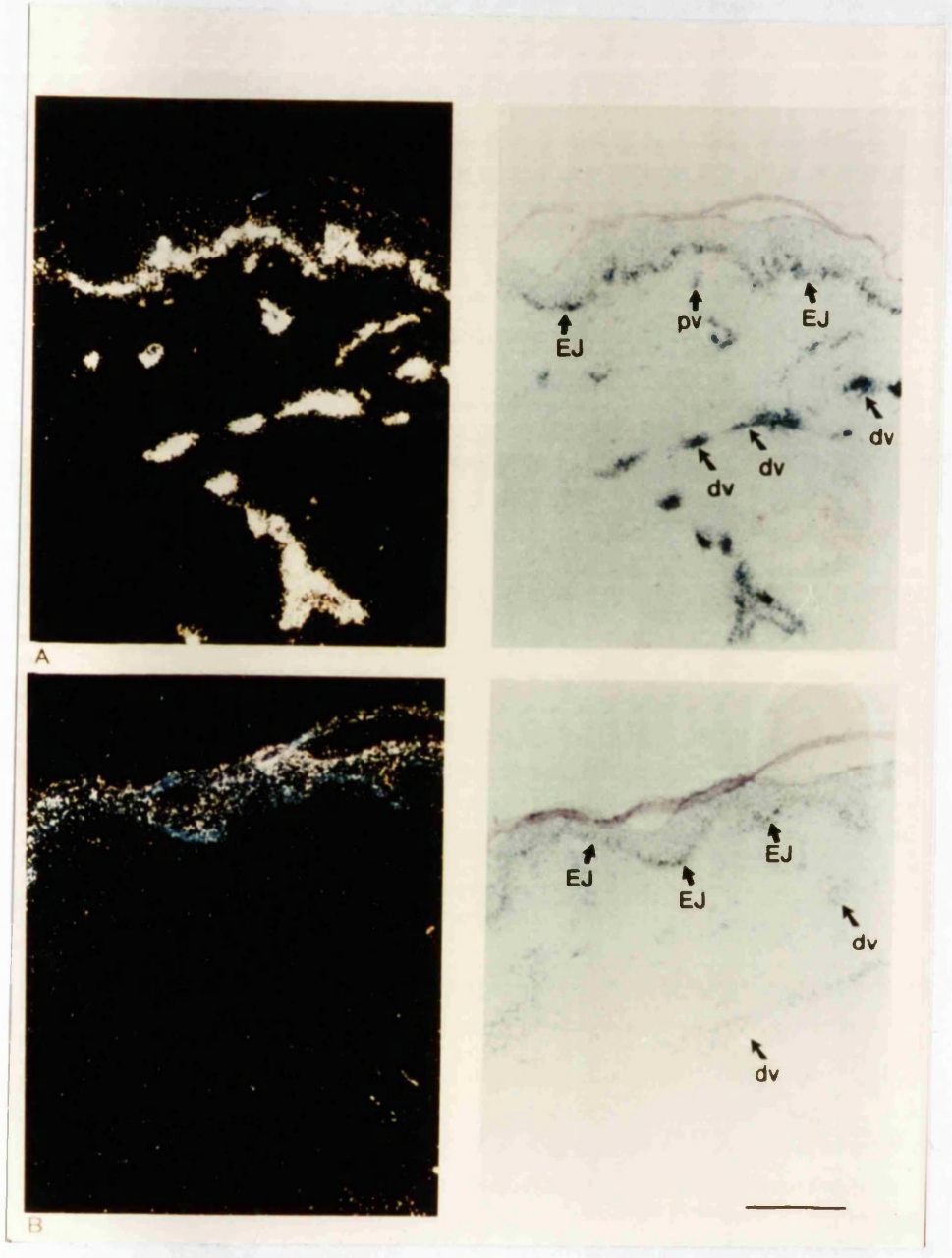
A, High resolution autoradiograph from slide-mounted normal skin section incubated in [^{125}I]ET-1 alone (total binding) and B, in the presence of excess unlabelled ET-1 (non specific binding). Note extensive grains associated with the papillary (pv) and deep dermal vessels (dv), the dermal/epidermal border (EJ), and to vascular layer around sweat gland. Scale bar in A and B = 500µm. C and D. High power photomicrographs of the same sections shown in A and B . Note grains associated with the dermal/epidermal border (EJ) and papillary vessel (PV) Scale bar = 100µm

Figure 4.3: ET-1 receptors in a clinically "uninvolved" SSc skin biopsy



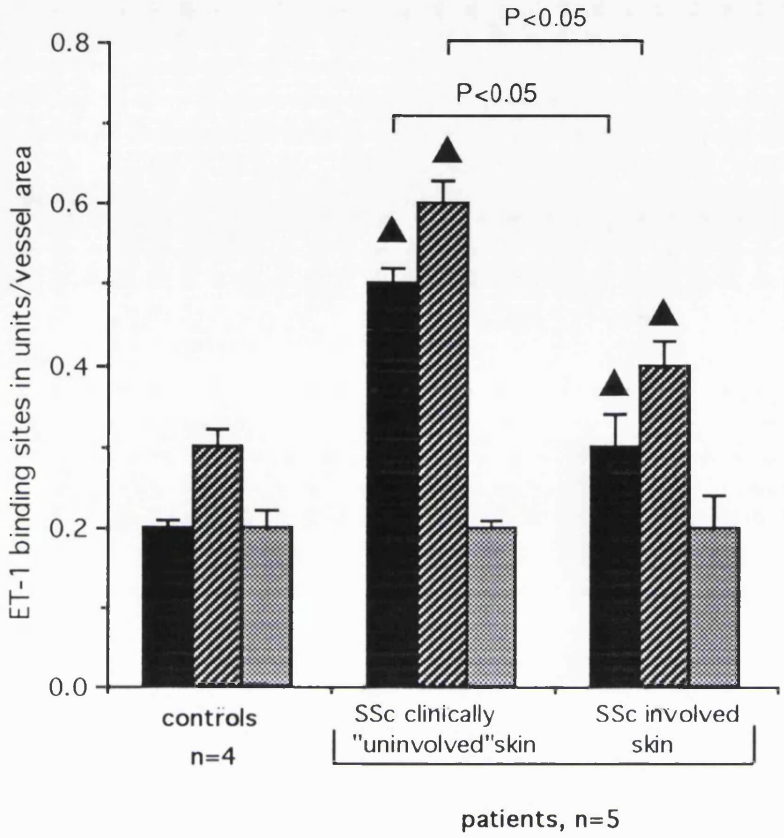
Dark-field illumination autoradiographs (left) of sections incubated in **A.** 150 pMol [^{125}I]ET-1 alone (total binding) and **B.** in the presence of 500 nMol unlabelled ET-1 (non-specific binding) and replicate sections (right) corresponding to A and B stained with haematoxylin and eosin. Note accumulation of white grains in A, to the dermal/epidermal border (EJ), papillary (pv) and deep dermal (dv) identified in replicate sections (right). Scale bar is 200 μm.

Figure 4.4: **ET-1 receptors in an involved SSc skin biopsy**



Dark-field illumination autoradiographs (left) of sections incubated in **A.** 150 pMol [^{125}I]ET-1 alone (total binding) and **B.** in the presence of 500 nMol unlabelled ET-1 (non-specific binding) and replicate sections (right) corresponding to A and B stained with haematoxylin and eosin. Note accumulation of white grains in A, to the dermal/epidermal border (EJ), papillary (pv) and deep dermal (dv) identified in replicate sections (right). Scale bar=200 μm.

Figure 4.5: Quantitative measurement of ET-1 receptors in normal and SSc skin



Quantitative measurement of ET-1 receptors (average units per 1900μm^2 area) associated with the dermal/epidermal border, papillary and deep dermal vessels in paired clinically uninvolved and involved skin of 5 SSc patients and 4 normal individuals (controls). The average number of receptors per unit area associated with the dermal/epidermal border (black) , papillary vessels (hatched bars) and deep dermal vessels (shaded bars) are shown. The vertical lines indicate the standard error of the mean.

Statistically significant results observed on comparison of normal control values with similar SSc dermal structures are shown by triangles and significant differences between SSc clinically uninvolved and involved data are shown by $p<0.05$.

4.3.2 LUNG

4.3.2.a Histology

In marked contrast to the normal histology of healthy lung biopsies (see fig 4.6A), all the FASSc biopsies had moderate to severe interstitial inflammatory infiltrates, lymphoid follicles, interstitial fibrosis with alveolar loss (fig 4.6B to 4.6D). Furthermore, a type II epithelial cell hyperplasia was evident lining the alveoli in all the FASSc biopsies: these cells expressed the MHC class II molecule HLA-DR, but were negative for the macrophage marker CD68 (as shown in fig 4.7A and 4.7B, respectively). The alveoli in normal lung biopsies were predominantly lined by type I epithelial cells which did not express HLA DR (data not shown). Very few type I epithelial cells (HLA DR negative) were seen lining the alveoli of FASSc biopsies (see fig 4.7).

4.3.2.b Immunocytochemistry.

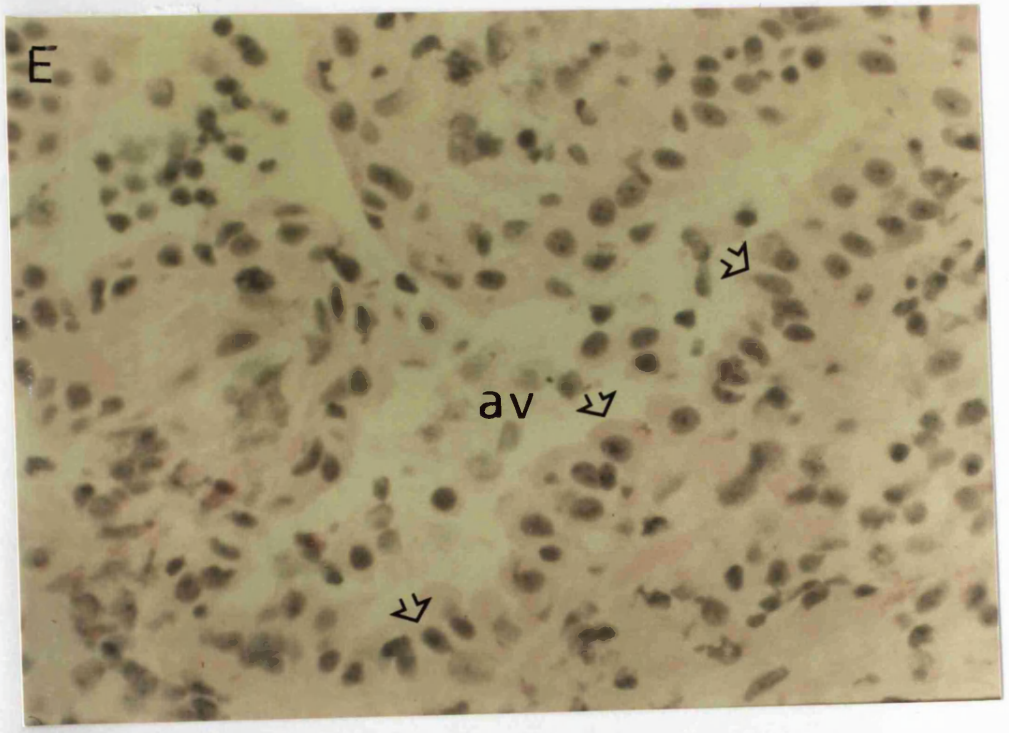
The localisation and level of ET-1 immunostaining was examined in normal and FASSc biopsies. The various pulmonary structures were identified in both biopsies using a number of markers. Bronchioles were delineated using antibodies against actin which stained smooth muscle and differentiated from large pulmonary vessels by the absence of staining with anti-QBEND 10, which is an endothelial cell marker. Pulmonary vessels were demarcated by staining with the mAb QBEND 10 and were further differentiated into large vessels (diameter of greater than 20μm) and small interstitial vessels. Furthermore, as it was difficult to clearly delineate small interstitial microvessels, fibroblasts and macrophages from each other, these cell types have been grouped into the "pulmonary interstitium".

Table 4.2 shows that increased levels of ET-1 staining were detected in association with a widespread number of pulmonary structures including the alveolar and bronchiolar epithelium (figs 4.8B and 4.8D), alveolar macrophages (not shown), the perivascular region in large pulmonary vessels (fig 4.9B) and the

interstitium in all 7 of the FASSc biopsies compared with barely detectable level of staining in 4 normal healthy lung biopsies (Table 4.2). ET-1 staining of the large pulmonary vessels was observed in the perivascular region in 3/7 FASSc biopsies but was not seen in association with vascular smooth muscle in any of the FASSc biopsies (fig 4.9B). Lymphoid follicles were not associated with ET-1 staining. The ET-1 staining pattern seen in sections from FASSc was not observed in comparable sections incubated with normal rabbit IgG (fig 4.8A and C)

Figure 4.6: Histological appearance of open lung biopsy specimens taken from normal controls and FASSc lesions

Haematoxylin and eosin stained normal lung (A) and FASSc cryostat sections (B-D). A. Note the presence of alveolar type I cells (small arrows) and normal alveolar architecture (av) in normal lung biopsy. B. shows marked alveolar loss, alveolar epithelial type II cells (open arrows) lymphoid follicles (LF), thickened blood vessels (V) and marked inflammation suggesting end stage disease in FASSc biopsy. C,D. Histology of 2 other FASSc biopsies showing inflammation, thickened blood vessels (V) and marked interstitial fibrosis in FASSc biopsies with loss of alveolar architecture. (Magnification X100). E. High power field of representative FASSc biopsy showing alveolus (av) lined by alveolar epithelial type II cells (open arrows) with characteristic cuboidal morphology.



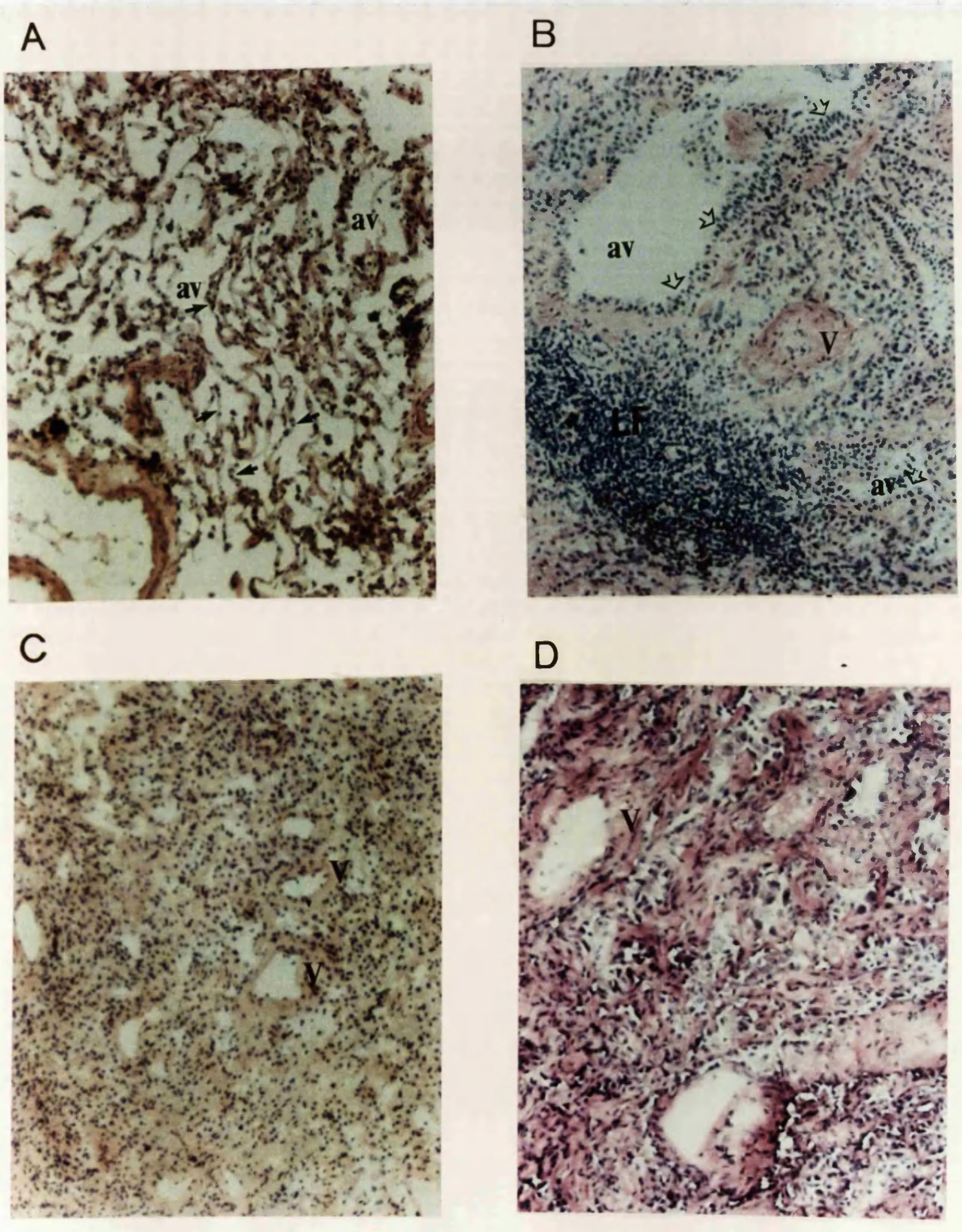

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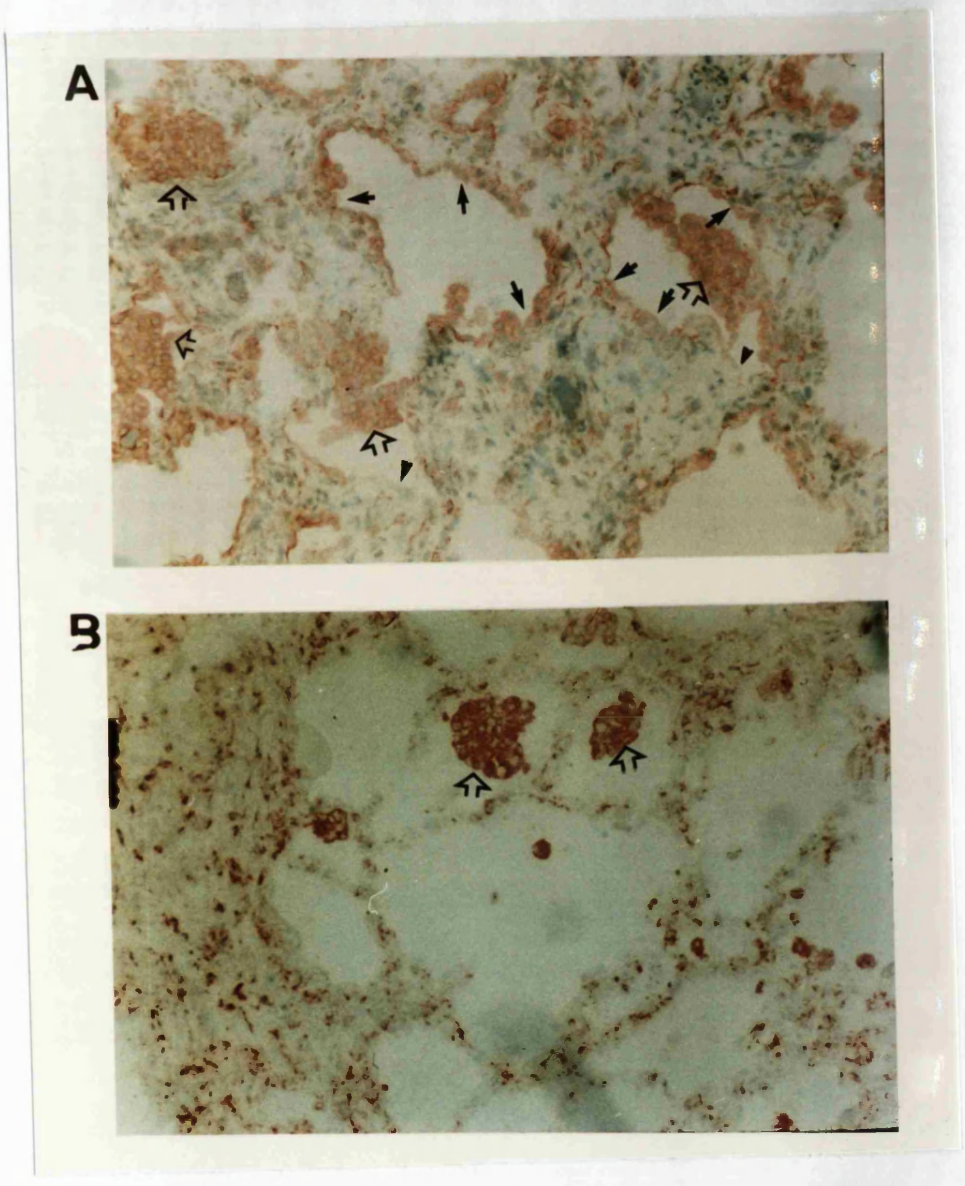
Figure 4.7: HLA DR expression in FASSc biopsies



A. Cryostat sections from a lung biopsy taken from a patient with FASSc from an area with CT evidence of disease (x 100 Magnification). Sections have been immunostained for (A) MHC Class II (HLA DR) expression and B. CD68 expression. Note the intense MHC Class II immunoreactivity of cells lining the alveoli (small arrows) but negative CD68 staining of alveolar epithelial cells delineating alveolar epithelial type II cells. Macrophages (large open arrows) expressed both MHC Class II and CD68. Very few HLA-DR negative cells (arrow head) were seen in the alveolar epithelial lining in A indicating replacement of normal alveolar epithelial type I cells by type II cells.

Table 4.2: ET-1 immunostaining in SSc lung and control biopsies

SSc patients	Alveolar epithelium	Bronchiolar epithelium	Alveolar macrophage	Large vessels (greater than 20μm in diameter)		Interstitium*
				Perivascular regions	Endothelium	
MB	+	+	++	±	±	+
KE	+	+	++	±	+	+
CL	±	+	+	-	-	+
NI	+	±	++	+	-	++
BA	+	+	+	+	±	+
WA	+	+	++	+	+	++
RL	+	+	+	±	-	++
Controls						
ST	-	-	-	-	-	±
BR	±	ND	-	-	±	-
BU	±	-	±	-	±	±
PL	±	-	-	-	-	±

* pulmonary interstitium comprised of small interstitial vessels, matrix cells and some immune cells.

ND = not determined. ET-1 immunoreactivity in biopsies was graded as follows: - = no staining, ± = minimal staining, + =moderate staining, ++ = marked staining.

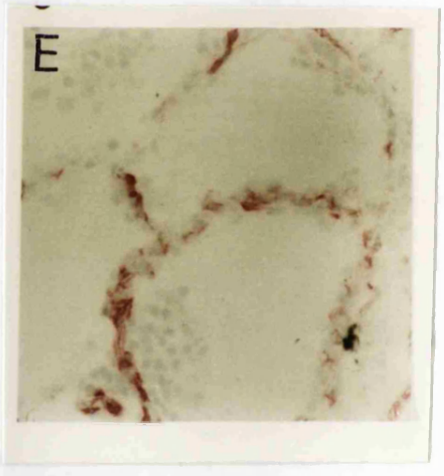

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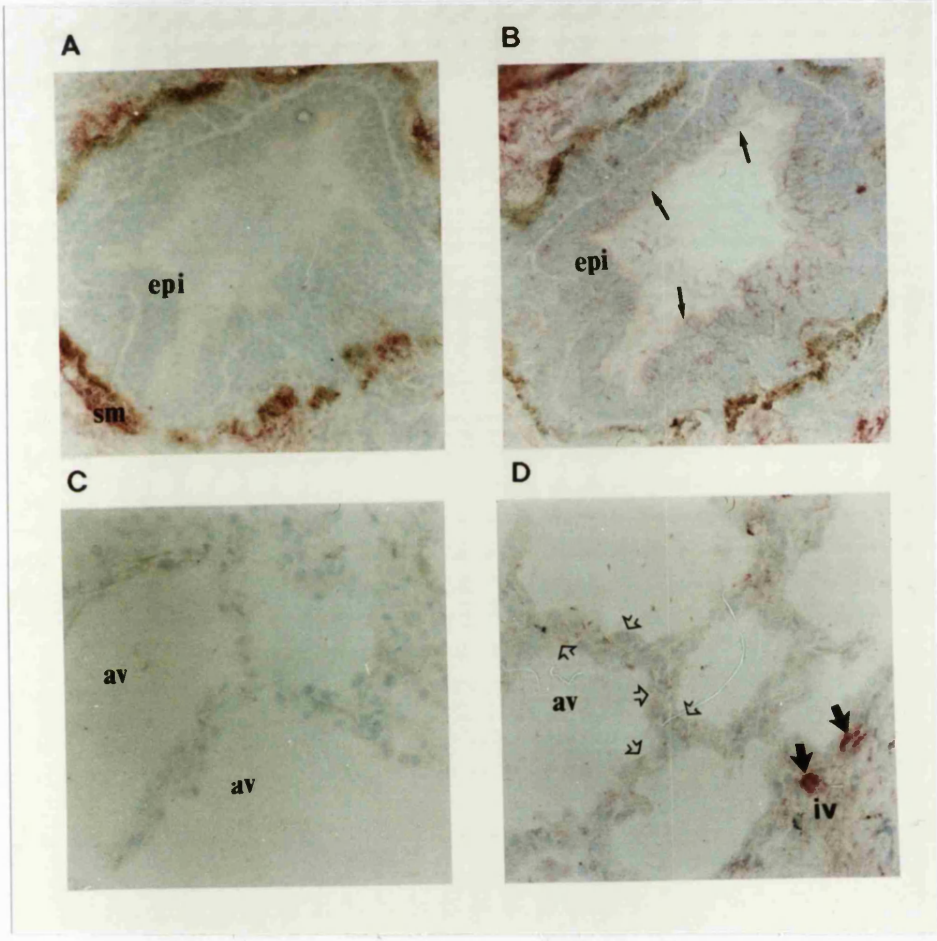
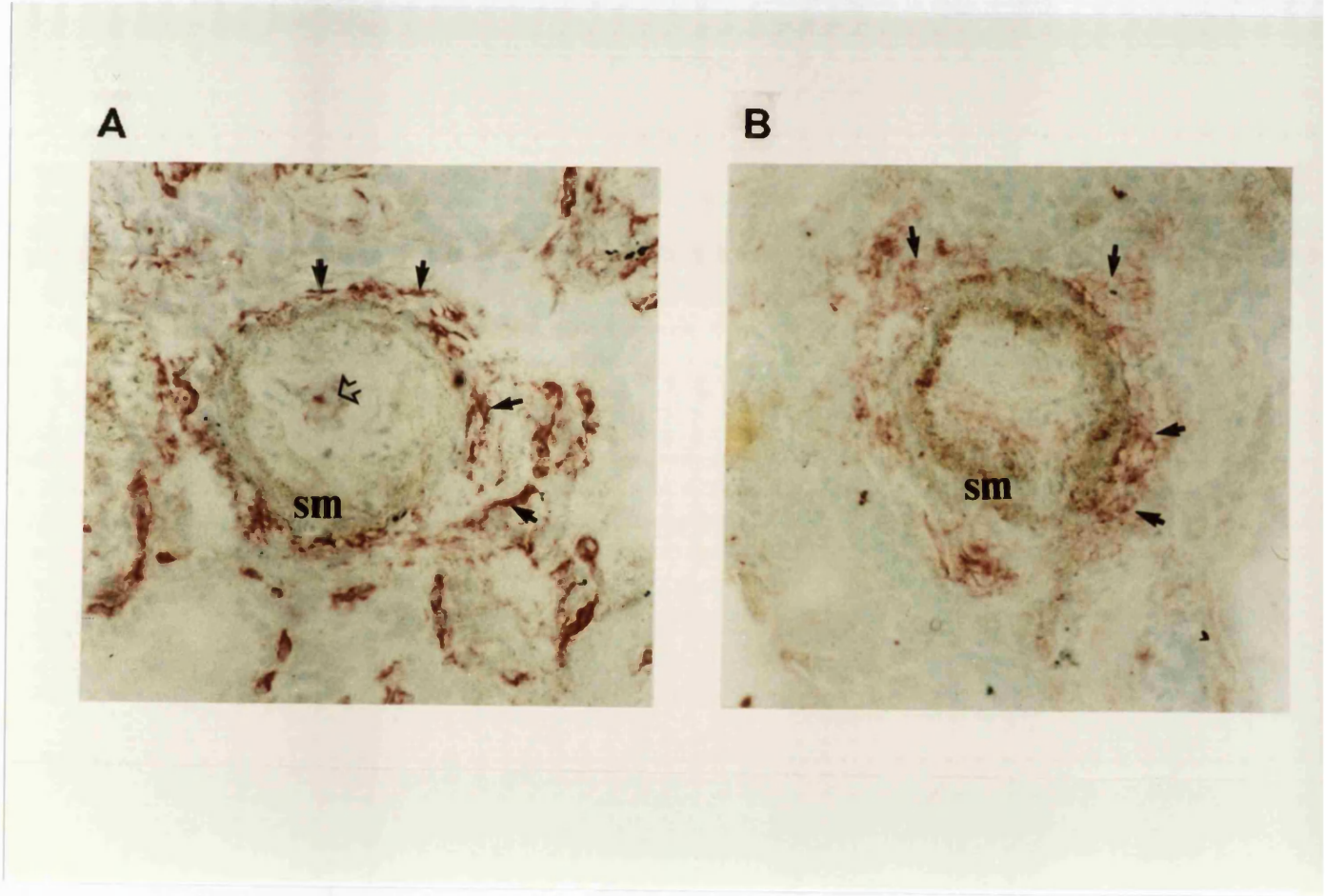
Figure 4.8: ET-1 staining in FASSc lesions



Cryostat sections from a representative FASSc biopsy incubated with; (A) mouse mAb to actin showing positive staining of smooth muscle (sm) around bronchioles and (B) replicate section to A. incubated with an ET-1 antibody showing positive ET-1 staining of bronchiolar epithelium (epi, small arrows). (C) normal rabbit IgG showing no staining in alveolar epithelium (av) and (D) replicate section to C incubated with an ET-1 antibody showing positive ET-1 staining of alveolar epithelial type II cells (large open arrows) and striking staining of interstitial vessels, (iv, large closed arrows) . (E) Replicate section to D delineating interstitial vessels as shown by the marker QBEND-10 (Magnification x200)

Figure 4.9: ET-1 staining of vessels



Cryostat sections of a lung biopsy from a FASSc patient. Shown are parallel sections illustrating; A. positive immunostaining of large pulmonary vessel with QBEND 10 (an endothelial cell marker). Note staining in the perivascular region (perivascular microvessels, small arrows) and endothelium of a large pulmonary vessel (large open arrow) by QBEND 10. B. positive ET-1 staining of large pulmonary vessel is predominantly associated with the perivascular region (small arrows) with minimal staining of smooth muscle (sm). Scale bar = 200μm

4.3.2.c ET-1 receptors

To evaluate the localisation of ET receptors in normal and FASSc lung, high and low resolution autoradiographs were generated from lung sections incubated with [^{125}I]ET-1. Representative examples of low resolution autoradiographs of total and non-specific binding of [^{125}I]ET-1 to FASSc and control biopsies are shown in fig 4.10. There was a striking increase in the binding of [^{125}I]ET-1 to FASSc sections compared with normal lung sections (see fig 4.10).

With high resolution microautoradiography, ET-1 receptors were demonstrated in the alveolar epithelium (fig 4.11A), bronchiolar smooth muscle and epithelium, large blood vessels and the pulmonary interstitium (see fig 4.12A). No receptors were noted in association with alveolar macrophages (data not shown).

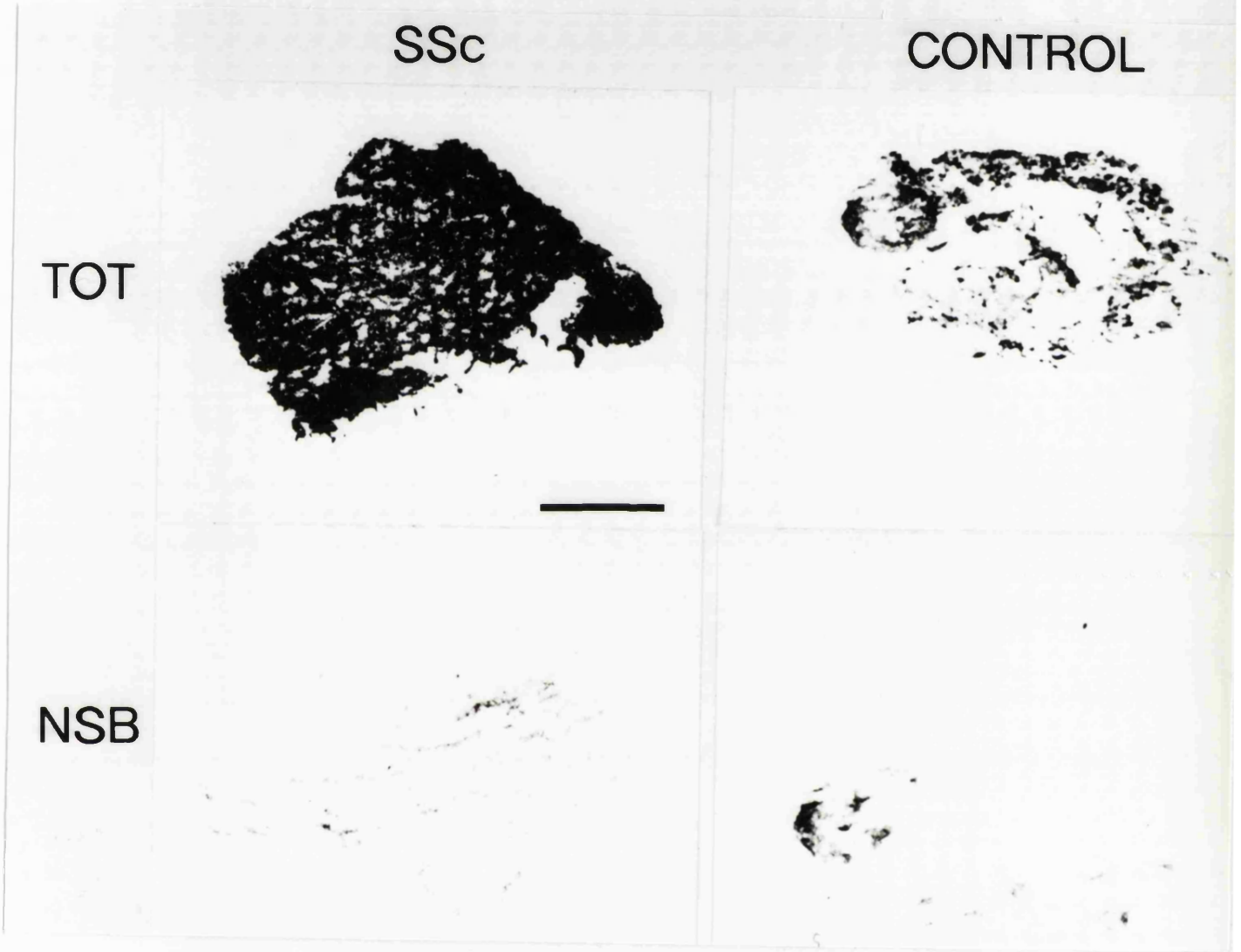
The quantitative assessment of high resolution autoradiographs indicated that there was a marked increase in the level of receptors associated with the alveolar epithelium in FASSc biopsies which comprised predominantly of alveolar epithelial type II cells compared with the alveolar epithelium (alveolar epithelial type I cells) in normal lung biopsies ($p<0.0001$) (Table 4.3). Similarly, a marked increase in ET-1 receptors was associated with the interstitium in FASSc compared with normal lung biopsies ($p<0.0001$). In contrast, no change was evident in the level of ET-1 receptors noted in bronchioles ($p=0.10$) and large vessels ($p=0.23$) in FASSc compared with biopsies obtained from normal lung.

Table 4.3: Expression of total ET-1 receptors

Patient group	Number of biopsies analysed†	Alveolar epithelium Median ± SE	Lung interstitium Median ± SE	Large vessels Median ± SE	Bronchioles Median ± SE
Control	4	0.195 + 0.05	0.18 + 0.01	1.04 + 0.12	1.03 + 0.18
FASSc	7	0.395 + 0.05*	0.60 + 0.01*	0.86 + 0.17	0.64 + 0.08

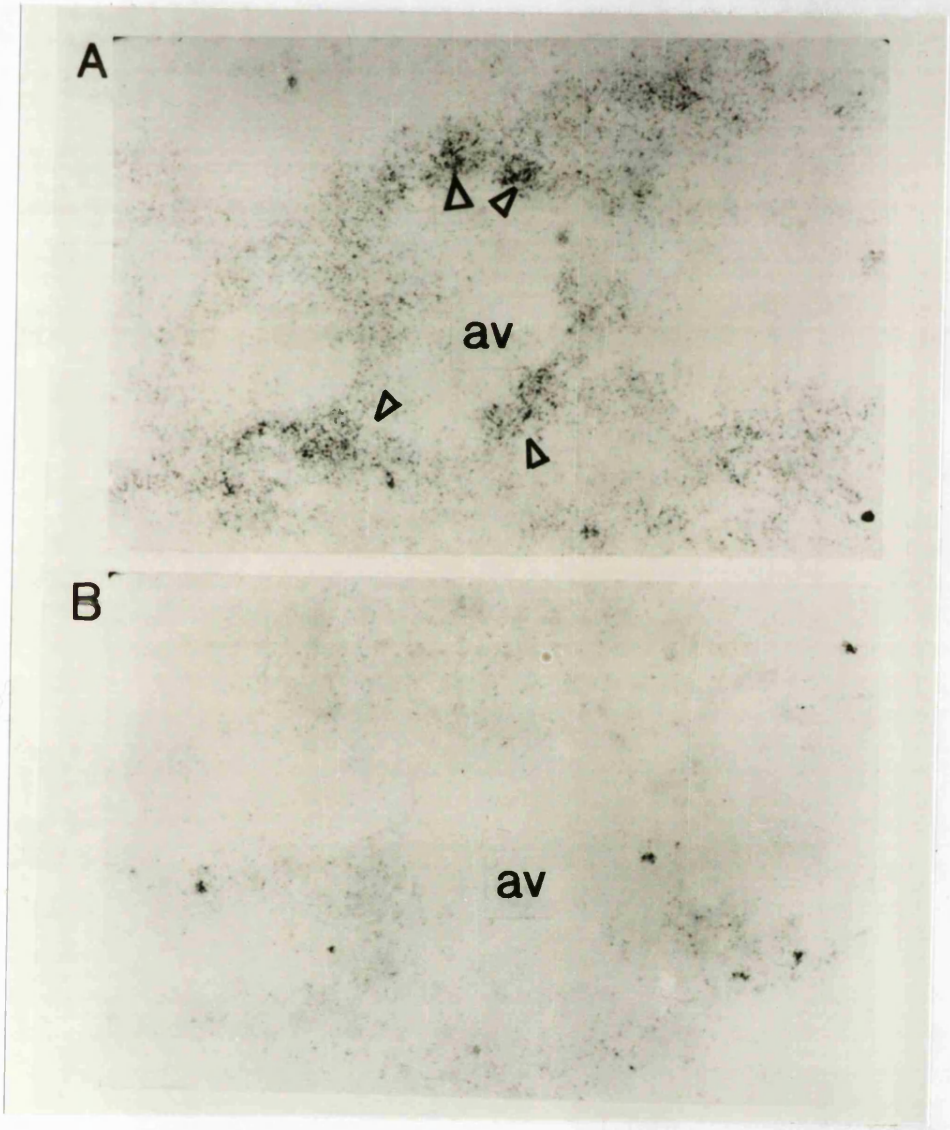
*- $p<0.05$ on comparison of patient with control group using Student's unpaired t-test .
†- 10 measurements were taken per structure per patient and control biopsy

Figure 4.10: Representative examples of total and non-specific binding of [^{125}I]ET-1 (receptor sites) to FASSc and control biopsies.



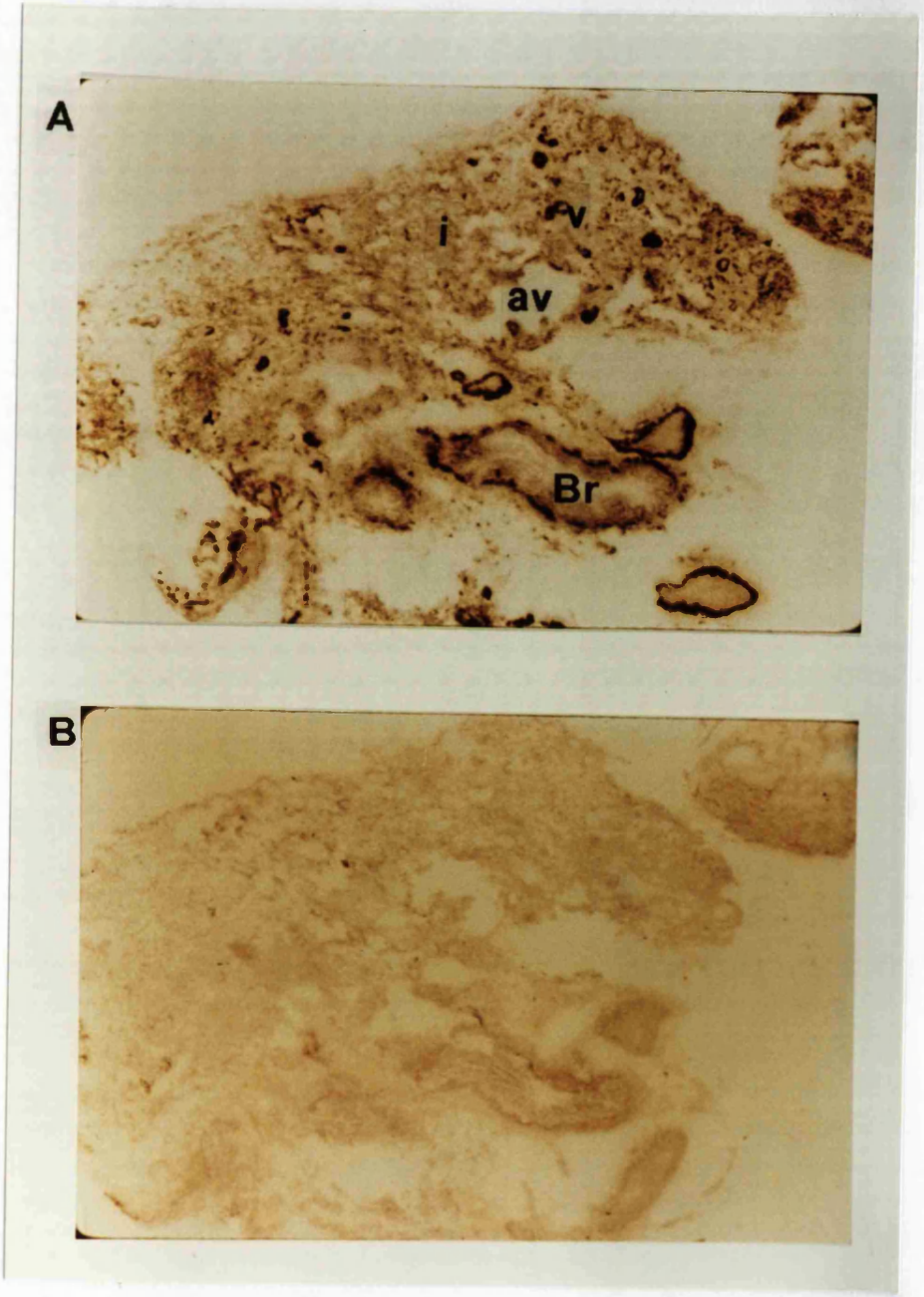
Low resolution autoradiographs generated on film, of FASSc (left) and normal control lung sections (right) incubated in 150 pM [^{125}I]ET-1 alone (total binding; TOT) and in the presence of 500 nMol unlabelled ET-1 (non-specific binding; NSB). Scale bar = 1mm

Figure 4.11: ET-1 receptors in alveolar epithelium



High resolution autoradiographs of consecutive FASSc sections incubated in **A.** 150 pM [^{125}I]ET-1 alone (total binding) and **B.** in the presence of 500 nMol unlabelled ET-1 (non-specific binding). Note accumulation of black grains as shown by open arrowheads in the cells lining the alveoli (av) in **A** and absence of such grains in **B**. Scale bar is 200 μm.

Figure 4.12: ET-1 receptors in FASSc



A, High resolution autoradiograph of representative FASSc section incubated in [^{125}I]ET-1 alone (total binding) and B, in the presence of excess unlabelled ET-1 (non specific binding). Note extensive grains in **A.** associated with the interstitium (i), alveolar walls (av), vessels (v) and bronchioles (Br). Magnification x 40

4.3.2.d Characterisation of different ET receptors

In order to establish the concentrations required to cause a greater than 50% inhibition of [^{125}I]ET-1 binding to all structures, the effect of a range of concentrations (10^{-11} to 10^{-5}M) of unlabelled ET-1, FR 139317, IRL 1620 and Ro 47-0203 were examined on [^{125}I]ET-1 binding to FASSc and control biopsies. Figure 4.13 shows film autoradiographs illustrating the dose dependant inhibition of [^{125}I]ET-1 binding by unlabelled ET-1, FR 139317(ETA selective ligand), IRL 1620 (ETB selective ligand) and Ro 47-0203 (mixed ET receptor selective ligand) to sections of a representative FASSc biopsy.

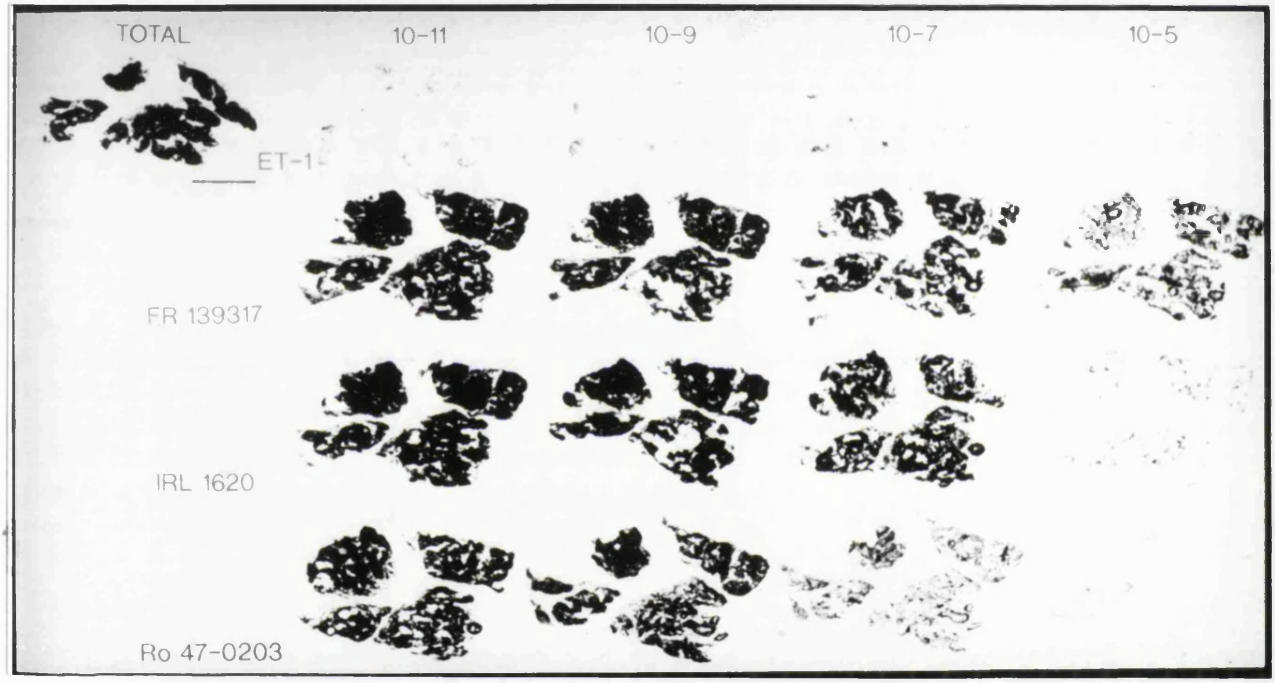
Since 500nMol of FR 139317, IRL 1620 and Ro 47-0203 was associated with a greater than 50% inhibition of [^{125}I]ET-1 binding to all structures, all subsequent experiments were performed at this concentration (see fig 4.14). Table 4.4 shows the effects of these ligands on total [^{125}I]ET-1 binding to alveolar epithelium, pulmonary interstitium, large vessels and bronchioles.

Incubation of sections with unlabelled ET-1 or the mixed ET receptor ligand (Ro 47-0203) was associated with marked inhibition (approximately 80% and 70%, respectively) of [^{125}I]ET-1 binding to all the pulmonary structures examined. In contrast, incubation with the ETA or ETB selective ligands resulted in a lesser level of inhibition of [^{125}I]ET-1 binding to various structures to both normal and FASSc biopsies.

The level of expression of ETA, ETB and other ET receptors were measured indirectly by subtraction of the percentage residual binding seen in the presence of the various ligands from the total [^{125}I]ET-1 binding (100%). Figure 4.15 shows that normal alveolar epithelium (epithelial type I cells) consisted of 26% ETA and 62% ETB receptors in contrast to that seen in FASSc alveolar epithelium (epithelial type II cells) which revealed a striking decrease in the number of ETA receptors (<2%, $p<0.05$), a reduced level of ETB receptors (29%) and an increased level of ET receptors recognised by the ligand Ro 47-0203 which was not ETA or ETB (70%, $p<0.05$). Normal control lung biopsies were found to consist of equal numbers of ETA and ETB receptors in the interstitium

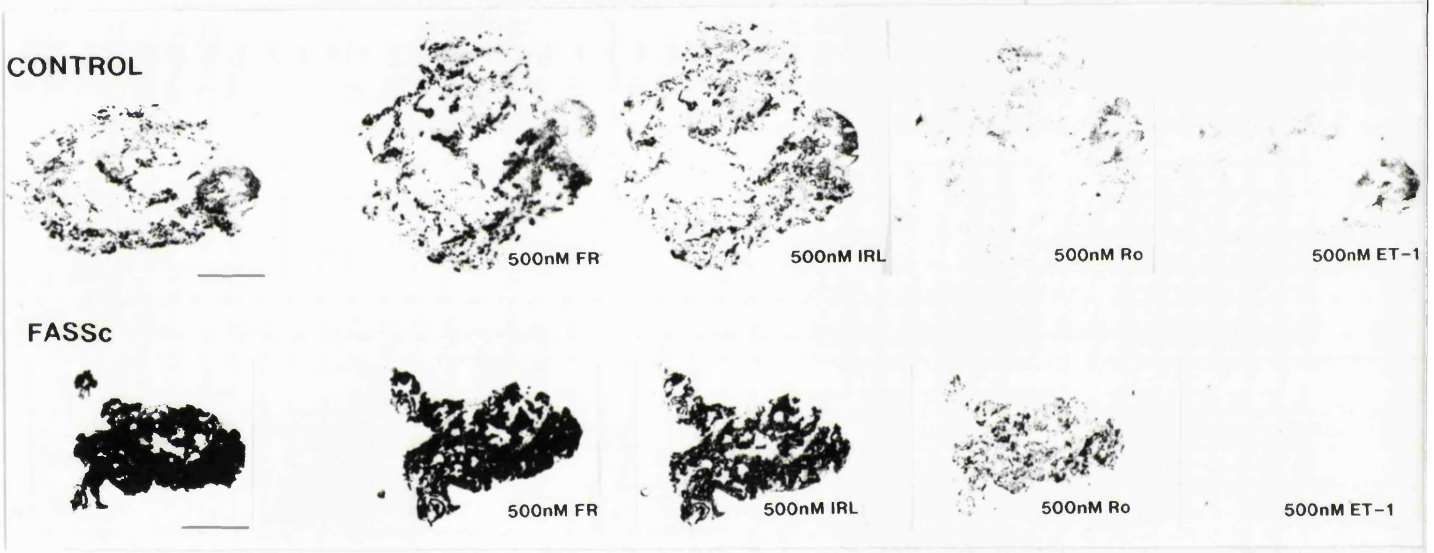
(41% and 41% respectively) and large pulmonary vessels (48% and 52%, respectively). This was markedly different to FASSc biopsies where ETA receptors were decreased while ETB receptors were increased in the interstitium (28% ETA and 60% ETB) and large vessels (24% ETA and 58% ETB). Bronchioles in FASSc biopsies comprised predominantly of ETB receptors (90%).

Figure 4.13 : Dose dependant inhibition of [^{125}I]ET-1 binding by 10^{-11} to 10^{-5}M of unlabelled ET-1, FR 139317, IRL 1620 and 47-0203 to a representative FASSc biopsy



Film autoradiographs of consecutive sections taken from a representative FASSc patient showing the dose dependant inhibition of [^{125}I]ET-1 binding by co-incubation of sections with a range of concentrations (10^{-11} to 10^{-5}Mol) of unlabelled ET-1, FR 139317 (ETA selective ligand), IRL 1620 (ETB selective ligand) and Ro 47-0203 (mixed ET receptor selective ligand) compared with section incubated with 150 pM [^{125}I]ET-1 alone (TOTAL). Scale bar =1mm

Figure 4.14: The effects of 500nMol of FR 139317, IRL 1620 and Ro 47-0203 on [^{125}I]ET-1 binding to a normal and FASSc biopsy



Low resolution film autoradiographs of sections taken from a normal lung (top) and FASSc biopsy (bottom) showing the residual binding of [^{125}I]ET-1 in the presence of 500nMol of FR 139317 (FR), IRL 1620 (IRL), Ro 47-0203 (Ro) and unlabelled ET-1 (ET-1) compared with total [^{125}I]ET-1 binding (far left). Note that residual [^{125}I]ET-1 binding is decreased in the following order in both normal and FASSc biopsies; FR 139317 (FR) > IRL 1620 (IRL) > Ro 47-0203 (Ro) > unlabelled ET-1 (ET-1). (Scale bar= 1mm)

Table 4.4: Percentage [^{125}I] ET-1 binding in the presence of 500nMol of various ET receptor ligands.

ET receptor ligands	Alveolar epithelium† (mean ± SEM)	Parenchyma‡ (mean ± SEM)	Large vessels (mean ± SEM)	Bronchioles (mean ± SEM)
Normal lung				
ET-1	5.7 ± 1.5	6.7 ± 0.6	5.1 ± 0.3	ND
FR 139317 (ETA)	73.9 ± 17.9	58.4 ± 11.1	52.2 ± 8.7	ND
IRL 1620 (ETB)	38.5 ± 17.9	58.6 ± 6.0	48.0 ± 6.2	ND
Ro 47 0203 (mixed)	15.4 ± 2.8	15.3 ± 6.7	10.2 ± 3.6	ND
FASSc lung				
ET-1	10.1 ± 0.8*	17.2 ± 0.2**	5.7 ± 0.8	9.5 ± 0.5
FR 139317 (ETA)	98.8 ± 10.1*	73.3 ± 10.0	76.1 ± 14.3	98.0 ± 17.2
IRL 1620 (ETB)	70.9 ± 12.7	40.0 ± 3.8*	42.3 ± 8.1	9.0 ± 0.9
Ro 47 0203 (mixed)	30.4 ± 5.0*	23.3 ± 8.3	20.9 ± 7.4	0.0 ± 0.7

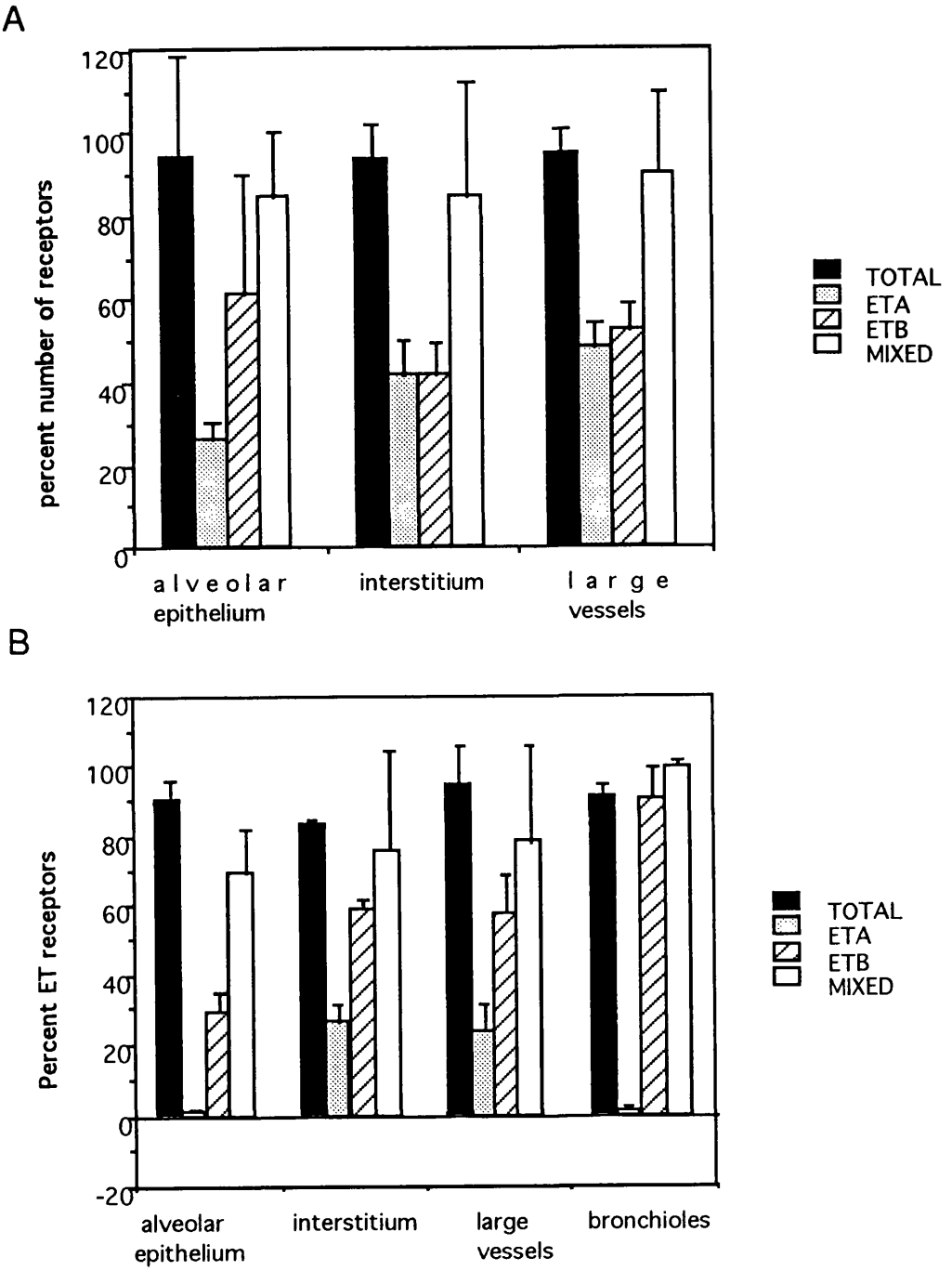
† The alveolar epithelium consisted predominantly of epithelial type I cells in normal biopsies, unlike in FASSc biopsies where alveolar epithelial type II cells were the predominant cell type

‡ The interstitium comprised of interstitial vessels, fibroblasts and immune cells.

* $p<0.05$, **$p<0.001$ in comparison of percentage residual ^{125}I ET-1 binding in FASSc compared with normal sections.

ND-not done

Figure 4.15: Endothelin receptor expression in normal and FASSc biopsies



The graph represents the percentage number of the different ET-1 receptors ± SEM in **A.** normal lung and **B.** FASSc biopsies. The total number of ET-1 binding sites (total -non specific binding) are shown by solid bars and the percentage number of ETA (dotted bars), ETB (hatched) and mixed ET (open) receptors have been calculated by subtracting the residual binding in the presence of the FR139317, IRL 1620 or Ro 47-0203 from total ET-1 binding (100%). Alveolar epithelium comprised predominantly of epithelial type I cells in normal lung biopsies and alveolar epithelial type II cells in FASSc biopsies.

4.4 DISCUSSION

This chapter provides novel data regarding the expression of ET-1 and ET-1 receptors in dermal and pulmonary lesions in SSc compared with normal biopsies taken from the skin and lung. The results of this part of the project showed that there was a marked increase in the staining of ET-1 and in the expression of ET-1 receptors in both dcSSc skin and FASSc biopsies compared with their respective controls. Although the increased expression of ET-1 and ET-1 receptors in the two most affected organs in SSc does not prove a cause-and effect relation, the involvement of ET-1 in the pathogenesis of SSc in both the skin and lung is biological plausible. Furthermore, the altered expression of ET-1 and ET-1 receptors in both SSc skin and lung biopsies where fibrosis was also present suggests that this peptide may play a role in the altered matrix expression in SSc and may explain the earlier observation of a relationship between circulating ET-1 levels and fibrosis.

Localisation and expression of ET-1

The increased expression of ET-1 in SSc was associated with the microvasculature in both SSc skin (papillary and deep dermal vessels) and lung biopsies (interstitial microvessels) compared with normal lung microvessels. Furthermore, the increased levels of ET-1 staining noted in the dermal vasculature in SSc was shown by both the involved and clinically uninvolved skin biopsies suggesting that the increased expression of ET-1 is an early pre-fibrotic event in SSc dermal lesions. ET-1 was barely detectable in SSc skin lesions characterised by marked fibrosis.

An increased expression of ET-1 was also shown by perivascular microvessels surrounding large muscular pulmonary vessels (greater than 20μm in diameter). This expression of ET-1 around large pulmonary vessels in FASSc biopsies was interesting and in view of the perivascular fibrosis noted in previous histological studies in some FASSc biopsies (Al-Sabbagh *et al.*, 1989), suggests a mechanism by which perivascular fibrosis may occur in FASSc and

result in PHT. The expression of ET-1 in large vessels in FASSc biopsies was also strikingly different to that reported previously by Giaid *et al.*, 1993a in biopsies taken from patients with primary PHT where an increased level of ET-1 staining was localised to the vascular smooth muscle of pulmonary arteries. It is thus conceivable that ET-1 may contribute to PHT in FASSc patients and also in those with isolated vascular disease by perivascular fibrosis or direct vasoconstriction, respectively, depending on it's site of expression.

Apart from the vasculature in FASSc biopsies, alveolar type II epithelial cells in FASSc biopsies revealed increased ET-1 staining. In an ultrastructural study of bleomycin induced fibrosis in the lung, Adamson *et al*, 1990, showed increased intercellular contacts between alveolar type II epithelial cells and lung fibroblasts. Recent experiments have also shown that alveolar epithelial type II cells markedly stimulate fibroblast proliferation and collagen secretion in co-culture experiments (Young L., *et al.*, 1993). Furthermore, previous studies on FASSc lesions have revealed that alveolar type II epithelial cells secrete increased levels of other growth factors including TGFβ and PDGF which are known to have potent synergistic effects on fibroblast proliferation and matrix synthesis when combined with ET-1 (Guidry *et al.*, 1991). Based on these observations, the demonstration that alveolar epithelial type II cells express increased levels of ET-1 in FASSc biopsies suggests a role for this peptide in the pathogenesis of pulmonary fibrosis in SSc.

ET-1 receptors

An indirect autoradiographic technique using [^{125}I]ET-1 was used to examine ET-1 receptors. This method was chosen as antibodies and cDNA probes for the different ET receptors were not available at the time of this study. Autoradiography however, is widely used to examine ET receptors. The specificity of [^{125}I]ET-1 binding (receptor sites) was established by the complete inhibition of [^{125}I]ET-1 binding with unlabelled ET-1 or a mixed ET receptor

ligand. Using this technique, an increased expression of ET-1 receptors was noted in both SSc skin and FASSc biopsies relative to their respective controls.

In normal skin, ET-1 receptors were localised to the dermal/epidermal border and the dermal vasculature. These results concur with previous data reported by Terenghi *et al.*, 1991, who revealed striking binding to all dermal blood vessels and to the dermal/epidermal border in normal dermal biopsies. In all the SSc biopsies, an increased level of ET-1 receptors were noted in association with papillary dermal vessels but not with the deep dermal vessels. An increased expression of ET-1 receptors was also observed in the dermal/epidermal border. Furthermore, this altered expression of ET-1 receptors was maintained with the progression of SSc dermal lesions.

The dermal microvasculature has been shown to be particularly sensitive to the constrictor action of ET-1 (Brain SD., *et al.*, 1988). In this respect, it is possible that the increased staining of ET-1 and the increased expression of ET-1 receptors in papillary vessels may result in an unbalanced microvessel tonal control favouring vasoconstriction and the Raynaud's component of SSc. Deep dermal vessels were however, not found to express a significant increase in ET-1 receptors despite the increased level of ET-1 staining described previously. This suggests an increased synthesis of ET-1 alone by deep dermal vessels which may contribute to the active fibrotic zone seen predominantly in the deep dermis of SSc skin lesions.

The observation of increased ET-1 receptors in the basal layer of the dermal/epidermal border was interesting. In view of the ability of ET-1 to increase the cellular proliferation of keratinocytes *in vitro* (Haynes *et al.*, 1993), the increased expression of ET-1 receptors in basal keratinocytes suggests abnormalities in this cell type in SSc which has hitherto not been investigated. Another notable observation in SSc skin biopsies was the absence of detectable ET receptors on matrix cells (fibroblasts and macrophages) despite the presence of increased ET-1 staining. Since *in vitro* experiments have shown that fibroblasts express ET receptors, it possible that the autoradiography technique used in this study was not able to detect ET receptor

expression by dermal fibroblasts *in vivo*. . It is also conceivable that the increased staining associated with fibroblasts in SSc suggest that these cells synthesise ET-1.

In normal lung biopsies, ET-1 receptors were localised to alveolar epithelial cells (epithelial type I cells) and the pulmonary interstitium, in addition to the pulmonary vasculature. These results concur with previous data reported by Maciniale *et al.*, 1992 and Nakamichi *et al.*, 1992 who examined normal human and rat lungs, respectively. Examination of FASSc lung biopsies showed a significantly increased expression of ET-1 receptors relative to controls particularly in association with the alveolar epithelium and the pulmonary interstitium. The expression of ET receptors in the alveolar epithelium in FASSc was considered to reflect alveolar epithelial type II cells as the predominant cell type lining alveoli in FASSc biopsies expressed HLA DR. The pulmonary interstitium was considered to reflect ET receptor expression by microvessels and matrix cells (fibroblasts, macrophages) as it was difficult to separate these structures.

There was no change in the number of ET-1 receptors localised to large pulmonary vessels in FASSc relative to control biopsies. The increase in ET-1 receptors noted in association with the microvasculature in SSc skin and FASSc biopsies and with alveolar epithelial type II cells was present concomitant with increased peptide levels.

Different ET receptor subtypes

ET-1 is known to mediate varied biological effects through different ET receptors and it is conceivable that an altered expression of the each of the ET receptors may be associated with fibrosis. A preliminary examination was therefore performed to assess the expression of each of the different ET receptors in FASSc compared with normal lung biopsies.

Using ET receptor selective ligands, two ET receptors were identified in the pulmonary alveolar epithelium, interstitium and vasculature in normal lung biopsies. A higher proportion of ETB receptors relative to ETA receptors were

present in normal alveolar epithelial type I cells and equal levels of ETA and ETB receptors were noted in the pulmonary interstitium (microvasculature and fibroblasts) and large pulmonary vessels.

In FASSc, there was a marked alteration in the expression of each of the different ET receptors particularly in the alveolar epithelium (predominantly consists of alveolar epithelial type II cells) and in the interstitium. Alveolar epithelial type II cells revealed a striking decrease in ETA receptors, no change in ETB receptors and a marked increase in an ET receptor not recognised by the ETA and ETB selective ligands. The identity and function of this receptor is not known but it may be similar to the ETC receptor postulated by Emori *et al.*, 1990. In addition, FASSc biopsies revealed a decrease in the level of ETA receptors and a marked increase in the ETB receptors in the pulmonary vasculature and interstitium. Thus altered levels of the different ET receptors in FASSc biopsies compared with normal lung biopsies and may play a role in the pathogenesis of fibrosis in FASSc.

In conclusion, the finding of striking increases in both the expression of ET-1 and it's receptors provides direct evidence for the involvement of ET-1 in both skin (dcSSc) and lung lesions (FASSc) in SSc. Although the microvascular expression of ET-1 and the level of expression of ET-1 receptors in both organs appears to be similar, the increased staining of ET-1 and expression of ET-1 receptors in alveolar epithelial type II cells in FASSc and the increased level of ET-1 receptors alone in basal keratinocytes in dcSSc suggests the involvement of other cell types in the expression of disease in the lung and skin respectively. Furthermore, an altered expression of different ET receptors was also noted in FASSc. These observations provide important new information about the expression of ET-1 and ET-1 receptors in the two most affected organs in SSc and strongly suggests the involvement of this peptide in the pathogenesis of the disease in both the skin and lung.

CHAPTER FIVE:

CIRCULATING AND TISSUE EXPRESSION OF ADHESION MOLECULES IN THE SKIN AND LUNG IN SSc

5.1 INTRODUCTION

Inflammatory cells, predominantly lymphocytes are present in the skin of SSc patients in a perivascular distribution in the early oedematous phase of the disease (Gruschwitz *et al.*, 1992). In marked contrast, inflammatory cells including lymphocytes, eosinophils, neutrophils and plasma cells are found in the lung of SSc patients throughout the course of fibrosis and disappear only at a very late stage in disease (Harrison *et al.*, 1991). Furthermore, studies performed on BALF in FASSc patients have revealed a correlation between the predominant inflammatory cell type and the stage of disease: lymphocytes are present in the early stage of disease followed by eosinophils and finally neutrophils when there is established disease (Wells *et al.*, 1992). The presence of inflammatory cells in both the skin and lung in SSc suggests altered leukocyte interactions in both organs. However, the significant differences in the temporal aspects of inflammation and in the cellular infiltrates in the skin and lung in SSc suggest that the mechanisms that mediate leukocyte adhesion in these two organs are different.

The mechanisms that control immune cell traffic to various tissues are largely considered to be due to a complex process which is mediated by three general classes of adhesion molecules - selectins, integrins and members of the immunoglobulin superfamily (Ruoslahti *et al.*, 1987; Springer 1990; Hynes 1992). Cellular interactions with leukocytes are predominantly mediated by E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Springer 1990; Norris *et al.*, 1991; Pilewski *et al.*, 1993).

E-selectin, a member of the selectin family is expressed predominantly on activated endothelial cells and is involved in leukocyte extravasation and migration to tissues (Bevilaqua *et al.*, 1989). This molecule is rapidly and often transiently upregulated on endothelial cells *in vitro* by cytokines such as IL-1β and TNF-α (Pober *et al.*, 1986) and mediates neutrophil (Bevilaqua *et al.*, 1989), monocyte (Hakkert *et al.*, 1991) and resting memory CD4+ T cell adhesion

(Picker *et al.*, 1991; Shimizu *et al.*, 1991). The low affinity of binding of leukocytes by E-selectin is thought to be involved in leukocyte rolling on the vessel walls and may also provide a triggering signal that promotes integrin-mediated firm adhesion and extravasation (Butcher 1991). Endothelial expression of E-selectin is particularly relevant to skin pathology since it is the counter receptor for T cells expressing the cutaneous lymphocyte antigen (CLA), which specifically migrate to skin (Picker *et al.*, 1991).

ICAM-1 acts as a counter-receptor for the β2 integrins such as LFA-1 expressed by lymphocytes and Mac-1 expressed by monocytes and neutrophils (Marlin *et al.*, 1987; Springer 1990). ICAM-1 is constitutively expressed by most cell types, with variations in the degree of basal expression and in responsiveness to TNF, IL-1, IFN-γ and other activating factors (Dustin *et al.*, 1986). β2 integrin interactions with ICAM-1 are important in mediating the secondary phase of leukocyte adhesion and migration to sites of inflammation (Lawrence *et al.*, 1991) and are also known to provide an additional stimulatory signal for T cell activation (Van Seventer *et al.*, 1990).

VCAM-1 is present mainly on cytokine-activated endothelial cells, dendritic cells and sub-populations of macrophages (Rice *et al.*, 1991). This molecule is induced on endothelial cells by the cytokines IL1-β, TNF-α and IL-4 and acts as the ligand for VLA-4 (very late antigen-4; α4/β1) and the α4/β7 integrins, which are expressed on lymphocytes, monocytes and eosinophils (Elices *et al.*, 1990; Ruegg *et al.*, 1992). VCAM-1/VLA-4 interactions are known to be the major mechanism that mediates endothelial-eosinophil binding (Walsh *et al.*, 1991; Weg *et al.*, 1993).

In order to examine the mechanisms involved the altered immune interactions in SSc skin lesions, previous investigators have shown both a normal and increased dermal expression of E-selectin (Claman *et al.*, 1991; Gruschwitz *et al.*, 1992), ICAM-1 (Majewski *et al.*, 1991; Gruschwitz *et al.*, 1992; Sollberg *et al.*, 1992), decreased levels of VCAM-1 (Koch *et al.*, 1993) in SSc *in vivo*, in semi-quantitative studies. There have however been no studies on the expression of

adhesion molecules in FASSc biopsies. Recent studies have also shown that various adhesion molecules exist in the circulation (Gearing *et al.*, 1993), are altered in disease (Carson *et al.*, 1993; Sfikakis *et al.*, 1993) and may modulate cellular interactions (Gearing *et al.*, 1993; Heufelder *et al.*, 1993). It would therefore be advantageous to examine both the dermal and pulmonary expression of the various adhesion molecules and their circulating forms in SSc. In this study, I have therefore examined the level of expression of the circulating forms of E-selectin, ICAM-1 and VCAM-1 and the dermal and pulmonary expression of these three adhesion molecules in SSc in order to investigate the mechanisms that mediate leukocyte adhesion in both organs.

5.2 PATIENTS AND STATISTICS

5.2.1 Patient samples

Serum samples were obtained from 116 patients and 155 normal healthy individuals (89 males and 66 females, aged 20 to 66 years, average 40 years). The SSc patient group consisted of: 35 patients with dcSSc and 39 patients with lcSSc. Other patient groups were also studied including 11 patients with "autoimmune" RP (RP patients with positive antinuclear antibodies and abnormal nailfold capillaries), 21 patients with primary RP and 10 patients with localised scleroderma (morphea) (see Table 5.1 for clinical details of patients).

Dermal punch biopsies ($4mm^3$) were obtained from the forearm of 4 normal individuals (1 male, 3 females; aged 24-70 years), 4 patients with primary RP (4 females; aged 27-38 years), 1 "autoimmune" RP patient (female, 33 years, ANA titre>1:1000) and from 10 dcSSc patients (3 male, 7 female; aged 26-55 years). In all the SSc patients, paired biopsies were taken from clinically "uninvolved" (lower back area) and from the most recently involved skin to examine the expression of E-selectin, VCAM-1 and ICAM-1 with disease progression. Normal lung biopsies were taken from the periphery of unaffected

regions of lobectomy or pneumonectomy samples from 4 age matched individuals undergoing thoracotomy for tumor resection (3 males, 1 female, average age was 48 years) and compared with the affected areas of 7 SSc patients with FASSc (all female, aged 37-56 years, average age was 44 years). In one patient, paired unaffected and affected lung biopsies were available and were used to examine the expression of adhesion molecules with disease progression (female, aged 42 years).

5.2.2 Statistical analysis

Statistical assessment of variation in levels of adhesion molecules within the disease groups was made using the ANOVA test. When significant, group comparisons were made using the Student's *t-test* with the Bonferroni correction. Possible relationships between the individual circulating adhesion molecules were studies by examining correlation coefficients. Similarly the relationship between adhesion molecules and clinical and laboratory parameters were sought by examining correlation coefficients. The dermal expression of E-selectin in paired SSc biopsies was compared using the paired Student's *t*-test. The level of significance was taken as $p<0.05$.

Table 5.1 Clinical data for the patients studied

Patient group	No	Mean age in years	% Female	Duration of Raynauds in months (Mean)	Duration of SSc or morphea in months (Mean)	Mean Raynauds grade (max=3)	Mean Skin score (max=60)	Average no of organs involved excluding skin+ (max=6)	% ANA	%Scl-70, ACA
DcSSc	35	44.3	63	99.9	52.9	2.0	24.3	1.6	90	40% scl-70
LcSSc	39	52.6	76	163.0	94.0	2.1	8.2	1.2	81	35% ACA
"Autoimmune" Raynauds phenomenon	11	48.4	57	48.0	0.0	2.6	0.0	0.0	82	9% scl-70 9% ACA
Primary Raynauds phenomenon	21	38.0	70	110.4	0.0	2.0	0.0	0.0	0	0 %
Morphea	10	53.4	100	0.0	148.0	0.0	0.0	0.0	30	0 %

"Autoimmune" Raynaud's phenomenon indicates patients with Raynauds phenomenon, positive antinuclear antibodies and abnormal nailfold capillaries.

+ The average number of organs includes documented clinical involvement of the gut, lungs, kidneys, heart and musculoskeletal system and symptoms of Sjogrens syndrome.

% ANA indicates percentage number of patients with positive antinuclear antibodies

% Scl-70, ACA indicates the percentage number of patients with either Scl-70 or anticentromere antibodies.

5.3 RESULTS

5.3.1 Measurement of circulating E-selectin, ICAM-1 and VCAM-1 levels in SSc, RP and morphea

Circulating levels of E-selectin, ICAM-1 and VCAM-1 were measured by ELISA in 155 normal healthy volunteers and 116 patients with various diseases including SSc. As shown in Table 5.2, 155 normal individuals had average circulating E-selectin levels of 79.6 ng/ml, circulating ICAM-1 levels of 82.0 ng/ml and circulating VCAM-1 levels of 592.8 ng/ml. The percentage of patients with levels of circulating adhesion molecules higher than the control mean + 2 SD and the average levels $\pm$ SEM in the different disease groups: dcSSc, lcSSc, "autoimmune" and primary RP and morphea, compared with normal individuals are shown in Table 5.2.

In comparison with the normal levels of circulating E-selectin, ICAM-1 and VCAM-1, all the SSc patient groups had significantly higher circulating levels of E-selectin and VCAM-1 than normal healthy volunteers. Significantly increased circulating levels of E-selectin levels and VCAM-1 were also seen in "autoimmune" RP, in contrast to primary RP where circulating VCAM-1 alone was raised. The "autoimmune" RP group was particularly interesting as 6/11 (55%) patients developed SSc within 3 years and 5 of the 6 patients who progressed to SSc had significantly raised circulating E-selectin levels (83%).

Circulating levels of E-selectin were also significantly higher in the SSc ($p=0.04$, 55% of patients) and "autoimmune" RP ($p<0.02$, 82%) groups compared with primary RP (14% patients). In morphea, circulating E-selectin were not significantly elevated compared with the control group and VCAM-1 was near normal. Levels of circulating ICAM-1 were not significantly raised in any of the disease groups. Furthermore, there was no significant difference in the circulating levels of E-selectin, ICAM-1 and VCAM-1 between dcSSc and lcSSc.

Table 5.2. Circulating levels of E-selectin, ICAM-1 and VCAM-1 in dcSSc, lcSSc, primary Raynaud's syndrome and Morphea

Patient Group	Circulating E-selectin			Circulating ICAM-1			Circulating VCAM-1		
	% positive†	Mean± SEM in ng/ml	p value*	% positive †	Mean± SEM in ng/ml	p value*	% positive †	Mean± SEM in ng/ml	p value*
Controls		79.6 ± 3.4			82.0 ± 5.3			592.8 ±13.8	
Diffuse SSc	66%	118.9 ± 9.9	**0.0014**	3%	97.2 ±10.1	>0.8	57%	883.3 ± 65.7	**0.0006**
Limited SSc	44%	115.6 ± 12.3	**0.0021**	8%	117.0 ± 18.8	0.07	59%	905.2 ±102.2	**0.008**
"Autoimmune" Raynauds phenomenon	82%	144.0± 19.5	**0.0014**	9%	93.4 ± 19.6	>0.8	55%	868.1 ±169.8	**0.04**
Primary Raynauds phenomenon	14%	89.9 ± 9.4	>0.8	5%	124.0 ± 31.6	0.21	43%	810.3 ±146.0	**0.05**
Morphea	30%	100.2 ± 22.5	>0.8	0%	129.4 ± 9.0	0.49	10%	473.8 ± 29.2	>0.8

* Significance levels have been calculated using the Student's unpaired t-test with the Bonferroni correction and are expressed in comparison with the normal control group. p values <0.05 are considered significant.

† Numbers of patients with levels higher than the control mean + 2 Standard deviations

5.3.2 Relationship between the circulating levels of E-selectin, ICAM-1 and VCAM-1 in normal individuals and disease

In the present study, when circulating levels of E-selectin were related to the circulating levels of ICAM-1 and VCAM-1 in 155 normal individuals, there was a significant correlation between levels of E-selectin and both ICAM-1 and VCAM-1, i.e., $r=0.21$, $p=0.007$ and $r=0.31$, $p<0.001$, respectively (see fig 5.1A). A significant relationship was also seen between circulating levels of ICAM-1 and VCAM-1 ($r=0.21$, $p=0.006$) in normal healthy individuals (fig 5.1A).

This apparent relationship between the circulating levels of the three adhesion molecules was altered with disease, as shown for dcSSc and lcSSc (see figs 5.1B and 5.1C) and for RP (both "autoimmune" and primary disease) and morphea (see fig 5.1D and 5.1E).

5.3.3 The relationship between circulating adhesion molecules and organ involvement

There was no significant difference in the circulating levels of E-selectin, ICAM-1 and VCAM-1 between patients with dcSSc and lcSSc (see Table 5.2). In addition, as shown in Table 5.3 , the circulating levels of E-selectin, ICAM-1 and VCAM-1 did not correlate with clinical evidence of lung, renal, oesophageal or muscle involvement ($p>0.10$ for all comparisons).

5.3.4 The relationship between circulating adhesion molecules and other clinical parameters

The circulating levels of E-selectin, ICAM-1 and VCAM-1 in the study group did not relate to demographic parameters including age or sex ($p>0.40$ for all comparisons). Furthermore, in the disease groups, no relationships were

observed between these three circulating adhesion molecules and parameters of disease activity such as duration of SSc and Raynaud's phenomenon, skin score, severity of Raynaud's and serology ($p>0.10$ for all comparisons).

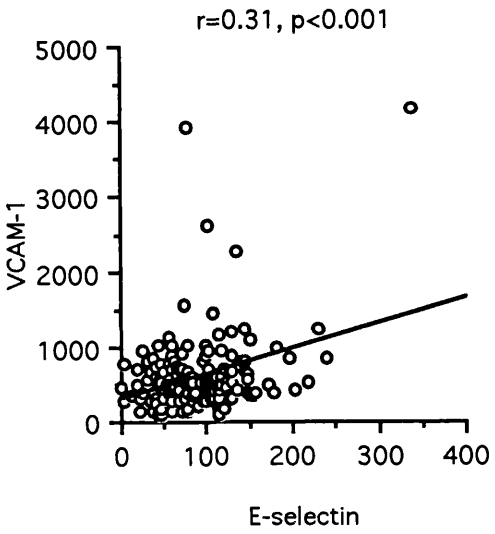
Figure 5.1. Relationship between the circulating adhesion molecules

Relationship between the circulating adhesion molecules in ng/ml in **A.** normal healthy volunteers **B.** patients with dcSSc **C.** lcSSc **D.** RP and **E.** morphea The relationships between circulating levels of (I) E-selectin and VCAM-1, (II) E-selectin and ICAM-1 and (III) VCAM-1 and ICAM-1 are shown statistically by the correlation coefficients (r value) and significance values (pvalue).

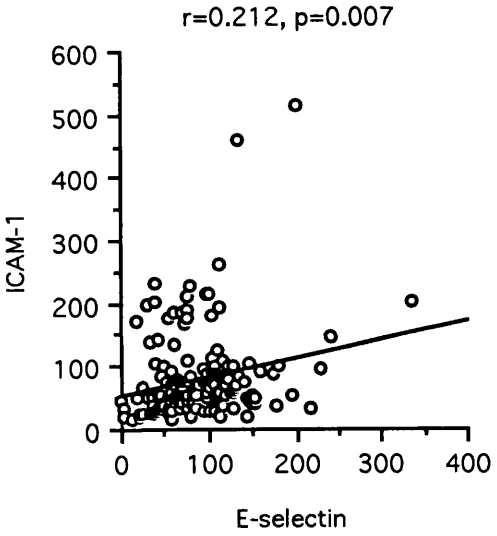
Figure 5.1: Relationship between the circulating levels of E-selectin, iCAM-1 and VCAM-1

A. CONTROLS

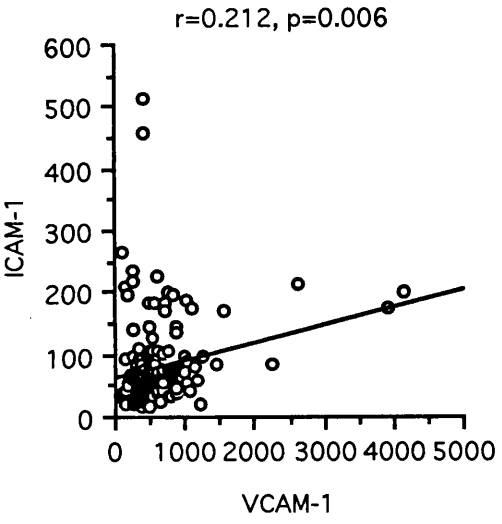
I



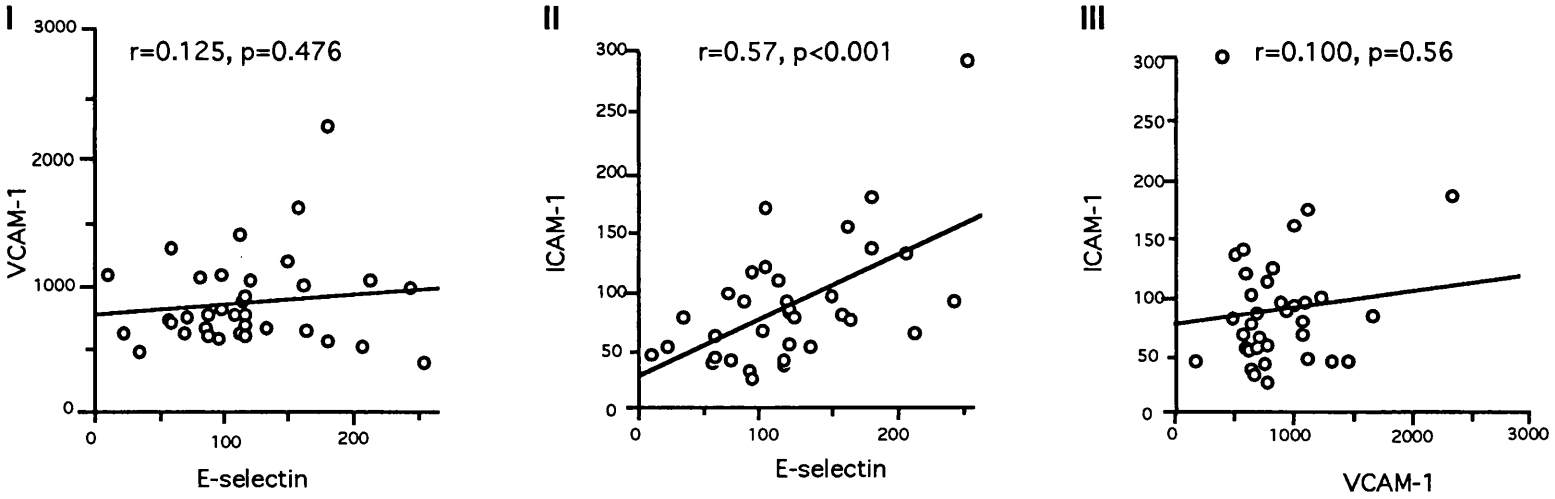
II



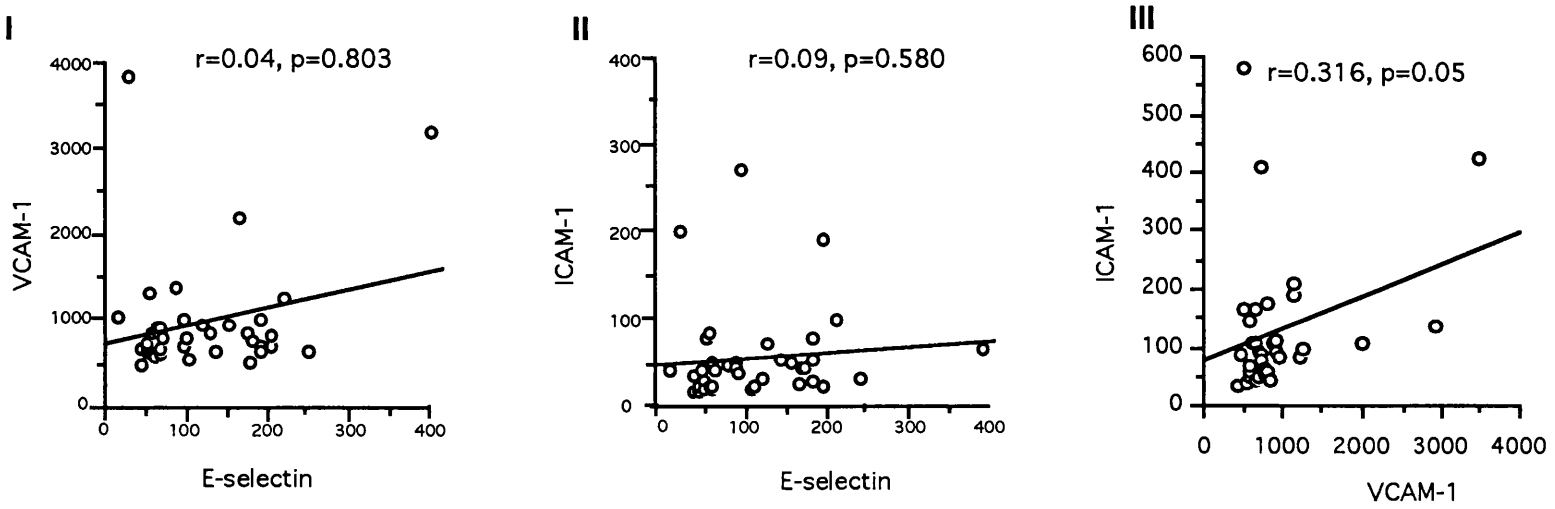
III



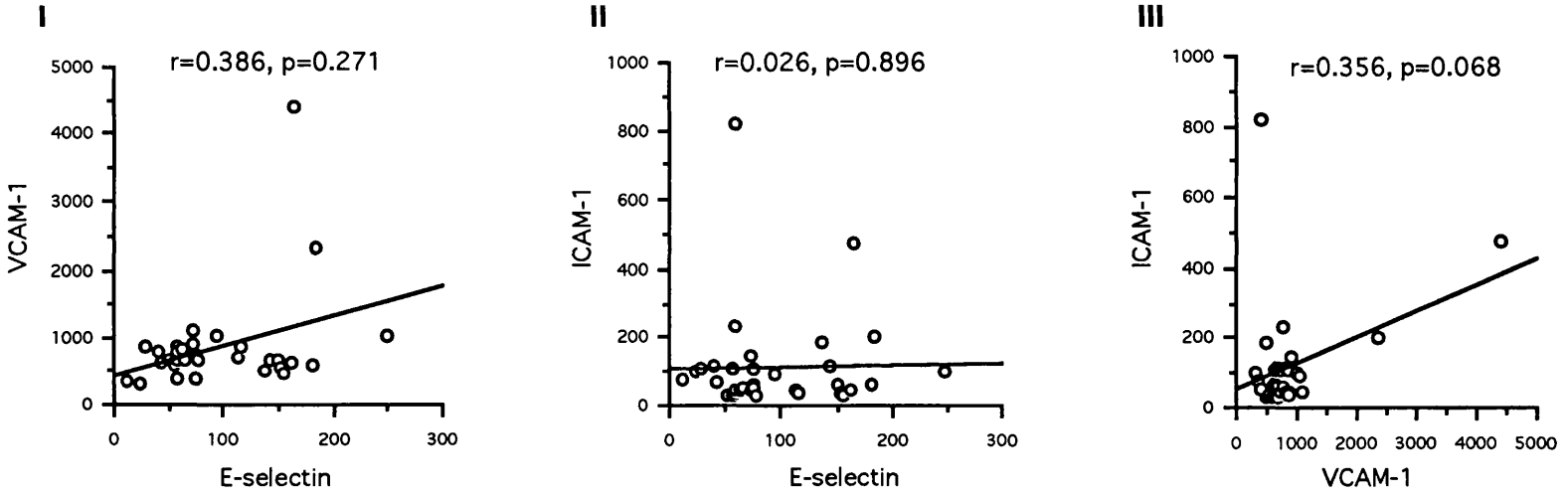
B. DIFFUSE SYSTEMIC SCLEROSIS



C. LIMITED SYSTEMIC SCLEROSIS



D PRIMARY RAYNAUD'S PHENOMENON



E MORPHEA

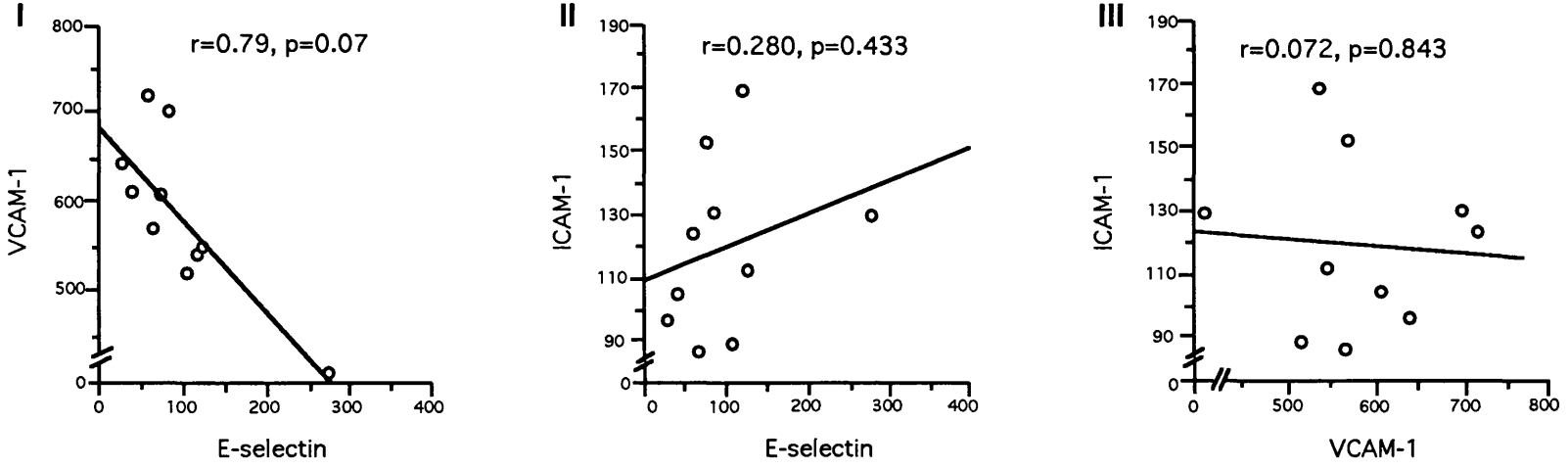


Table 5.3 Circulating levels of E-selectin, ICAM-1 and VCAM-1 and organ involvement

SSc with type of organ involvement	Circulating E-selectin Mean ± SEM	Circulating VCAM-1 Mean ± SEM	Circulating ICAM-1 Mean ± SEM
Lung involvement			
+	108.7 ± 11.1	919.7 ± 92.3	94.2 ± 12.6
-	134.4 ± 9.7	853.5 ± 65.7	89.4 ± 13.1
Renal involvement			
+	117.7 ± 19.7	1226.9 ± 149.5	67.8 ± 13.9
-	119.1 ± 8.1	857.9 ± 60.3	92.3 ± 9.7
Oesophageal involvement			
+	123.5 ± 8.7	896.5 ± 79.6	72.4 ± 6.70
-	108.8 ± 16.7	857.4 ± 98.2	101.8 ± 21.2
Muscle			
+	119.0 ± 8.7	890.2 ± 69.2	94.0 ± 10.5
-	119.3 ± 16.0	909.2 ± 104.4	74.1 ± 13.3

+ indicates clinical evidence of organ involvement
- no clinical evidence of organ involvement

5.3.5 Dermal expression of ICAM-1, VCAM-1 and E-selectin in RP and SSc

Routine histologic assessment of haematoxylin and eosin stained sections was performed on 10 paired clinically "uninvolved" and involved dcSSc skin biopsies, 1 "autoimmune" RP, 4 primary RP and 4 control skin biopsies. Light microscopic examination of all the dcSSc biopsies revealed varied thickening of the dermal collagen network, flattening of the dermal-epidermal junction, perivascular and periappendageal infiltrates (10/10 involved and 8/10 clinically "uninvolved" biopsies) and variable loss of dermal adnexal and microvascular structures (data not shown). In contrast, the RP and normal skin biopsies showed normal-appearing dermal and epidermal features with no perivascular and periappendageal inflammatory infiltrates.

In order to examine the dermal expression of ICAM-1, VCAM-1 and E-selectin in consecutive sections taken from the above biopsies, immuno-histochemical staining was performed using mAbs to ICAM-1, VCAM-1 and E-selectin. The expression of ICAM-1 in normal skin was lower than that seen in clinically "uninvolved" and involved dcSSc and "autoimmune RP" biopsies and was restricted to endothelial and matrix associated cells i.e. fibroblast or dermal macrophages. There was no detectable staining of ICAM-1 on keratinocytes (fig 5.2A) in normal skin. Staining with the mAbs to VCAM-1 and E-selectin were barely detectable in normal control skin (fig 5.2B and 5.2C for E-selectin, VCAM-1, respectively). Similar staining patterns for all three molecules were seen in the primary RP skin biopsies.

Visual appraisal of the sections from the clinically "uninvolved" and involved dcSSc biopsies clearly showed the increased expression of all three adhesion molecules compared with normal control sections. Fig 5.2D,E and F show the presence of ICAM-1, VCAM-1 and E-selectin respectively, localised to the vascular areas in clinically "uninvolved" skin. Fig 5.2G, H and I show the presence of ICAM-1, VCAM-1 and E-selectin in involved dcSSc skin. Although the expression of E-selectin was limited to endothelial cells only (see figs 5.2F and 5.2I), ICAM-1 and VCAM-1 were expressed on endothelial cells and by closely associated perivascular cells. Additionally, ICAM-1 was detected on basal keratinocytes and matrix cells ie, fibroblasts, dendritic cells and dermal macrophages (figs 5.2A, D and G). ICAM-1 was found expressed on more dermal structures than E-selectin or VCAM-1.

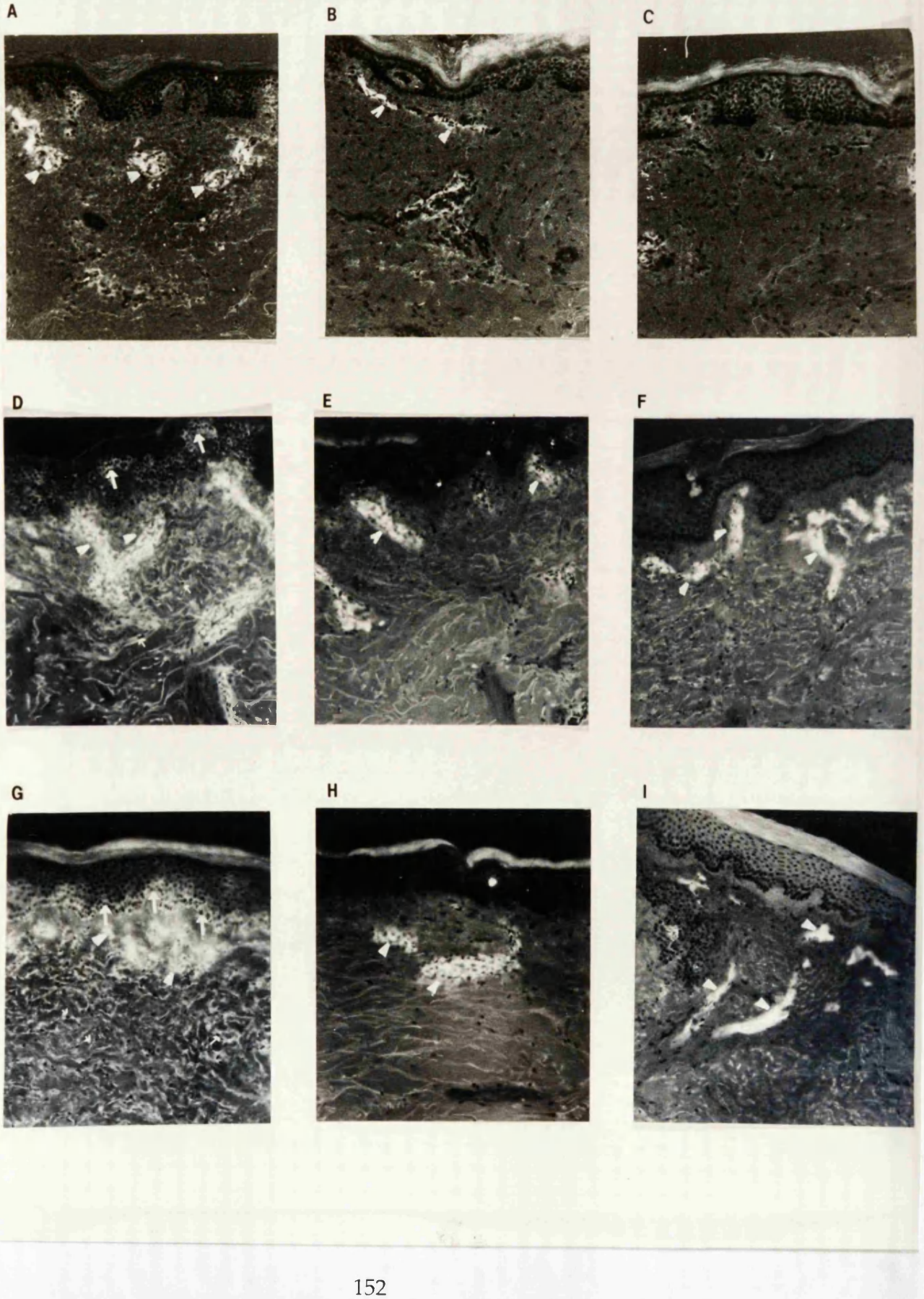
The pattern of expression of ICAM-1, VCAM-1 and E-selectin in early active involved SSc skin showed a similar localisation to that seen in clinically "uninvolved" skin (fig 5.2G,H and I). All the SSc dermal sections had higher levels of E-selectin (5.2F and 5.2I) and VCAM-1 staining (5.2E and 5.2H) in comparison with normal control skin (5.2C and 5.2B, respectively) and primary RP skin (not shown). The expression of ICAM-1, VCAM-1 and E-selectin in the

"autoimmune" RP biopsy was similar in distribution to the SSc skin biopsies (not shown).

Figure 5.2: The expression of ICAM-1, VCAM-1 and E-selectin in normal and SSc dermal biopsies

Immunofluorescent detection of the ICAM-1, VCAM-1 and E-selectin in the normal healthy skin (A-C), clinically "uninvolved" skin (D-F) , early actively involved SSc skin (G-I). Scale bar = 200μm. Immunofluorescent staining of normal skin with; **A.** mAb to ICAM-1 showing staining of blood vessels only (white arrowheads). **B.** mAb to VCAM-1 showing some vascular staining (white arrowheads) and **C.** mAb to E-selectin. Note minimal staining.

Parallel cryostat sections from clinically "uninvolved" skin; **D.** with mAb to ICAM-1 showing staining of dermal vessels (white arrowheads) and perivascular infiltrates, basal keratinocytes (large white arrows) and matrix cells (small white arrows). **E.** with mAb to VCAM-1 showing positive staining of blood vessels only (white arrowheads). **F.** with mAb to E-selectin showing intense staining of vascular endothelium (white arrowheads). Immunofluorescent staining of lesional skin; G. with mAb to ICAM-1 revealed intense staining of blood vessels (white arrowheads), matrix cells (small white arrows) and keratinocyte layer (large white arrows). H with the mAb to VCAM-1 showing marked staining of dermal vessels and perivascular infiltrates (white arrowheads). I. with mAb to E-selectin showing intense staining of vessel endothelium (white arrowheads).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5.3.6 Pulmonary expression of ICAM-1, VCAM-1 and E-selectin in normal and FASSc biopsies

Histological assessment of FASSc and normal lung biopsies was performed after haematoxylin and eosin staining of sections from 7 FASSc and 4 normal lung biopsies. Light microscopic examination revealed that 6/7 FASSc biopsies, except one biopsy taken from a clinically "unaffected" part of the lung (no CT evidence of disease) revealed moderate to marked interstitial fibrosis, alveolar loss, alveolar epithelial cell hyperplasia, moderate to severe interstitial inflammatory infiltrates and lymphoid follicles. Although the unaffected FASSc biopsy did not show marked abnormal features, some hypercellularity and mild alveolar wall thickening were present suggesting early pathologic involvement. In contrast, the normal lung biopsies showed normal alveolar architecture with no features of intra-alveolar or interstitial inflammation. The affected FASSc biopsies were the same as those used previously when ET-1 and ET receptors were examined.

Immunohistochemical staining was performed using mAbs to ICAM-1, VCAM-1 and E-selectin to examine the expression of these major adhesion molecules in SSc and normal lung biopsies. The expression of ICAM-1 in normal lung (fig 5.3 A) was shown by a number of structures including vessels, alveolar epithelium and scattered interstitial cells. There was no detectable staining of VCAM-1 and E-selectin in normal healthy lung (fig 5.3B and 5.3C, respectively).

In contrast to the expression in normal lung, sections from all the FASSc biopsies showed an increased expression of ICAM-1 and VCAM-1 compared with normal control sections. E-selectin was detectable in 4/7 FASSc biopsies. Fig 5.3 D,E and F show the presence of ICAM-1, VCAM-1 and E-selectin, respectively, in a representative FASSc biopsy. ICAM-1 was widely expressed by a large number of structures in both the unaffected and affected FASSc biopsies in association with the alveolar epithelium (alveolar epithelial type II cells), vessels (both small interstitial vessels, large vessels and vessels associated with

lymphoid follicles) and interstitial cells (fibroblasts, macrophages). A marked increase in the expression of ICAM-1 was shown by alveolar epithelial cells with a plump cuboidal morphology characteristic of type II alveolar epithelial cells (fig 5.3D and 5.4A). This was the predominant epithelial cell type lining alveoli in FASSc biopsies.

While moderate VCAM-1 staining was detected in all 7 of the FASSc biopsies in scattered interstitial vessels, it was particularly noted in association with lymphoid follicles (see fig 5.3E). In the biopsies where E-selectin was detected, staining was shown by endothelial cells in scattered interstitial vessels. E-selectin staining was not detected in the endothelium of large pulmonary vessels or vessels associated with lymphoid follicles.

Figure 5.3: Pulmonary expression of ICAM-1, VCAM-1 and E-selectin in normal and FASSc biopsies

Immunohistochemical detection of the ICAM-1, VCAM-1 and E-selectin in normal healthy lung (A-C) and FASSc (D-F). Scale bar = 100μm. A. Note widespread ICAM-1 staining localised to the alveolar epithelium (small arrows) and endothelium (large open arrows). B and C. No staining of normal control sections with mAbs to VCAM-1 and E-selectin, respectively. D. Note the intense localisation of ICAM-1 in FASSc to alveolar type II epithelial cells (small arrows), matrix cells (arrow heads) and vessels (large open arrows). E. Note VCAM-1 localisation in FASSc with small blood vessels (small arrows) and lymphoid follicle (large open arrow). F. Note barely detectable expression of E-selectin (small arrows) in FASSc.

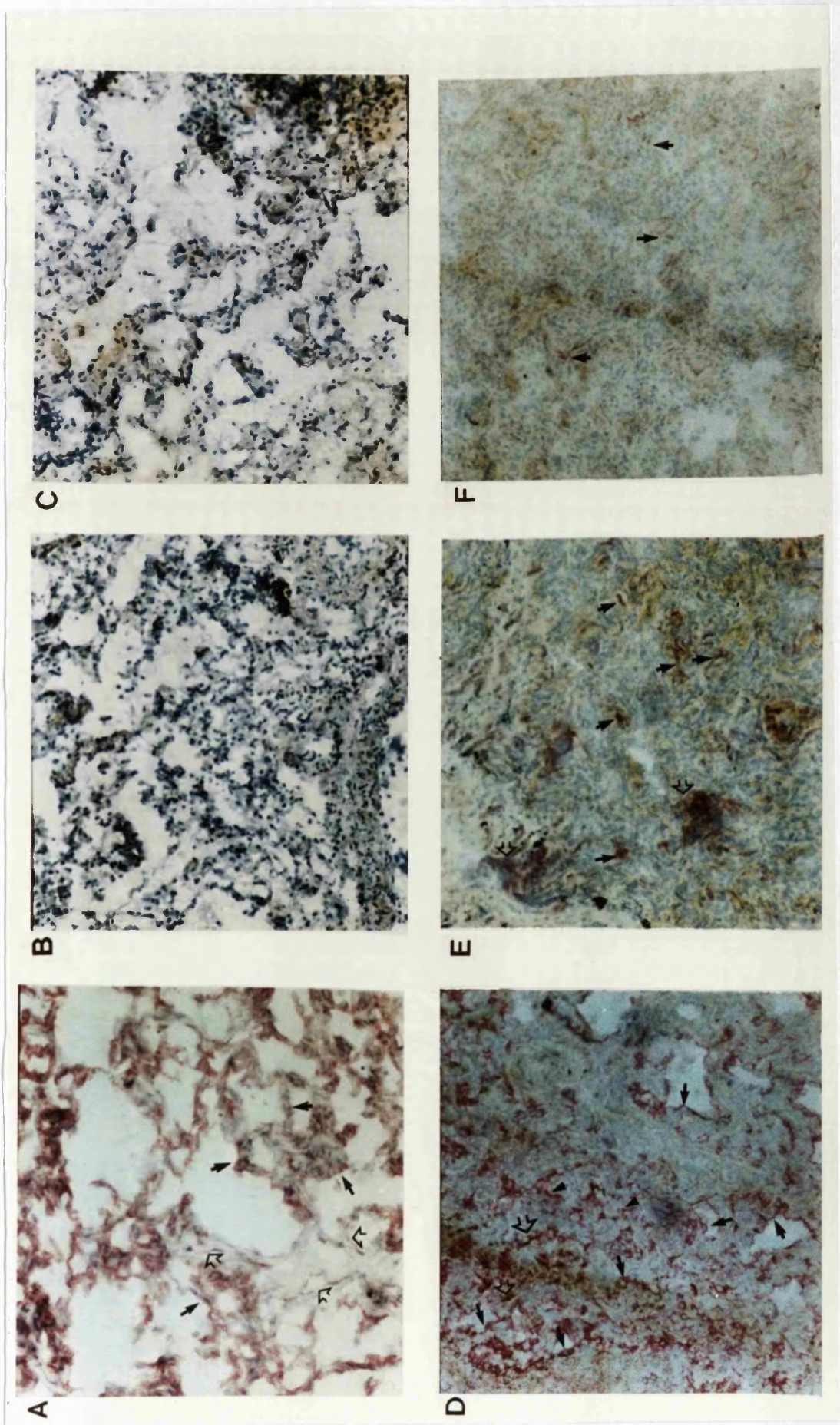
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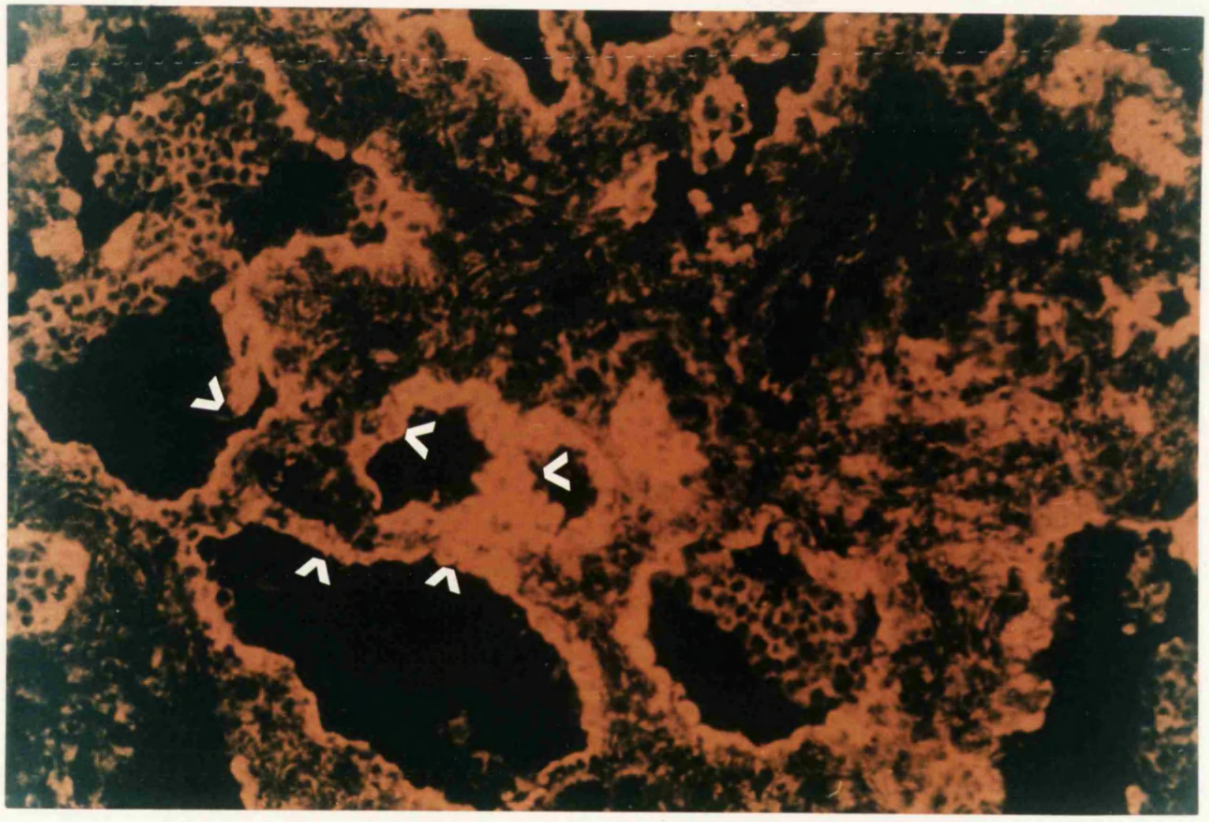
Figure 5.4: Pulmonary epithelial cell expression of ICAM-1



Cryostat section from FASSc biopsy immunostained with mAb to ICAM-1. Note immunofluorescence particularly associated with alveolar epithelial type II cells (small white arrowheads). Scale bar = 200μm.

5.3.7 Quantitation of ICAM-1, VCAM-1 and E-selectin expression in SSc skin and lung.

The fluorescent intensity of vascular expression (marked by QBEND 10^+ endothelial cells) of ICAM-1, VCAM-1 and E-selectin in the SSc, RP and control skin biopsies were measured using a KONTRON IPS image analysis system and are expressed in mean fluorescent units (MFU) $\pm$ SEM in Table 5.4. Similarly, the vascular expression of ICAM-1, VCAM-1 and E-selectin in interstitial vessels, large pulmonary vessels and vessels associated with lymphoid follicles in FASSc and normal lung biopsies were measured and are shown in Table 5.5. Lastly, the alveolar epithelial cell expression of ICAM-1 was also analysed in FASSc biopsies and normal biopsies and is expressed as MFU $\pm$ SEM per cell (see fig 5.5).

5.3.7.a Dermal vascular expression of ICAM-1, VCAM-1 and E-selectin

An approximately 4 fold higher vascular expression of ICAM-1 was observed in dcSSc biopsies compared with normal vessels. Primary RP vessels did not show a significant increase in the vascular expression of ICAM-1. The dermal vascular expression of VCAM-1 was significantly increased in clinically "uninvolved" dcSSc skin biopsies only (approximately 2 fold) but not in involved dcSSc and RP compared with normal biopsies.

The dermal vascular expression of E-selectin in clinically "uninvolved" and involved dcSSc skin was markedly higher (26 and 42 fold, respectively) than that observed in primary RP and normal control biopsies (see Table 5.4). A high expression of E-selectin was also seen in the "autoimmune" RP biopsy (ANA positive RP patient) similar to the dcSSc biopsies. Additionally, involved skin biopsies showed a further increase in the expression of E-selectin compared with "uninvolved" SSc skin ($p=0.07$).

Lastly, there was no difference in the expression of the E-selectin, VCAM-1 and ICAM-1 between papillary and deep dermal vessels in any of the patient biopsies (data not shown).

5.3.7.b Pulmonary vascular expression of ICAM-1, VCAM-1 and E-selectin

The fluorescent intensities of expression of ICAM-1, VCAM-1 and E-selectin were also examined in vessels in FASSc and normal lung biopsies (see Table 5.5). The level of ICAM-1 expression was significantly increased in all vessels in FASSc biopsies relative to controls: interstitial vessels (2.6 fold, $p<0.002$), vessels associated with lymphoid follicles (1.9 fold, $p<0.05$) and large pulmonary vessels (approximately 2 fold, $p=0.015$). A significantly increased expression of VCAM-1 was also shown by small interstitial vessels (approximately 4 fold, $p=0.022$) and vessels associated with lymphoid follicles (5 fold, $p=0.003$) in FASSc biopsies relative to controls. Large pulmonary vessels of greater than 20μm in diameter did not reveal significantly raised VCAM-1 levels ($p=0.08$). Examination of both the clinically unaffected and affected FASSc biopsy pair, revealed no difference in the vascular expression of ICAM-1 or VCAM-1.

While the levels of expression of ICAM-1 and VCAM-1 were both significantly raised in FASSc vessels relative to controls, the average vascular expression of E-selectin in FASSc biopsies was not significantly increased compared with normal control vessels (1.6 fold, $p>0.05$).

5.3.7.c Alveolar epithelial cell expression of ICAM-1

Alveolar epithelial cells were found to express ICAM-1 in both normal and FASSc biopsies. The fluorescent intensity of expression of ICAM-1 was measured in alveolar epithelial cells and divided by the cell number. As shown in fig 5.5, all seven FASSc biopsies revealed a striking increase in the expression of ICAM-1 by alveolar epithelial cells (alveolar epithelial type II cells) compared with normal lung biopsies (epithelial type I cells) (approximately 3 fold increase, $p<0.001$). Furthermore, the expression of ICAM-1 by epithelial cells was not different between the unaffected and affected FASSc biopsy pair (average fluorescence units of 128 ± 10.0 versus 96.0 ± 4.0, respectively).

Table 5.4: Dermal vascular expression of ICAM-1, VCAM-1 and E-selectin

Patient group	n	ICAM-1 MFU ± SEM	VCAM-1 in MFU ± SEM	E-selectin in MFU ± SEM
Healthy controls	4	13.0± 6.70	12.9 ± 4.50	1.0 ± 3.42
Involved SSc skin	10	50.2± 6.90†	20.2 ± 4.70	46.0 ± 6.70*†
Clinically "uninvolved" skin	10	49.1 ± 5.90†	29.4 ± 4.40†	26.4 ± 7.40*†
"Autoimmune" Raynaud's	1‡	28.0 ± 0.00	16.0± 0.00	27.8 ± 0.00
Primary Raynaud's	4	25.0 ± 8.30	17.5 ± 5.00	9.5 ± 3.80†

n- number of biopsies

* - $p<0.05$ in comparison with dermal biopsies taken from patients with primary Raynaud's phenomenon.

† - $p<0.05$ in comparison with normal control biopsies.

‡ - no statistical analysis was performed as only 1 biopsy

Table 5.5: Pulmonary vascular expression of ICAM-1, VCAM-1 and E-selectin

Patient group	n	ICAM-1 in MFU ± SEM	VCAM-1 in MFU ± SEM	E-selectin in MFU ± SEM
Healthy controls	4			
Small interstitial vessels		34.0 ± 11.5	19.1 ± 5.1	2.7 ± 1.7
Large vessels+		32.8 ± 14.8	18.2 ± 4.8	2.3 ± 1.2
Lymphoid follicles++		ND	ND	ND
All FASSc biopsies	7			
Small interstitial vessels		88.6 ± 6.5‡	71.1 ± 3.7‡	4.6 ± 1.3
Large vessels+		67.0 ± 2.9‡	44.3 ± 9.2	3.5 ± 0.8
Lymphoid follicles++		63.0 ± 3.7‡	85.8 ± 5.1‡	ND
Unaffected FASSc biopsy*	1			
Small interstitial vessels		77.0 ± 5.0	56.0 ± 5.0	6.7 ± 0.6
Large vessels+		58.0 ± 3.0	41.0 ± 6.0	6.7 ± 1.0
Lymphoid follicles++		67.0 ± 10.3	74.0 ± 5.0	ND
Affected FASSc biopsy*	1			
Small interstitial vessels		73.3 ± 3.0	38.0 ± 4.0	1.9 ± 0.7
Large vessels+		68.0 ± 4.0	36.0 ± 5.0	1.5 ± 0.9
Lymphoid follicles++		62.0 ± 7.9	97.0 ± 6.0	ND

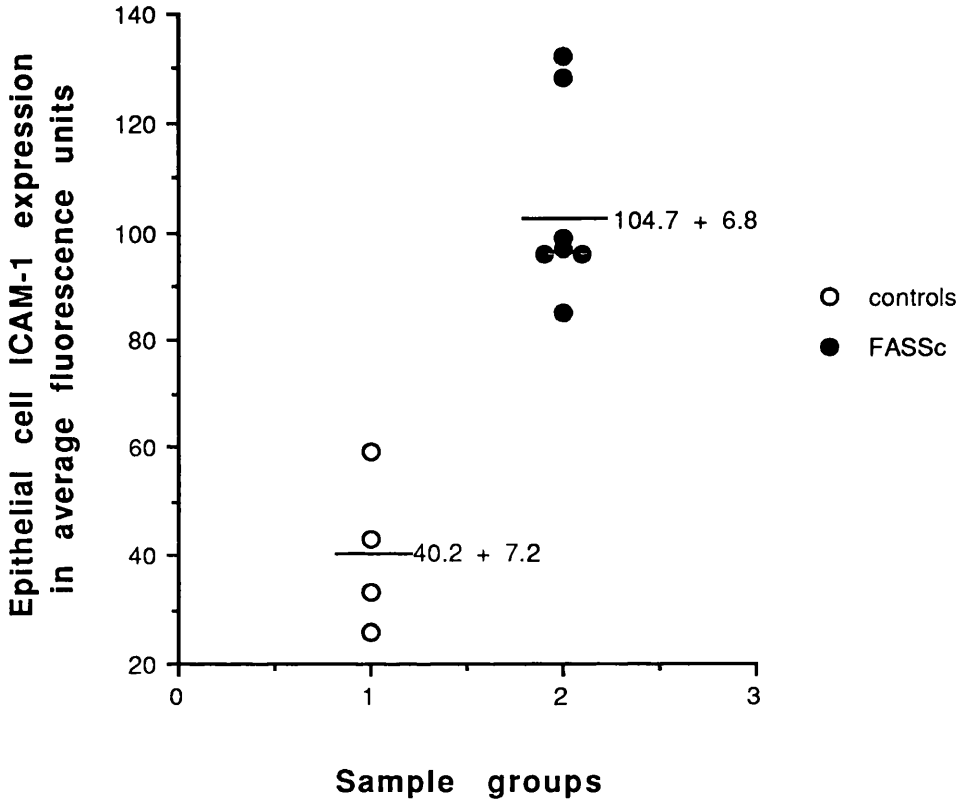
+ Vessels were considered large when their diameter was greater than 20μm

++ Vessels associated with lymphoid follicles

* These results are derived from the paired biopsies obtained from one FASSc patient.

‡ $p<0.05$ on comparison of FASSc results with matched normal control data. No statistical comparisons were made between the data derived from the paired affected and unaffected FASSc biopsies

Figure 5.5 : ICAM-1 expression in alveolar epithelial cells in normal and FASSc lung biopsies



The average fluorescent intensities of ICAM-1 expression by alveolar epithelial cells was quantified and is shown for 4 normal lung biopsies (open circles) and for 7 FASSc biopsies (closed circles). Normal lung epithelial cells were predominantly alveolar epithelial type I cells while FASSc cells were predominantly alveolar epithelial type II cells.

5.4 DISCUSSION

The cellular infiltrates and the timing of the inflammatory process are strikingly different between SSc dermal and lung lesions. In order to gain insight into the mechanisms that contribute to inflammation in the skin and lung in SSc, I have examined the pattern of expression of the major adhesion molecules in both the circulation and in tissues (skin and lung) in SSc. The results of this chapter provide evidence for the increased expression of circulating E-selectin and circulating VCAM-1 in SSc. In addition, while a striking increase in the vascular expression of E-selectin and ICAM-1 was observed in dcSSc skin lesions, FASSc biopsies revealed no change in the expression of E-selectin but a significant increase in the expression of VCAM-1 and ICAM-1. These results provide evidence for the expression of altered levels of adhesion molecules in SSc which are known to mediate immune interactions and additionally show a significant variation in the type of adhesion molecules that mediate leukocyte interactions in the skin compared with the lung.

Circulating adhesion molecules

Examination of the circulating levels of E-selectin, ICAM-1 and VCAM-1 revealed similar levels of E-selectin and ICAM-1 and a seven fold higher expression of VCAM-1 is present in normal healthy volunteers. The physiological role of circulating adhesion molecules in normal individuals in unknown but these levels were found to be comparable to a number of other studies where the levels of circulating adhesion molecules were measured (Gearing *et al.*, 1993). The mechanisms by which these constitutive levels are maintained in normal sera is also unclear as are the tissue source. The generally higher basal levels of circulating VCAM-1 in normal sera may suggest an important physiological role for this molecule. The cell types which contribute to the pool of circulating pool of VCAM-1 may include bone marrow stromal cells and dendritic cells in lymphoid tissues (Rice *et al.*, 1991; Ryan *et al.*, 1991). An

apparent relationship was also observed between the circulating levels of E-selectin, ICAM-1 and VCAM-1 in healthy volunteers which was differentially altered with disease.

In SSc, significantly elevated levels circulating levels of E-selectin and VCAM-1 and a modest increase in ICAM-1 (not significant) were present. Although, previous investigators have also reported similar results in independent smaller studies (Carson *et al.*, 1993; Sfikakis *et al.*, 1993), this chapter additionally provides novel data showing raised levels of circulating VCAM-1 in SSc. The results shown here also provide data for all three circulating adhesion molecules within the same study.

Significantly raised circulating levels of E-selectin and VCAM-1 in 82% and 55%, respectively were also seen in "autoimmune" RP patients, in contrast to primary RP where circulating E-selectin was not raised. In morphea characterised by predominantly localised dermal lesions, although a modest increase in circulating E-selectin and ICAM-1 was present, the levels of VCAM-1 was near normal. Since over 95% of SSc patients present with RP as the first symptom, raised levels of circulating E-selectin in patients with "autoimmune" RP who consequently progressed to SSc compared with those with primary RP alone may suggest that this molecule may provide an additional marker for the early detection of SSc in patients with RP. The presence of increased levels of circulating E-selectin in "autoimmune" RP (prescleroderma), dcSSc and lcSSc further suggests the presence of early sustained vascular activation throughout the course of SSc. The differential increase in the circulating forms of E-selectin, ICAM-1 and VCAM-1 in SSc, RP and morphea suggest that diverse pathophysiological mechanisms are involved in these related disorders .

The mechanism by which adhesion molecules are released into the circulation in disease is still unknown, and it is not clear whether they have a function in regulating cellular interactions. Gearing *et al.*, 1993 postulated that they may competitively inhibit binding of leukocytes to their membrane bound counterparts and thus regulate cellular adhesion . In contrast, Heufelder *et al.*,

1993, revealed that circulating ICAM-1 present in patients with Graves ophthalmopathy promotes binding of mononuclear cells to fibroblasts. There is no evidence so far in humans for alternatively spliced forms of E-selectin, ICAM-1 or VCAM-1 lacking transmembrane domains and it is therefore probable that the presence of circulating adhesion molecules is due to shedding from cell surfaces. Since the cellular expression of each of the three molecules is upregulated by cytokines and other factors, levels of circulating adhesion molecules may reflect inflammatory activity, or the rate of membrane turnover and clearance from the circulation.

A number of *in vitro* reports have shown increased ICAM-1 expression by dermal fibroblasts in SSc (Needleman 1990a; Abraham *et al.*, 1991; Shi-Wen *et al.*, 1994). It is therefore plausible that circulating levels of ICAM-1 could reflect increased fibroblast activation. Since circulating levels of ICAM-1 were similar in both dcSSc and lcSSc, diseases characterised by significantly different levels of fibrosis, such a mechanism appears unlikely. This observation is consistent with a previous report showing a lack of correlation between circulating ICAM-1 levels and skin score (Sfikakis *et al.*, 1993). Other likely sources of circulating ICAM-1 in SSc include vascular endothelial cells and mononuclear cells (Rothlein *et al.*, 1991; Pigott *et al.*, 1992; von Asmuth *et al.*, 1992). These levels of these molecules also did not correlate with visceral organ involvement. These negative results were unexpected as previous reports have shown increased levels of circulating ICAM-1 in idiopathic pulmonary fibrosis, and a close correlation between circulating VCAM-1 and renal function (Shijubo *et al.*, 1992; Gearing *et al.*, 1993).

Dermal expression of E-selectin, ICAM-1 and VCAM-1

In addition to our observations on the circulating forms of E-selectin, ICAM-1 and VCAM-1, I also studied the level of expression of these adhesion molecules in dermal biopsies obtained from SSc, "autoimmune" (prescleroderma) and primary RP patients. At the light microscopic level, the pattern of

expression of E-selectin, ICAM-1 and VCAM-1 in the "autoimmune" RP biopsies was similar to that seen in clinically "uninvolved" and involved dcSSc skin biopsies in our study and in previous reports on SSc (Claman *et al.*, 1991; Majewski *et al.*, 1991; Gruschwitz *et al.*, 1992; Sollberg *et al.*, 1992; Koch *et al.*, 1993). E-selectin was expressed by dermal endothelial cells only, while VCAM-1 was expressed by vessels and perivascular cells. The expression of ICAM-1 was more widespread and was expressed by vessels, perivascular cells, keratinocytes and matrix cells. Although an increased expression of ICAM-1 is shown by keratinocytes in SSc skin, previous investigators have consistently noted the lack of intraepidermal β1 integrin expressing leukocytes (Gruschwitz *et al.*, 1992; Sollberg *et al.*, 1992).

In semi-quantitative studies in SSc, previous investigators have shown that ICAM-1 and E-selectin expression in SSc skin may be unchanged (Claman *et al.*, 1991; Gruschwitz *et al.*, 1992) or increased (Claman *et al.*, 1991; Majewski *et al.*, 1991; Sollberg *et al.*, 1992; Koch *et al.*, 1993) and VCAM-1 decreased (Koch *et al.*, 1993) with disease progression. Upon quantitation of the vascular expression of E-selectin, ICAM-1 and VCAM-1, the expression of E-selectin was strikingly increased in dcSSc lesions (20 to 40 fold) while ICAM-1 was modestly raised in biopsies from both clinically "uninvolved" and involved dcSSc skin. An approximately 20 fold higher expression of E-selectin was also observed in "autoimmune" RP skin in contrast to primary RP suggesting that E-selectin is involved early in the expression of dermal SSc. Increased VCAM-1 levels were present only in clinically "uninvolved" dcSSc suggesting an early transient event in disease. The striking increase in E-selectin in all the dcSSc biopsies may suggest that this molecule predominantly mediates the lymphocyte accumulation in skin in SSc. This is plausible based on the observation that a subset of T cells which express the CLA, a counter-receptor for E-selectin, migrate specifically to the skin.

It should be noted that this quantification was made on overall vascular staining in the case of ICAM-1 and VCAM-1, as it was impossible to separate the

staining of endothelial cells from that of sub-endothelial structures such as pericytes and smooth muscle cells. It is apparent from the study of "autoimmune" RP and clinically "uninvolved" skin that even normal appearing skin can exhibit increased adhesion molecules particularly E-selectin, perhaps reflecting early events in disease pathogenesis.

Pulmonary expression of E-selectin, ICAM-1 and VCAM-1

The pulmonary expression of E-selectin, VCAM-1 and ICAM-1 was also measured in SSc and normal lung biopsies as virtually nothing is known about the expression of adhesion molecules in FASSc lesions. Examination of normal lung biopsies revealed a minimal expression of E-selectin and VCAM-1 while ICAM-1 was detected in alveolar epithelial cells and endothelial cells.

Examination of FASSc biopsies revealed a barely detectable expression of E-selectin in 4 out of 7 biopsies in striking contrast to dcSSc skin biopsies. In addition, VCAM-1 was significantly expressed by vessels and perivascular cells and the expression of ICAM-1 was more widespread and was shown by vessels, alveolar epithelial type II cells and matrix cells.

Quantitation of the vascular expression of E-selectin, ICAM-1 and VCAM-1 in FASSc and normal lung biopsies revealed a significantly increased level of expression of VCAM-1 and ICAM-1 in small pulmonary vessels: interstitial vessels and vessels associated with lymphoid follicles, with no change in E-selectin. Large pulmonary vessels revealed a significant increase in ICAM-1 only. The raised expression of ICAM-1 and VCAM-1 in vessels associated with lymphoid follicles was interesting and suggests that leukocytes expressing β1 and β2 integrins may be involved in the formation of lymphoid follicles in FASSc.

The increased vascular expression of VCAM-1 in FASSc was notable particularly in view of the increased number of eosinophils seen in FASSc lesions and the observation that VCAM-1/VLA-4 interactions predominantly mediate endothelial-eosinophil binding (Walsh *et al.*, 1991; Weg *et al.*, 1993). The

predominant adhesion molecules that mediate neutrophil binding are E-selectin and ICAM-1. The results of this study showed that ICAM-1 alone is significantly raised in FASSc biopsies suggesting that ICAM-1 and not E-selectin may mediate neutrophil adhesion in FASSc. Gimbrone *et al.*, 1989 revealed that the affinity of neutrophil binding to endothelial cells can be modulated by IL-8 by reducing the level of E-selectin and upregulating the level of ICAM-1/CD11b mediated binding. It is therefore possible that a similar mechanism may regulate the affinity of neutrophil adhesion in the lung in SSc as increased levels of IL-8 are present in FASSc biopsies (Southcott *et al.*, 1993b). Additional support for ICAM-1 dependant neutrophil accumulation in lung disease is provided by antibody blockade experiments where mAbs to ICAM-1 and CD18 were found to predominantly inhibit neutrophil accumulation in animal models of pulmonary inflammation (Barton *et al.*, 1989; Argenbright *et al.*, 1991).

The expression of adhesion molecules in FASSc noted in this chapter is similar to that shown by Georas *et al.*, 1992, following segmental antigen challenge in animal models where increased eosinophils, neutrophils and basophils were seen in BALF. Eosinophils expressed significantly higher CDw49d (VLA-4) levels, the ligand for VCAM-1, while neutrophils expressed 2-3 fold higher CD11b with a marked decrease in L-selectin, a counter receptor for E-selectin.

The marked increase in ICAM-1 expression by alveolar epithelial type II cells in all the FASSc biopsies suggests that epithelial type II cells may also mediate immune cell interactions in FASSc. Increased mononuclear cells, and granulocytes cells are present in close proximity to the alveolar epithelium and have been shown to precede fibroblast activation in pulmonary fibrotic disorders (Vyalov SL., *et al.*, 1993). Furthermore, in this study, both the unaffected and affected FASSc biopsies appeared to have alveolar epithelial type II cells which expressed markedly high levels of ICAM-1. Based on these observations, it is possible that this cell plays a significant role in maintaining the inflammation seen throughout the pulmonary disease process in contrast to the transient

inflammation seen in SSc skin lesions. Increased ICAM-1 expression by type II alveolar epithelial cells is not peculiar to FASSc and has been noted in other idiopathic pulmonary fibrotic disorders (Shijubo *et al.*, 1992).

In conclusion, the results of this chapter show novel data with respect to the significant increase in the circulating levels of E-selectin in both patients with "autoimmune" RP who later progressed to SSc and in all the SSc patient groups in contrast to primary RP which suggests the use of this molecule as an early marker of progression to SSc. It also shows that circulating VCAM-1 is significantly raised in SSc. Perhaps the most interesting observations made in this chapter was the very striking increase in the dermal vascular expression of E-selectin in dcSSc skin biopsies in contrast to FASSc biopsies where no change in vascular expression of E-selectin was present. In addition, while the vascular expression of VCAM-1 was transiently increased in clinically "uninvolved" dcSSc skin biopsies, there was a significant increase in the expression of VCAM-1 in FASSc. ICAM-1 was similarly elevated in both skin and lung biopsies in SSc patients with alveolar epithelial type II cells providing an additional cell type which contributes to the pulmonary expression of ICAM-1 in SSc.

This chapter provides important new information on the circulating, dermal and pulmonary expression of E-selectin, ICAM-1 and VCAM-1 in SSc and shows that significant differences in the expression of E-selectin and VCAM-1 are present in the two most affected organs in SSc. These differences may contribute to the previously documented variation in inflammation in the skin and lung and in the expression of SSc in these organs.

CHAPTER SIX:

PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF NORMAL SKIN AND LUNG FIBROBLASTS

6.1 INTRODUCTION

Fibroblasts and their extracellular matrix products play a critical role in maintaining the structural integrity of organs, in reparative processes and also in a number of pathological disorders. In the study of SSc and of fibrotic disorders in general, considerable attention has been directed to one facet of disease: the immune events which are thought to be responsible for altering fibroblast metabolism. In these studies, the fibroblast is viewed as a passive cell responding to changes in the physiological environment. An alternative, and not mutually exclusive hypothesis suggests abnormalities in the metabolic properties of fibroblast populations or fibroblast sub-populations. Sub-populations of normally quiescent fibroblasts which produce high levels of matrix may be selected and expanded by cytokines in disease. Alternatively, an intrinsically abnormal sub-population(s) of fibroblasts which has undergone a regulatory genetic mutation may express high levels of matrix components in disease.

Fibroblasts are generally identified by morphological and functional characteristics which are shared among a class of mesenchymal cells including not only the prototypic dermal fibroblast but also lung, synovial, tendon, periosteal, gingival and other fibroblasts (Korn *et al.*, 1992). A diverse number of diseases are characterised by fibrosis occurring in specific sites such as keloids, interstitial lung disease, rheumatic heart disease, cirrhosis of the liver, renal interstitial fibrosis, periodontal fibrosis (Dalvi 1993). Furthermore, in SSc, fibrosis is only targeted to selective organs. The reason for the development of organ-specific fibrosis has been suggested to be due to the presence of different fibroblast populations in different organs (Korn *et al.*, 1992; Dalvi 1993).

So far, the evidence for organ-specific fibroblast heterogeneity has been largely conflicting in studies which have examined adult human fibroblasts derived from different organs within the same study. Investigators have documented substantial differences between fibroblasts from the skin and lung with respect to growth kinetics (Schneider *et al.*, 1977) and steroid hormone

metabolism (Kondo *et al.*, 1985; LeRoy 1985). In contrast, Rodemann *et al.*, 1989 provided little evidence for differences in ultrastructure and protein synthesis between both normal embryonic skin and lung fibroblasts.

A second, more subtle type of heterogeneity has been shown to exist among fibroblasts within a tissue. Such studies are more numerous and show that sub-populations of human diploid fibroblasts differ with respect to their morphology (Goldring *et al.*, 1990; McCulloch *et al.*, 1991; Atherton *et al.*, 1992), synthetic function (Botstein *et al.*, 1982; Goldring *et al.*, 1990), growth kinetics (Hassell *et al.*, 1983), receptor expression (Akamine *et al.*, 1992; Elder *et al.*, 1992) and response to cytokines and the complement component C1 (Brinkerhoff *et al.*, 1981; Korn *et al.*, 1984; Korn 1985a; Korn *et al.*, 1985b; Feldman *et al.*, 1993). Fibroblasts isolated from pathologic sites, such as dermal SSc lesions, keloids and rheumatoid synovium appear generally activated but show a variation in their biosynthetic capacity supporting the concept of an expansion of certain fibroblast sub-populations in disease (Korn *et al.*, 1992).

Evidence suggests therefore that the development of fibrosis may depend on the expansion and activation of a normal/abnormal sub-population of fibroblasts and also on the activation of certain organ-specific cells. Despite the ease of propagation of fibroblasts *in vitro*, there has been very little definitive information on the characteristics of fibroblasts derived from different tissues and their possible heterogeneity which may be important in the expression of disease in different organs. This is partly because of the lack of monoclonal antibodies to specific antigens which are unique to fibroblasts. In this chapter, in order to delineate the characteristics of fibroblasts derived from normal skin and lung, I have measured a measured the rate of cell proliferation, matrix expression and the expression of a number of cell surface antigens in fibroblasts cultured from normal adult skin and lung biopsies. In addition, I have examined the *in vitro* and *in vivo* expression of a number of thus far unidentified antigens using four mAbs originally obtained using foreskin fibroblasts. Lastly, in order to compare cell-cell interactions in both cell

types, I have also measured the level of T lymphocyte interactions with skin and lung fibroblasts.

6.2 PATIENTS AND STATISTICS

6.2.1 Samples

Dermal punch biopsies ($4mm^3$) were taken from the forearm of 14 normal healthy volunteers (5 females, 9 males, aged between 35 and 52 years; average age was 41 years). Normal lung biopsies were taken from the periphery of unaffected regions of lobectomy or pneumonectomy samples from 8 patients undergoing thoracotomy for tumor resection (2 females, 6 males, aged between 35 and 77 years, average age was 56 years). These biopsies were considered unaffected after histological examination revealed no evidence of pathology.

6.2.2 Statistical analysis

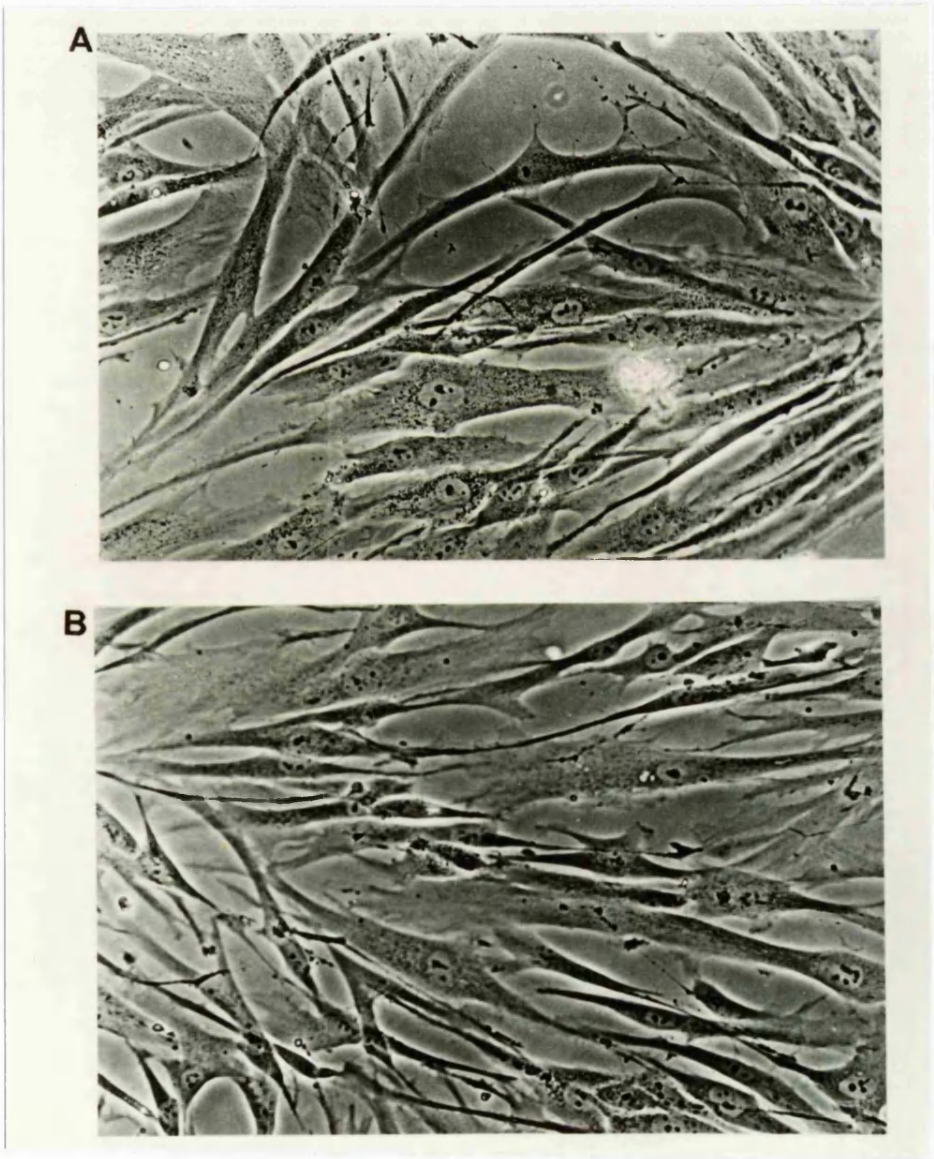
Statistical comparisons between the various phenotypic parameters measured in normal skin and lung fibroblasts were made using the Student's t-test.

6.3 RESULTS

6.3.1 Cell morphology

As shown in fig 6.1, all the cells in cultures of the skin and lung biopsies had the characteristic spindle-shaped morphology of fibroblasts.

Figure 6.1: Morphologic appearance of normal skin and lung fibroblasts in culture

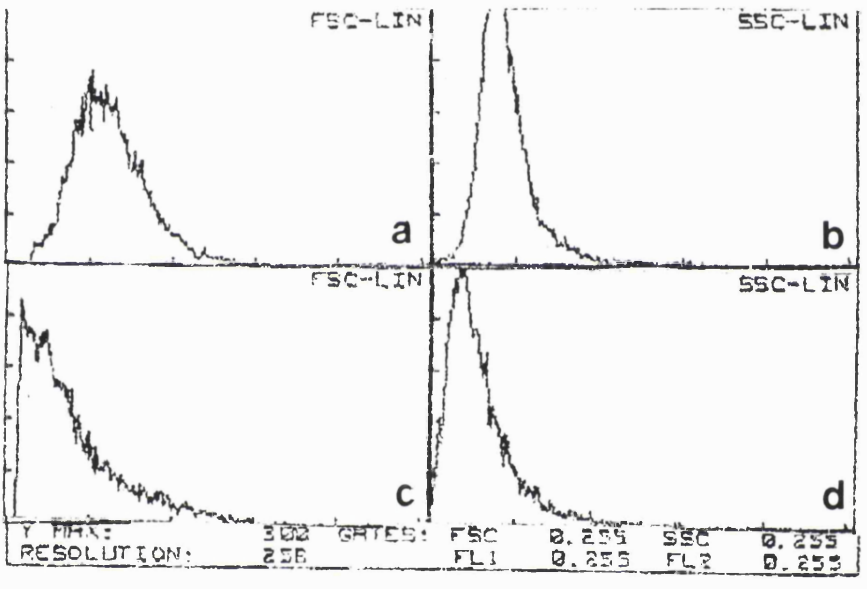


Phase contrast photomicrographs of (a) skin and (b) lung fibroblasts showing the spindle shaped morphology of both cell types. (x200 Magnification)

6.3.2 Phenotypic characteristics of cultured fibroblasts from normal skin and lung

The FCM parameters of forward and side scatter (90°), which reflect cell size and cytoplasmic granularity, respectively, (Carter NP., *et al.*, 1990) were also compared between both skin and lung fibroblasts. As shown in fig 6.2, dermal fibroblasts were found to be 29% larger compared with lung fibroblasts (forward scatter of 56 $\pm$ 7 compared with 40 $\pm$ 3, p=0.0009). Although dermal fibroblasts were also slightly more granular than lung cells, this difference was not statistically significant (side scatter of 14 $\pm$ 1 compared with 11 $\pm$ 1, p>0.05, respectively) (see fig 6.2).

Figure 6.2: Light scattering profiles of normal skin and lung fibroblasts

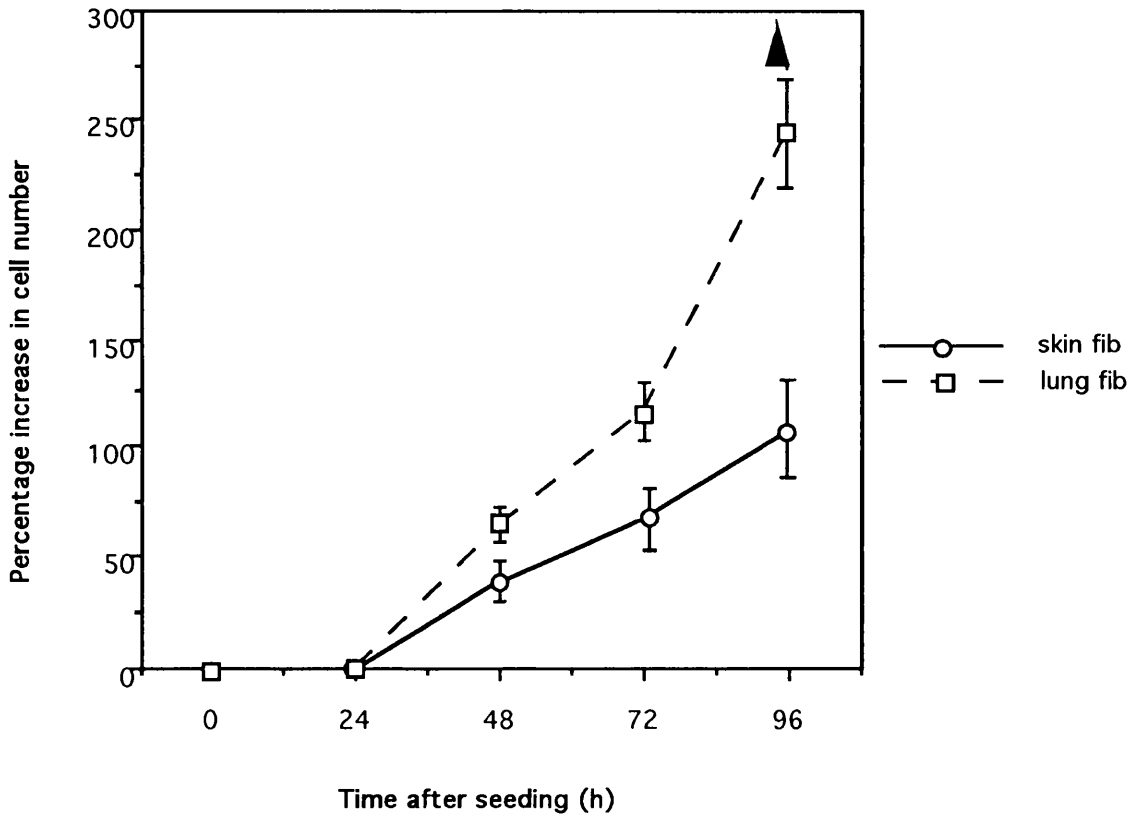


FCM profiles of forward scatter (FSC; a,c) and side scatter (SSC; b,d) of normal skin fibroblasts (top panels) and normal lung fibroblasts (lower panels). Note the markedly increased FSC in skin fibroblasts (a) compared with lung fibroblasts (c). Although the SSC was also slightly increased in dermal fibroblasts (b) compared with lung fibroblasts (d), this difference was not statistically significant.

6.3.3 Cell proliferation

Comparison of the rate of proliferation of the normal skin and lung fibroblasts revealed that while there was no difference in average cell number at 24 h after seeding, the lung fibroblasts were more numerous by 48 h (62%) and markedly higher by 72 h (153%) and 96 h (164%, $p<0.05$), compared with normal skin fibroblasts (see fig 6.3). Thus, the rate of proliferation of the lung cells was clearly greater than that of the dermal fibroblasts, with the number of lung fibroblasts being twice as high at 96 h.

Figure 6.3: Growth characteristics of skin and lung fibroblasts

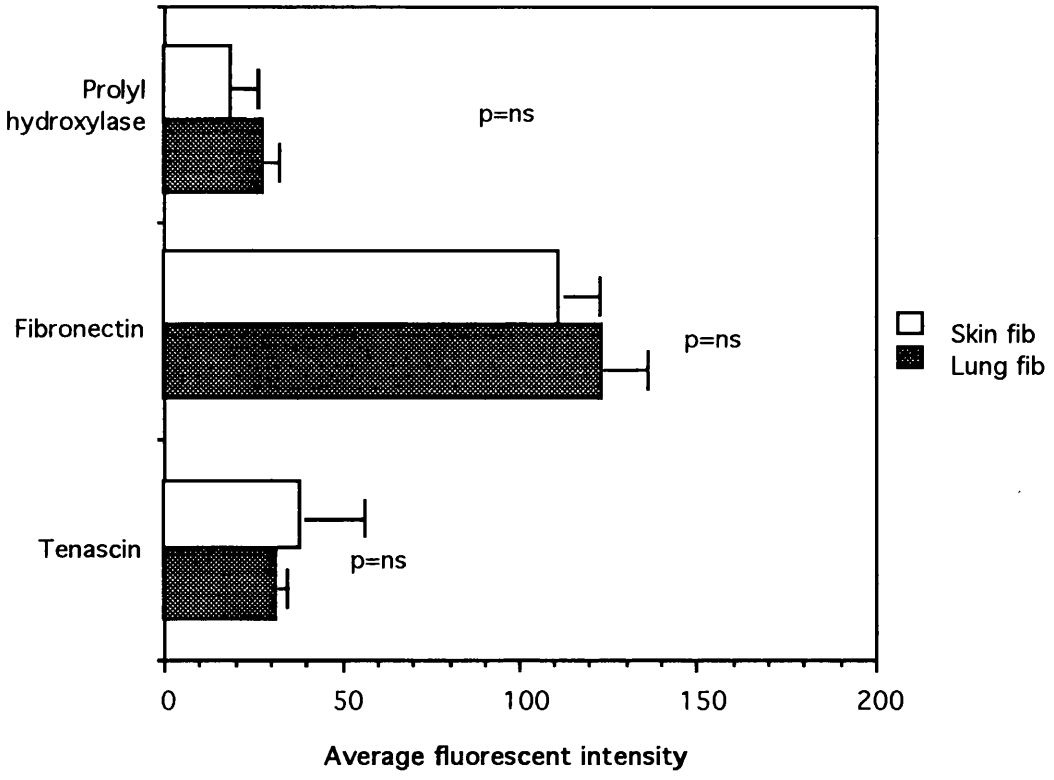


At time points indicated, the average percentage increase in cell number are shown for skin fibroblasts (open circles, solid line) and lung fibroblasts (open squares, dashed line) are shown above. Significant differences ($p<0.05$) between the two cell lines are shown by a closed triangle.

6.3.4 Total cellular matrix expression

The total cellular levels of matrix expression was measured by FCM and the results are shown in fig 6.4. Although normal lung fibroblasts had slightly higher levels of total cellular fibronectin (AFIs of 124 ± 12 versus 112 ± 24), prolyl hydroxylase (AFIs of 28± 3 versus 19 ± 6) and lower levels of tenascin (BC4) (AFIs of 31 ± 16 versus 37 ± 6) compared with normal dermal fibroblasts, these differences were not statistically significant.

Figure 6.4: Total cellular matrix expression

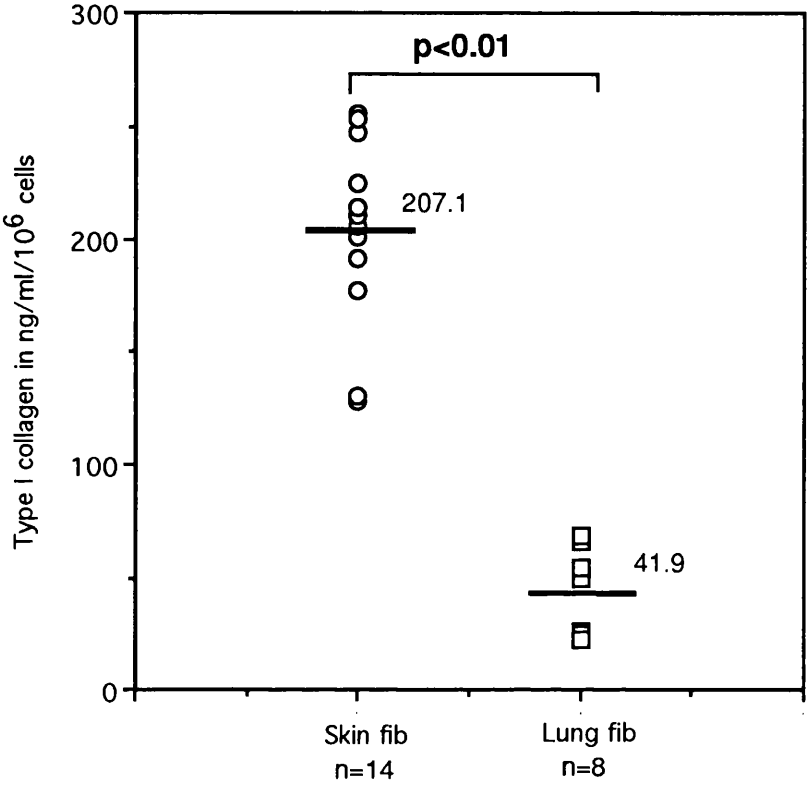


The expression of total cellular matrix proteins in skin fibroblasts (open bars) and lung fibroblasts (shaded bars) and the standard errors are shown above. The comparison of all three parameters in the two types of fibroblasts were not significant (p=ns)

6.3.5. Type I collagen secretion

The secretion of type I collagen *in vitro* was measured in the normal skin and normal lung fibroblast cultures. Using a competition ELISA, approximately 5 times more type I collagen was secreted by dermal fibroblasts in 24 h compared with lung cells (207.10 $\pm$ 31.8 ng/ml per million cells versus 41.9 $\pm$ 9.5 ng/ml per million cells) (see fig 6.5). This 500% difference in type I collagen secretion between normal skin and normal lung fibroblasts was interesting, particularly in view of the similar cellular levels of expression of other matrix components including fibronectin, tenascin and prolyl hydroxylase between the two types of cells.

Figure 6.5: Type I collagen secretion by normal skin and lung fibroblasts.



The mean values of replicate experiments are shown for normal skin fibroblast cultures (open circles) and for normal lung fibroblast cultures (open squares). The mean value for each group is shown by a bar.

6.3.6 Expression of cell surface antigens in cultured fibroblasts from normal skin and lung biopsies

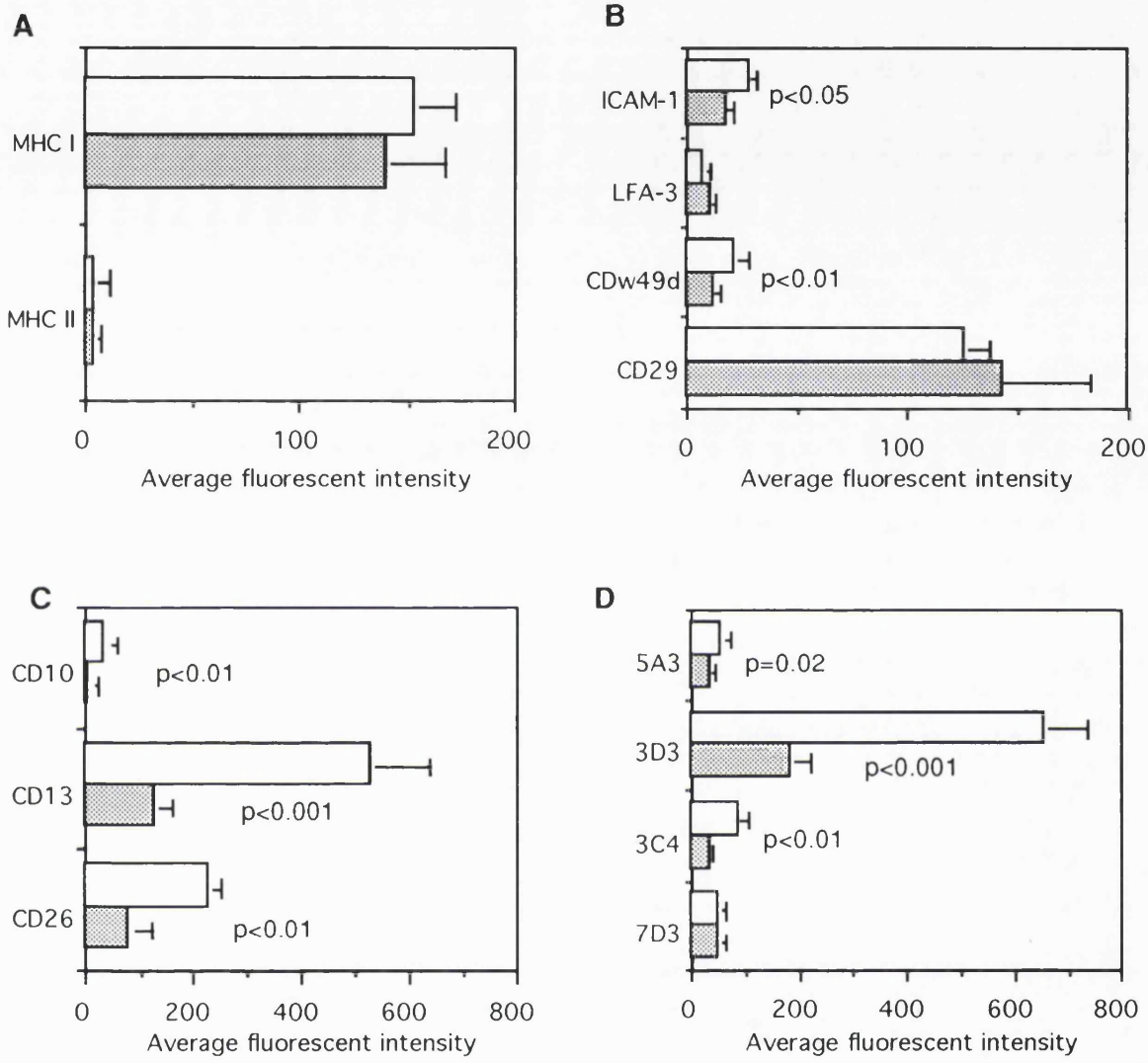
FCM was used to measure the expression (average fluorescent intensity; AFI) of MHC classes I and II, the adhesion molecules ICAM-1, LFA-3, CD29 and CDw49d, the surface ectoenzymes CD10, CD13 and CD26 and the fibroblast antigens identified by the mAbs 5A3, 3D3, 3C4 and 7D3.

The results in fig 6.6A and 6.6B, show that there were no significant differences in the levels of MHC I and II between skin and lung fibroblasts (153 $\pm$ 17 compared with 140 $\pm$ 25 and 5 $\pm$ 4 versus 4 $\pm$ 1, respectively) and in the levels of CD29 and LFA-3 (AFIs of 126 $\pm$ 10 versus 143 $\pm$ 38 and 7 $\pm$ 2 compared with 10 $\pm$ 2, respectively). However, skin fibroblasts did express significantly higher levels of ICAM-1 and CDw49d (AFIs of 36 $\pm$ 2 compared with 26$\pm$ 4, $p=0.02$ and AFIs of 22 $\pm$ 3 versus 12 $\pm$ 1, $p<0.01$, respectively).

Moreover, in addition to the differences noted between skin and lung fibroblasts for ICAM-1 and CDw49d, the level of expression of all the cell surface ectoenzymes were strikingly higher in the dermal compared with pulmonary fibroblasts. As shown in fig 6.6C, CD10 was approximately 500% higher (AFIs of 36 $\pm$ 11 compared with 8 $\pm$ 2, $p<0.01$), CD13 was 400% higher (AFIs of 526 $\pm$ 96 versus 123 $\pm$ 22, $p<0.001$) and CD26 was 300% higher (AFIs of 226 $\pm$ 30 compared with 78 $\pm$ 28, $p<0.01$) in skin fibroblasts compared with lung cells.

Three of the mAbs, 5A3, 3D3 and 3C4, also showed that dermal fibroblasts expressed higher levels of antigen relative to lung cells (AFIs of 54 $\pm$ 11 compared 32 $\pm$ 4, $p=0.02$; 651 $\pm$ 71 versus 184 $\pm$ 25, $p<0.001$ and 84 $\pm$ 12 compared with 28 $\pm$ 3, $p<0.01$, respectively) in contrast to the mAb 7D3, which was similar in both skin and lung cells (AFIs of 49 compared with 46, respectively, see fig 6.6D).

Figure 6.6: Comparative expression of fibroblast cell surface antigens on normal skin and lung fibroblasts



Average Fluorescent Intensities (AFIs) of expression of phenotypic antigens on skin (open bars) and lung fibroblasts (shaded bars) stained with mAbs to; (**A**) MHC I and II (**B**) ICAM-1; CD54, LFA-3; CD58, CD29; β1 integrin, CDw49d; α4 integrin (**C**) CD10; neutral endopeptidase 24.11, CD13; aminopeptidase-N, CD26; dipeptidyl peptidase IV (**D**) 5A3, 3D3, 3C4 and 7D3. Significant differences between skin and lung fibroblasts for the various antigens are shown by p values which are less than 0.05.

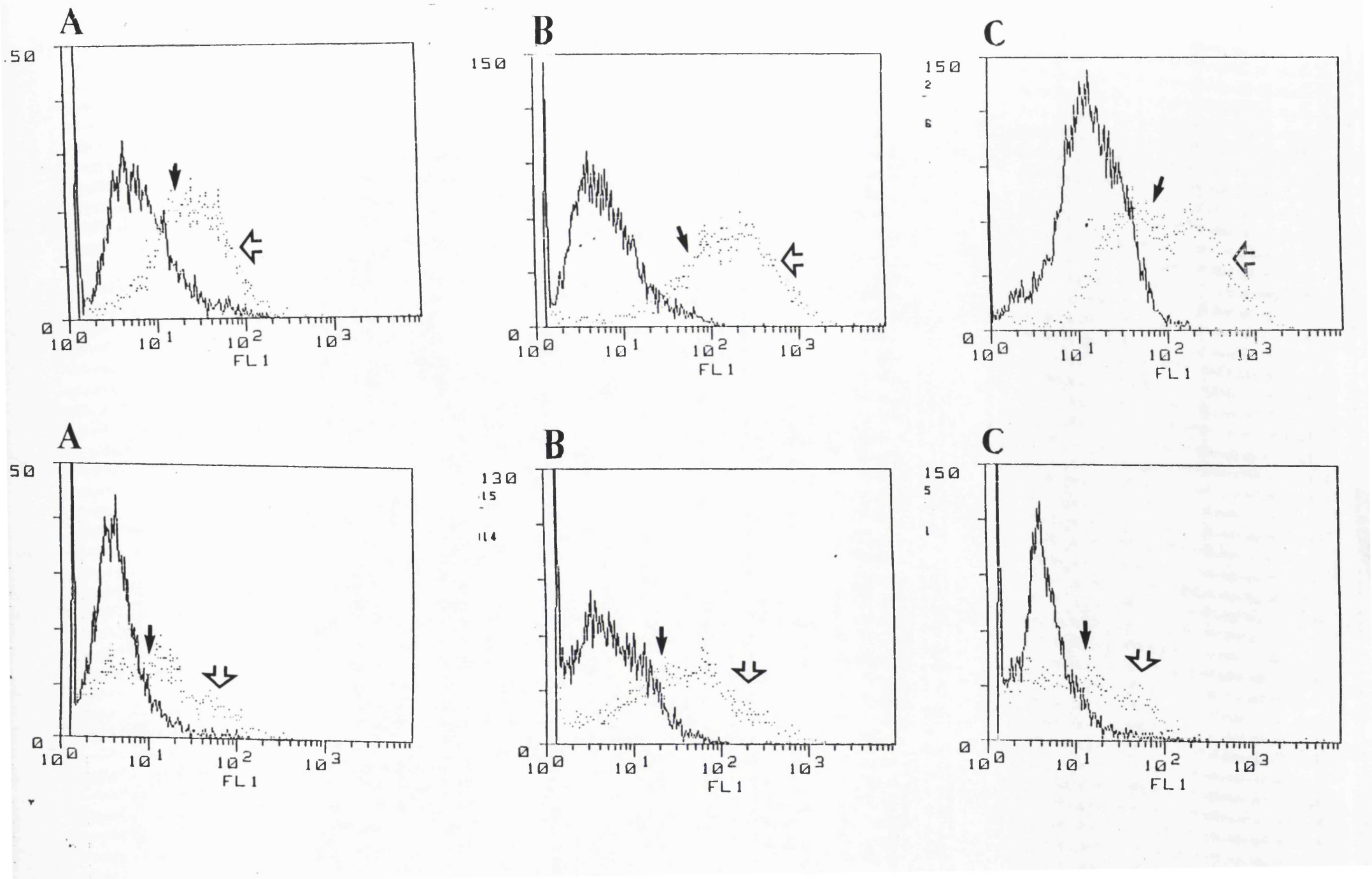
6.3.7 ICAM-1, CD26 and 3C4 distribution in skin and lung fibroblasts

Examination of the FCM profiles of all the mAbs used in this study revealed distinct sub-populations of fibroblasts when the expression of ICAM-1 and CD26, and 3C4 were examined (see fig 6.7). These sub-populations of fibroblasts were present in both dermal and pulmonary fibroblast cultures. Dermal fibroblast sub-populations were classified into ICAM-low cells on the basis of having an AFI less than 30 and ICAM-1-high cells based on having an AFI between 30 and 300. Similarly, CD26-low and 3C4-low fibroblasts had AFIs less than 100 and CD26-high and 3C4-high cells had AFIs between 100 and 2000, respectively. As shown in fig 6.7, pulmonary fibroblasts were also classified into distinct sub-populations by using different AFI values to distinguish the subsets. Thus, an AFI of 20 was used to divide the low and ICAM-1 high sub-populations and an AFI of 30 was used to classify low and high CD26- and 3C4- expressing cells.

The results in Table 6.1 show not only that there are 2 distinct sub-populations in the skin and lung which expressed low and high levels of each of the 3 antigens, but also that the proportion of low and high expressing cells differed between the skin and lung, at least for CD26 and 3C4. Thus, twice as many cells expressed high levels of CD26 in skin as in the lung (30% compared with 14% of the total population) and almost twice as many also expressed high 3C4 levels (40% compared with 23% of the total). However, the proportion of high and low ICAM-1 expressing cells were the same in both the pulmonary and dermal fibroblast populations (35% and 37% respectively).

Figure 6.7 Flow cytometric profiles of ICAM-1, CD26 and 3C4 expression in normal skin and lung fibroblast cultures.

FCM profiles of (A) ICAM-1, (B) CD26 and (C) of staining of the mAb 3C4 in normal dermal fibroblasts (top panel) and normal lung fibroblasts (lower panel). Note the presence of sub-populations of fibroblasts in both cultures (low antigen-expressing cells are shown by small, solid arrows and high antigen-expressing cells are shown by large, open arrows) The dotted lines are the FCM profiles of cells stained with mAbs to ICAM-1, CD26 and 3C4 while control cells stained with isotype-matched mAbs are shown by the continuous lines. The cut-off point for high and low ICAM-1 expressing dermal fibroblasts is an AFI of 30 while pulmonary fibroblasts can be divided on the basis of an AFI of 20. The cut-off points for low and CD26 high cells and low and 3C4 high expressing dermal fibroblasts is an AFI of 100 while pulmonary fibroblasts can be divided by using an AFI of 30.


A
B
C
A
B
C
FL1
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FL1

Table 6.1: Expression of ICAM-1, CD26 and 3C4 in normal skin and lung fibroblast sub-populations

Fibroblast sub-populations	Normal dermal fibroblasts		Normal lung fibroblasts	
	AFI ± SEM	% cells ± SEM	AFI ± SEM	% cells ± SEM
ICAM-1				
Low fibroblasts	8 ± 1	60 ± 5	6 ± 1	65 ± 14
High fibroblasts	89 ± 18	37 ± 5	69 ± 7	35 ± 10
CD26				
Low fibroblasts	51 ± 7	70 ± 4	21 ± 2	86 ± 4
High fibroblasts	665 ± 56	30 ± 4	436 ± 65	14 ± 4
3C4				
Low fibroblasts	23 ± 3	59 ± 2	18 ± 3	75 ± 8
High fibroblasts	182 ± 29	40 ± 2	72 ± 5	23 ± 4

6.3.8 Immunohistochemical staining by novel fibroblast mAbs

In view of the unknown cellular specificity of the mAbs 5A3, 3D3, 3C4 and 7D3, immunohistochemical staining was performed to examine the expression of the respective antigens identified by the mAbs in skin and lung biopsies *in vivo*. As shown in table 6.2, while the mAb 3D3 stained a number of cell types in both skin and lung, 5A3, 3C4 and 7D3 had a far more limited cellular localisation. The mAb 5A3 stained all fibroblasts from both the skin and lung as well as a band of matrix in the sub-epithelial or epidermal-dermal junction (see fig 6.8B and 6.9B) In the skin, the mAb 3D3 stained keratinocytes, endothelial cells, macrophages, adipocytes, smooth muscle cells and papillary, dermal and perivascular fibroblasts (see fig 6.8 A). This was in contrast to the less marked staining seen in alveolar and airway epithelial cells, smooth muscle cells, interstitial fibroblasts and endothelial cells in pulmonary biopsies (see fig 6.9A). Staining with the mAbs 3C4 and 7D3 was barely detected in normal skin

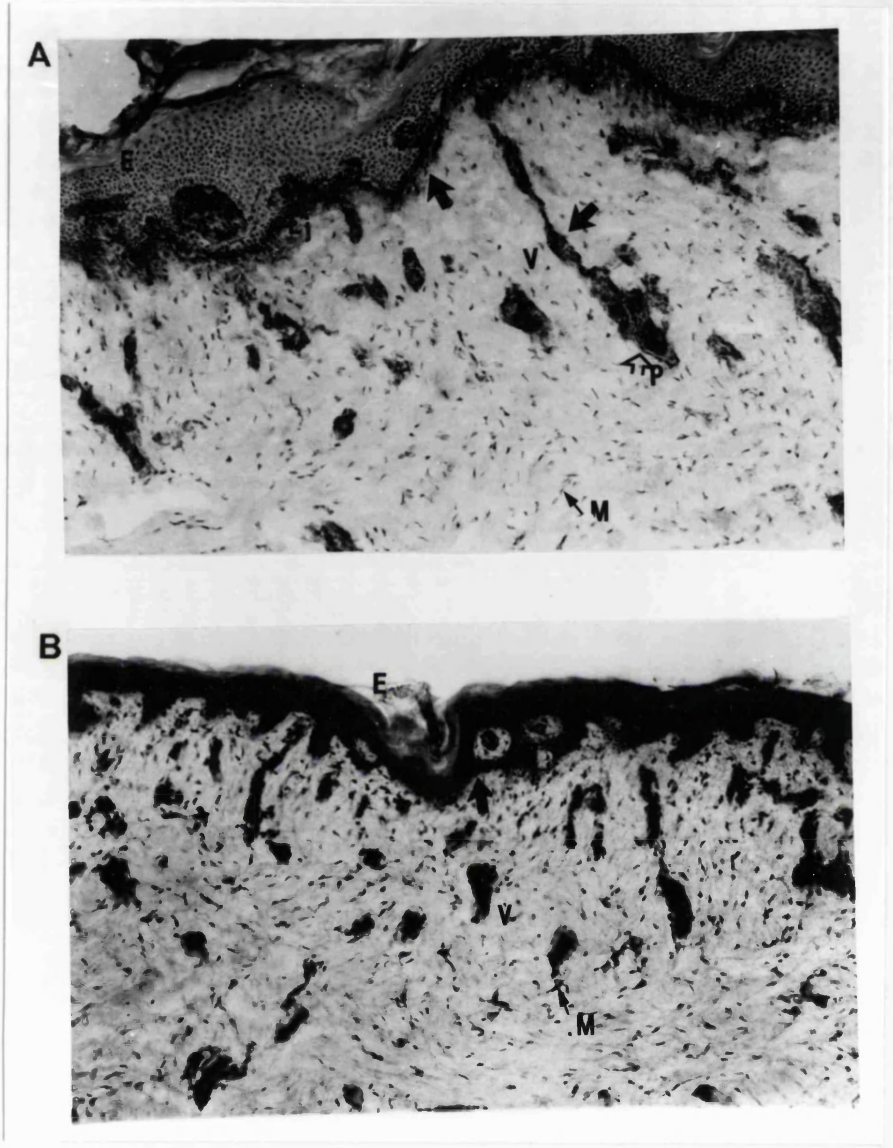
biopsies, in contrast to lung biopsies in where 7D3 staining was localised to interstitial fibroblasts (not shown). Both 3C4 and 7D3 additionally stained smooth muscle cells and endothelial cells, respectively, suggesting a shared phenotype with other cell types.

Table 6.2: Reactivity of cell types in normal adult skin and lung to novel mAbs 5A3, 3D3, 3C4 and 7D3

Type of biopsy	5A3	3D3	3C4	7D3
Skin				
keratinocytes	-	+	-	-
epidermal-dermal junction	+	-	-	-
endothelial cells	-	+	-	±
vascular smooth muscle	-	+	±	-
perivascular fibroblasts	+	+	±	±
papillary fibroblasts	+	+	-	-
deep-dermal fibroblasts	+	+	±	±
macrophages	-	+	-	-
adipocytes	-	+	-	-
Lung				
alveolar epithelium	-	±	-	-
sub-epithelial junction	+	-	-	-
endothelial cells	-	+	-	+
interstitial fibroblasts	±	±	±	+
interstitial macrophages	-	-	-	-
vascular smooth muscle	-	±	+	-
bronchial epithelium	-	±	-	-
bronchial smooth muscle	-	±	+	-

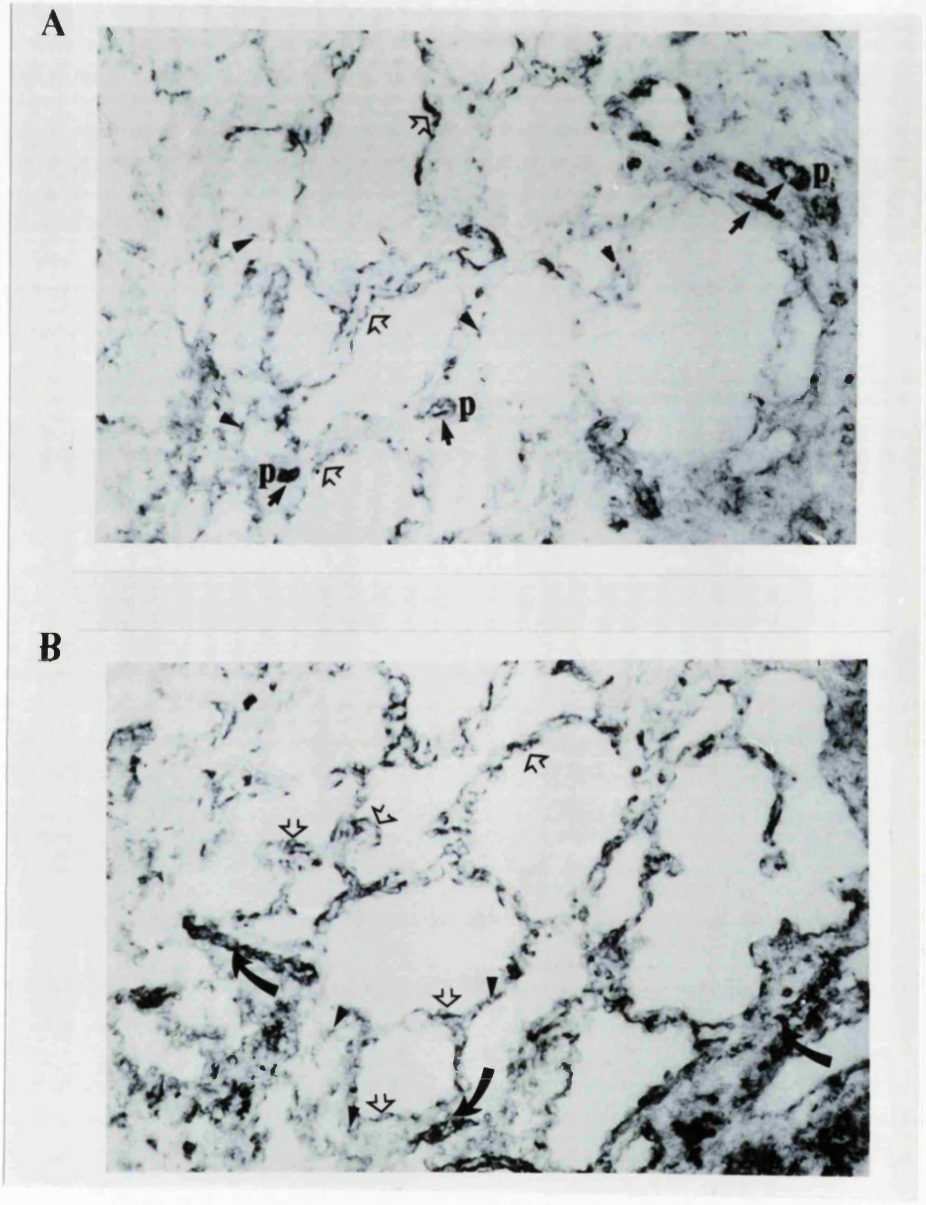
+ positive staining, ± moderate staining, - no staining

Figure 6.8: Reactivity of the mAbs 5A3 and 3D3 in cryostat sections from normal skin



Immunohistochemical localisation of antigens in normal skin identified by the mAbs 5A3 (**A**) and 3D3 (**B**) (X100 magnification). **A**. Note mAb 5A3 staining of the epidermal-dermal junction (Ej, large arrow), perivascular region (P; open arrow) and matrix associated cells (M, small arrows). **B**. Staining of skin with mAb 3D3. Note positive reactivity of matrix associated cells (M), keratinocytes (K), endothelial cells (V) and perivascular regions (P).

Figure 6.9: Reactivity of the mAbs 5A3 and 3D3 in cryostat sections from normal lung



Immunohistochemical localisation of antigens in normal lung identified by the mAbs 5A3 (**A**) and 3D3 (**B**) (X200 magnification). **A**. Note mAb 5A3 staining of the sub-epithelial junction (large open arrow), perivascular regions (P, small arrow) and matrix associated cells (arrowheads). **B**. Staining of lung with mAb 3D3. Note positive reactivity of matrix associated cells (arrowheads), alveolar epithelial cells (large open arrows), endothelial cells (curved arrows) and perivascular region around vessels.

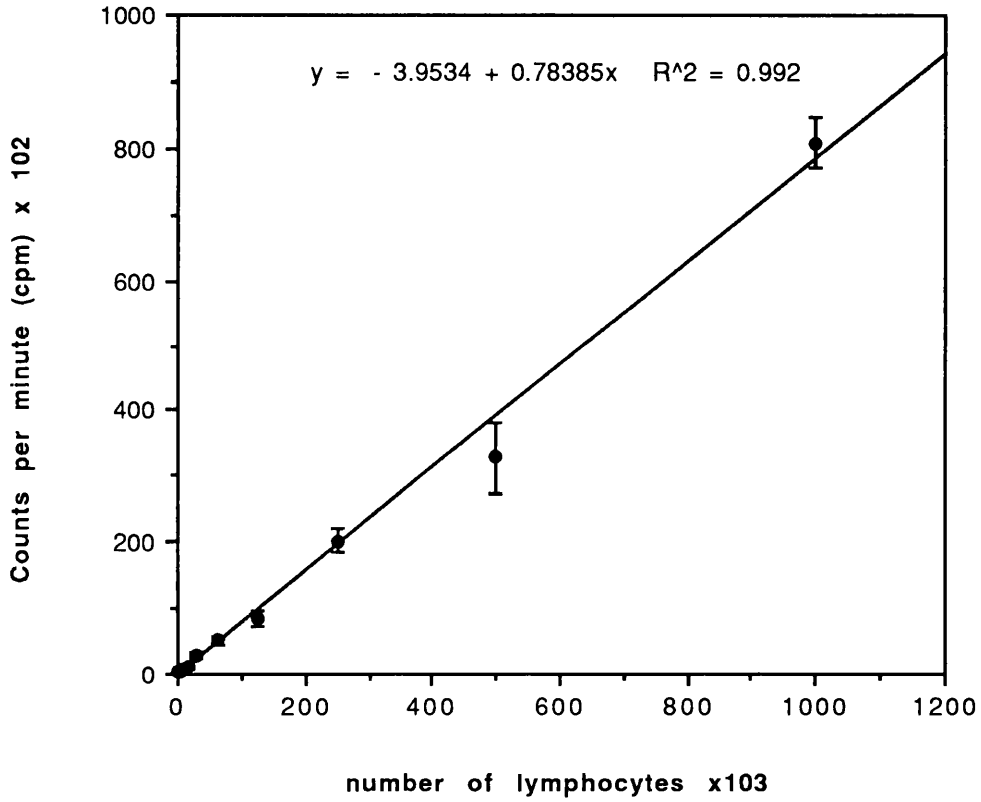
6.3.9 Binding of T lymphocytes to dermal and lung fibroblasts *in vitro*

The adhesion of activated T lymphocytes to dermal and lung fibroblasts was examined by measuring the number of lymphocytes which remained attached to fibroblast monolayers after extensive washing, as described previously in chapter 2. The number of lymphocytes bound to a known number of fibroblasts was determined by comparison with a standard curve of known numbers of labelled lymphocytes. A representative standard curve is shown in fig 6.10 which shows that the uptake of [^{3}H] leucine was directly proportional to the number of lymphocytes present (r=0.996).

The results in fig 6.11 show that the binding of T cells to the dermal fibroblasts was only very slightly higher than the adhesion to lung cells (2.5 $\pm$ 0.2 versus 2.2 $\pm$ 0.2, respectively, P>0.05). However, antibody blockade experiments demonstrated important differences in the molecular mechanisms which mediate the adhesive interactions of T lymphocytes with skin and lung cells.

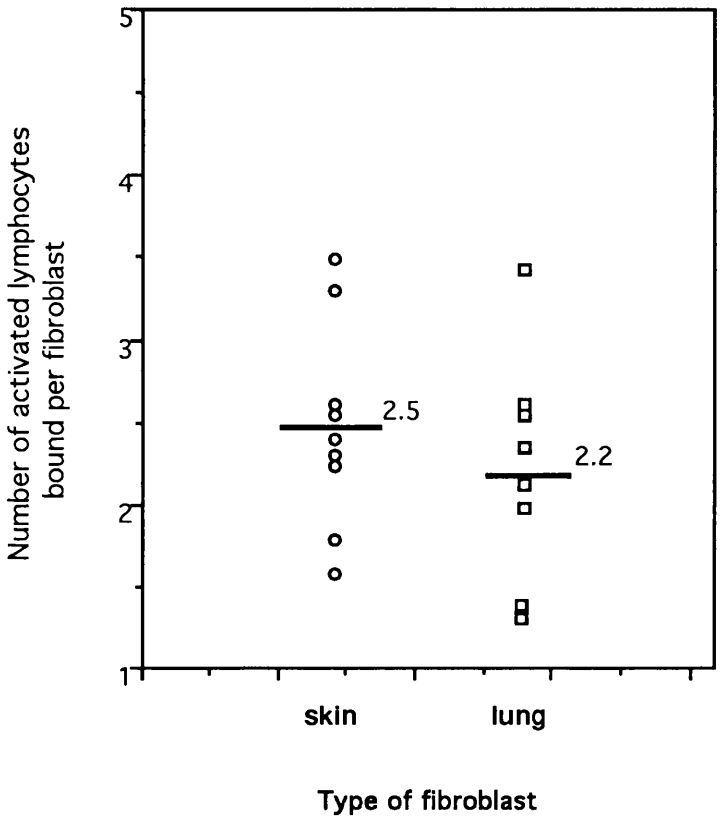
Fig 6.12 shows that despite the very similar levels of binding of T cells to skin and lung fibroblasts, prior incubation of these cells with saturating concentrations of mAbs against ICAM-1 resulted in a far greater reduction of lymphocyte binding to skin cells (45%) compared with a less marked inhibitory effect of lung fibroblasts (20%). In contrast, antibody to LFA-3 inhibited both skin and lung fibroblasts equally (approximately 28%). MAbs against MHC classes I and II and saturating concentrations of control IgG reduced T cell adhesion by less that 5% (data not shown).

Figure 6.10: Standard curve showing the uptake of [^{3}H] leucine by activated T lymphocytes



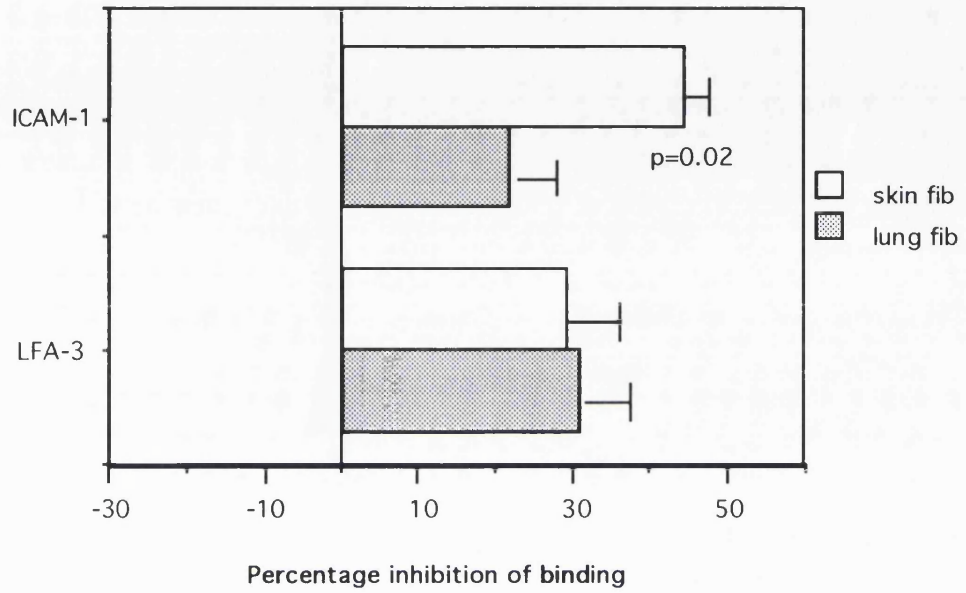
The standard curve shown above represents the average [^{3}H] leucine uptake with standard error bars shown by 4 different activated lymphocyte cultures. [^{3}H] leucine uptake was directly proportional to the number of cells (r=0.996)

Figure 6.11: T lymphocyte binding to skin and lung fibroblasts.



Activated lymphocytes were radio-labelled with [^{3}H] leucine and their adhesion to fibroblasts was measured as described in the Materials and Methods. Each data point represents the average binding to triplicate wells of a fibroblast . The results obtained with different skin fibroblast cultures are shown by open circles while lung fibroblast cultures are shown by open squares. The mean level of binding for each group is shown by a bar with a value.

Figure 6.12. Effects of mAbs on T cell adhesion to skin and lung fibroblasts



The role of ICAM-1 and LFA-3 in T cell binding to skin (open bars) and lung (dotted bars) fibroblasts was measured by antibody blockade experiments as described previously in chapter 2. The bars represent the average of the percentage inhibition of binding of lymphocytes to 5 normal skin fibroblasts (open bars) and 5 normal lung fibroblast (dotted bars) cultures. Prior incubation of fibroblasts with saturating concentrations of the mAbs to MHC classes I and II and non-specific IgG caused less than 5% inhibition of T cell binding to fibroblasts.

6.4 DISCUSSION

The results of this chapter show that there are important differences between the characteristics of fibroblasts obtained from normal skin and lung biopsies. Examination of the rate of cellular proliferation in both cell types revealed that lung cells have a markedly higher rate of proliferation compared with those from skin. Although total cellular levels of fibronectin, tenascin and prolyl hydroxylase (an enzyme involved in collagen synthesis) were nearly the same in both types of fibroblasts, type I collagen secretion was markedly lower in lung cells compared with skin cells. The expression of various surface antigens on both types of fibroblasts revealed that normal skin and lung cells could be readily distinguished by the level of expression of ICAM-1 and CDw49d, reactivity with the mAbs 5A3, 3D3 and 3C4 and the expression of surface proteases.

The increased rate of cellular proliferation observed in pulmonary fibroblasts compared with dermal fibroblasts has been found in a previous study which examined embryonic lung and skin cells (Schneider *et al.*, 1977). These embryonic lung cells were also shown to have a faster DNA synthetic rate, decreased cellular RNA and protein contents and lengthened *in vitro* life spans compared with similar dermal cells.

Examination of the expression of total cellular fibronectin and tenascin showed similar levels of both matrix proteins in both lung and skin fibroblasts. The function of tenascin in normal fibroblasts is unclear but this molecule has been reported to inhibit cell adhesion to fibronectin (Borsi *et al.*, 1992; Carnemolla *et al.*, 1992). Levels of prolyl-4-hydroxylase (Kawaguchi *et al.*, 1992b) were measured since this enzyme is involved in the conversion of prolyl residues to 4-hydroxyproline, an essential metabolic event in collagen synthesis. The expression of this enzyme were therefore used as an indicator of total collagen expression in fibroblasts. Levels of prolyl-4-hydroxylase were similar in lung

fibroblasts versus skin cells, suggesting that similar levels of collagen expression in both cell types.

Although the levels of prolyl 4-hydroxylase were similar in both skin and lung fibroblasts, the levels of type I collagen secretion into culture supernatants was markedly different in both types of fibroblasts. The anomaly between levels of prolyl 4-hydroxylase and type I collagen secretion is interesting and may be explained by a higher associated collagen degradation in lung fibroblasts relative to dermal cells or an increased synthesis of other types of collagens in the lung. Previous investigators have shown a high collagen turnover in the lung compared with other tissues but the intracellular degradation of collagen in pulmonary fibroblasts relative to dermal cells has not yet been examined (Laurent 1986; Harrison *et al.*, 1991).

The presence of different levels of inflammatory infiltrates between SSc skin and SSc lung lesions and the significant differences in ICAM-1 noted between normal skin and normal lung cells prompted the examination of the level of T lymphocyte interactions with both types of normal fibroblasts. These experiments showed that although the extent of T cell interactions with both cell types was similar, the contribution of the ICAM-1 cell adhesion pathway in skin and lung was markedly different, consistent with the difference noted in the level of surface ICAM-1 expression which was higher in skin that lung cells. The contribution of the ligand LFA-3 to interactions of lymphocytes with skin and lung fibroblasts was similar. Thus, the binding of activated T cells to dermal fibroblasts was found to be mediated predominantly via the ICAM-1/LFA-1 pathway, similar to a previous study (Abraham *et al.*, 1991) whereas lung fibroblasts had far lower levels of ICAM-1-dependant adhesion. This observation suggests that dermal and pulmonary fibroblasts utilise different pathways of interactions with lymphocytes.

The most striking difference between dermal and pulmonary fibroblasts was the markedly lower expression of the cell surface ectoenzymes in lung cells compared with skin cells. The physiological implication of lower levels of CD10

(neutral endopeptidase -24.11; NEP; CALLA), CD13 (aminopeptidase-N) and CD26 (dipeptidyl peptidase, DPPIV) in normal lung fibroblasts is unclear since the functions of these plasma membrane ectoenzymes are not fully characterised. CD10 is involved in the hydrolysis of biologically active peptides, particularly neuro-peptides such as bradykinin, angiotensin I and II, atrial natriuretic peptide, enkephalins and ET-1 (Murphy *et al.*, 1988; Bathon *et al.*, 1992; Gardiner *et al.*, 1992) while CD13 completes the hydrolysis of fragments produced by endopeptidase attack (Kenny *et al.*, 1987). CD26 cleaves X-proline dipeptides from the amino terminus of peptides, has a role in promoting cell surface fibronectin and collagen mediated cellular interactions with the extracellular matrix and has also been implicated in cytokine inactivation (Atherton *et al.*, 1992).

Very few mAbs are currently available which delineate the fibroblast phenotype. The present chapter describes the reactivity of four new mAbs derived against foreskin fibroblasts, and shows that each of the mAbs 5A3, 3D3, 3C4 and 7D3 produce a unique staining profile of cells in dermal and lung sections. Although the mAbs 5A3, 3C4 and 7D3 have a limited tissue distribution compared with 3D3, none of the mAbs used in this study were fibroblast-specific as their respective antigens were present on other cells. Moreover, while the mAbs 5A3 and 3D3 showed a significantly decreased level of staining on lung fibroblasts compared with skin fibroblasts *in vivo*, the mAb 7D3 was more readily detected on lung cells compared with skin cells *in vivo*. These observations suggest that there are organ-specific differences in fibroblast phenotypes *in vivo*.

Perhaps the most interesting observations made in this chapter were shown by examination of the FCM profiles of expression of ICAM-1, CD26 and 3C4. These profiles revealed that sub-populations of fibroblasts were present within both skin and lung cultures. Sub-populations of fibroblasts which express different levels of ICAM-1 and CD26 have been shown in previous studies in dermal fibroblasts and breast fibroblasts, respectively (Needleman

1990a; Atherton *et al.*, 1992), and the present data demonstrates that lung fibroblasts are also comprised of sub-populations of different cells. In addition, the staining pattern observed with the mAb 3C4 suggests that this additional new marker may be used to delineate distinct sub-populations of fibroblasts in both skin and lung cultures. The proportion of cells which expressed high levels of ICAM-1 in the lung was similar to that in the skin, while conversely the percentage of cells expressing high levels of the antigens CD26 and 3C4 was much lower in the lung that in skin. These observations provide support for the view that heterogeneous fibroblast sub-populations are present in both tissues and may result in organ-specific fibrotic reactions.

In conclusion, this study provides evidence for the presence of fibroblast heterogeneity and fibroblast sub-populations in both the skin and lung which could lead to differential organ-specific fibroblast responses in disease. Although a number of phenotypic and functional differences exist between these two fibroblast types with respect to cell size, cell proliferation, levels of matrix expression, levels of cell surface antigen expression, both skin and lung fibroblasts express show type of characteristics. It is unclear whether the differences noted between normal skin and lung cells arise because of the presence of extrinsic factors within different organs or if they represent intrinsic differences between resident fibroblasts in the two tissues. It is also unclear, if these heterogeneous organ-specific fibroblast populations respond differently to cytokines and growth factors in disease states.

CHAPTER SEVEN:

PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF FIBROBLASTS DERIVED FROM THE SKIN AND LUNG IN SSc

7.1 INTRODUCTION

The data shown in the last chapter provided evidence that normal skin and lung fibroblasts are significantly different with regard to the level of expression of a number of phenotypic and functional characteristics. The fibrotic process in SSc is believed to result from the local activation of collagen synthesis in dermal and organ based fibroblasts (Scharfetter *et al.*, 1988; Harrison *et al.*, 1991; Kahari 1993). So far, the examination of fibroblast abnormalities in SSc has been almost entirely restricted to studies on dermal cells chiefly as the skin is the most accessible organ to biopsy and very few studies have examined fibroblasts from lung lesions *in vitro*.

The studies on fibroblasts derived from SSc skin lesions have documented a significantly increased synthesis of several extracellular matrix proteins, including types I, III, IV, V and VI collagen, fibronectin and laminin (Xu *et al.*, 1991; Mauch *et al.*, 1993). An elevated expression of the intercellular adhesion molecule-1 (ICAM-1) (Needleman 1990a; Abraham *et al.*, 1991; Shi-Wen *et al.*, 1994) and reduced levels of the surface protease dipeptidyl peptidase IV (DPPIV; CD26) (Bou-Gharios *et al.*, 1995) have also been observed in SSc dermal fibroblasts relative to normal dermal cells.

The selection and activation of an normal/abnormal fibroblast subpopulation which produces very high levels of extracellular matrix proteins has been suggested as a fundamental pathogenic mechanism in SSc (Botstein *et al.*, 1982). This is based on (i) observations of elevated procollagen mRNA only in certain fibroblasts present in SSc dermal biopsies (Kahari *et al.*, 1988), (ii) the identification of selected clones of SSc fibroblasts which produce large amounts of collagen (Korn *et al.*, 1984; Korn *et al.*, 1985b; Whiteside *et al.*, 1988) and (iii) the presence of sub-populations of SSc cells which are more granular (Needleman *et al.*, 1990b) and (iv) the expression of high levels of ICAM-1 (Needleman 1990a) in a sub-population of SSc cells. It is still unclear whether such cells are abnormal i.e. whether they arise only in the disease situation, or whether they represent a

clonal imbalance of resident normal fibroblasts that have been expanded under the influence of other factors such as hormones and inflammatory mediators which are known to be increased in SSc lesions (Mauch *et al.*, 1990).

Since fibrosis occurs in selected organs in SSc, it has been postulated that the expansion of a sub-population of organ-specific cells may play an important role in disease. The availability of fibroblasts from both skin and lung biopsies provided a unique opportunity to investigate fibroblast abnormalities in both organs in SSc. Therefore, the parameters examined in this chapter included the rate of cell proliferation, matrix production and the expression of a number of surface antigens including adhesion molecules, ectopeptidases and the antigens identified by the mAbs 5A3, 3D3, 3C4 ad 7D3. In addition, the level of T lymphocyte binding to both SSc skin and SSc lung fibroblasts and the molecular pathways utilised in these interactions was studied.

7.2 PATIENTS AND STATISTICS

7.2.1 Samples

Dermal punch biopsies were taken from the lesional skin of 25 patients with SSc (6 males, 19 females, aged between 24 to 67 years; average age was 44 years) and from open lung biopsies taken from 9 FASSc lesions (7 females, 2 males, aged between 32 and 65 years, average age was 44 years).

7.2.2 Statistical analysis

Statistical comparisons between the various parameters measured in normal and SSc fibroblasts were made using the Student's t-test.

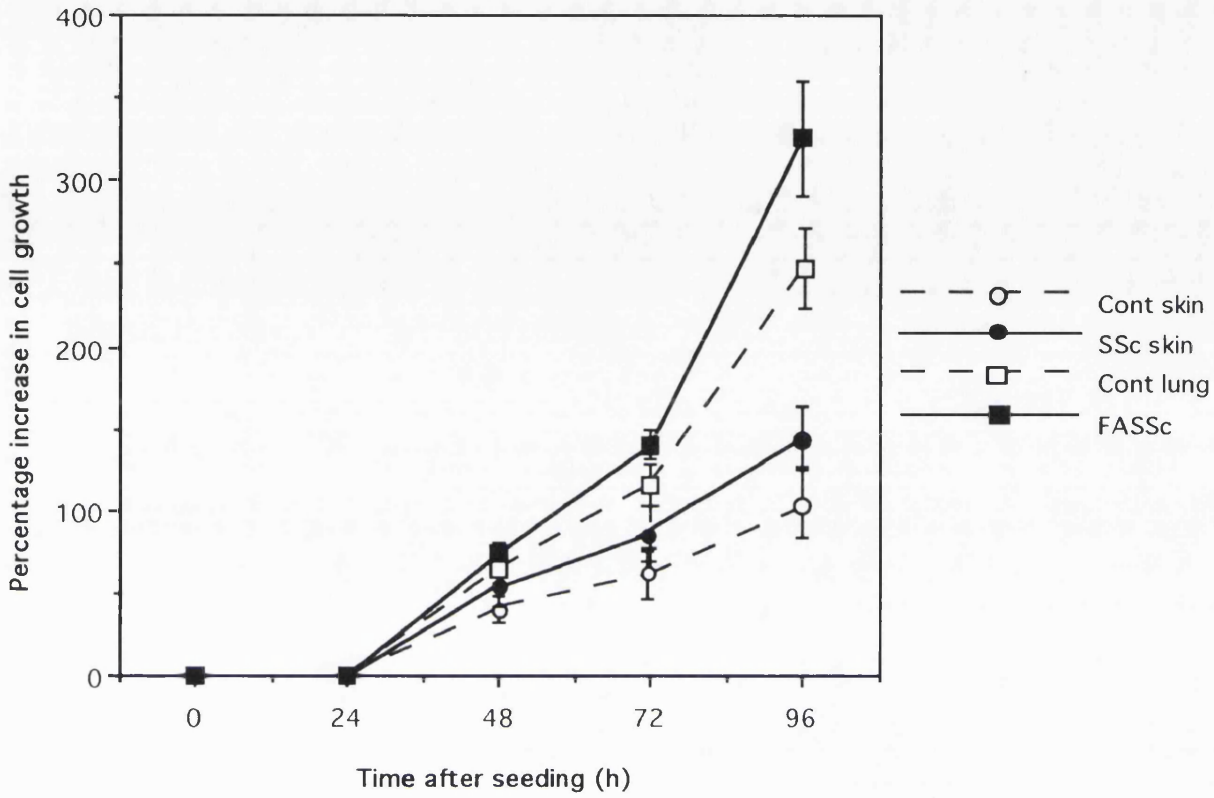
7.3 RESULTS

7.3.1 Cellular morphology and growth

SSc skin and SSc lung cells had a similar spindle-shaped morphology as normal fibroblasts. However, while both SSc fibroblasts had slightly higher levels of forward scatter, indicating cell size, compared with normal dermal and pulmonary cells (10% and 12%, respectively, $p>0.10$ in both cases), side scatter was altered in SSc cells by less than 5% compared with normal control cells.

Fig 7.1. shows the average rate of proliferation of SSc and normal fibroblasts from the skin and lung. Although both SSc skin and SSc lung cells appeared to have a slightly increased rate of cell proliferation at 48, 72 and 96h after seeding compared with their matched normal cells, these differences were not statistically significant. Both SSc lung and normal lung cells proliferated faster than skin fibroblasts.

Figure 7.1: Rate of cell proliferation



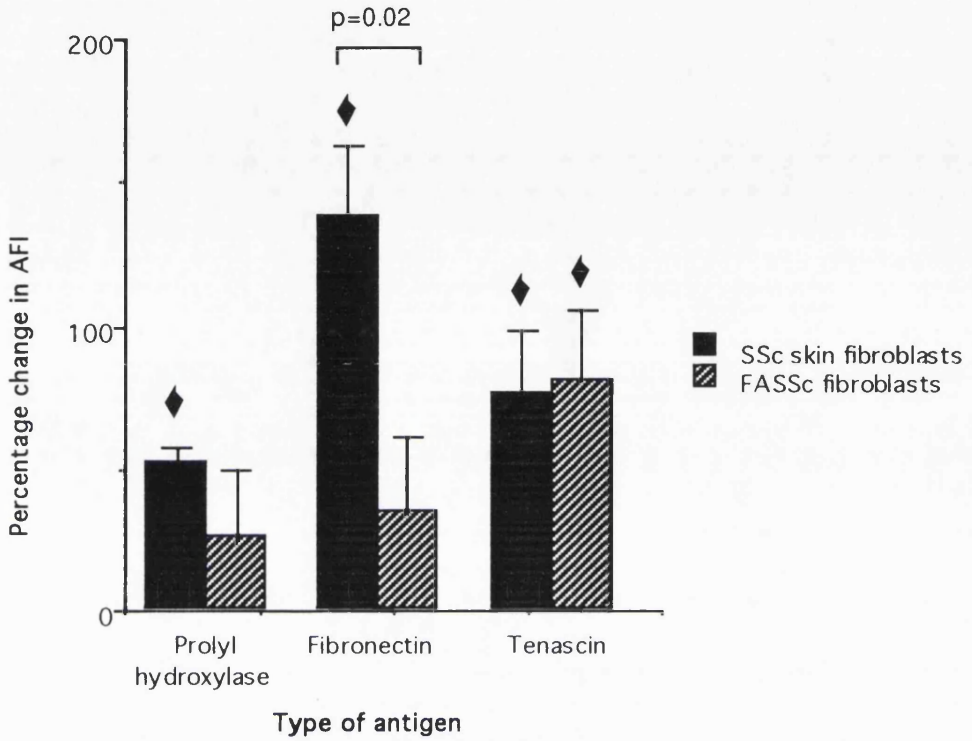
At the culture periods indicated the cells were counted as previously described in the Methods and Materials section. The average percent increase in cell number is shown for normal (dashed lines) and SSc fibroblasts (continuous lines). Skin fibroblasts are shown by circles while lung fibroblasts are shown by squares. No significant differences were in the rate of cell growth between the matched control and SSc cell cultures.

7.3.2 FCM analysis of total cellular matrix expression

The cellular expression of prolyl-4-hydroxylase, fibronectin and tenascin were measured by FCM after cellular permeabilisation. Figure 7.2 shows the percentage change in these matrix antigens in SSc dermal fibroblasts compared with normal dermal cells and in FASSc fibroblasts compared with normal lung cells. The SSc dermal fibroblasts had significantly higher levels of all three

antigens compared with normal dermal cells (AFIs of prolyl hydroxylase were of 29 compared with 19, levels of fibronectin were 266 compared with 112 and tenascin showed AFIs of 65 versus 37, respectively). In contrast, FASSc fibroblasts showed significantly raised levels of tenascin (AFIs of 54 compared with 31, $p<0.05$). The slight increases in SSc lung cells of prolyl hydroxylase and fibronectin were not significant (AFIs of 35 versus 28 and 166 versus 124, respectively).

Figure 7.2: FCM analysis of cellular matrix expression



Matrix expression in SSc skin and lung fibroblasts. The percentage increase in the cellular levels of prolyl hydroxylase, fibronectin and tenascin shown by SSc dermal fibroblasts (solid bars) compared with FASSc fibroblasts (hatched bars) compared with their respective normal cells, are represented as bars $\pm$ Standard Errors. Significant changes ($p<0.05$) in the level of matrix expression in disease fibroblasts compared with matched controls are shown by diamonds.

7.3.3 Type I collagen secretion by SSc dermal and FASSc fibroblasts

The secretion of type I collagen in SSc skin and SSc lung fibroblasts was measured by a competition ELISA assay. SSc dermal cells were found to secrete 153% higher levels of type I collagen compared with normal dermal cells (317.1 $\pm$ 76.9 ng/ml/million cells versus 207.10 $\pm$ 31.8 ng/ml/million cells, $p<0.05$), while SSc lung cells secreted 200% higher levels of type I collagen compared with normal lung fibroblasts (84.7 $\pm$ 18.0 versus 41.9 $\pm$ 9.5, $p<0.05$, respectively). It is notable that SSc skin fibroblasts secreted approximately 100% more collagen than FASSc cells.

7.3.4 Expression of cell surface antigens

FCM was used to measure the expression of MHC Classes I and II, the adhesion molecules ICAM-1, LFA-3, CD29 and CDw49d, the ectoenzymes CD10, CD13 and CD26 and the fibroblast antigens identified by the mAbs 5A3, 3D3, 3C4 and 7D3. The results in Table 7.1 show that while there was no significant difference in the levels of the MHC antigens and LFA-3 and CD29, the expression of ICAM-1 and 3C4 was significantly higher in both SSc skin and lung fibroblasts compared with matched normal fibroblasts. SSc dermal cells also revealed a significantly higher level of 5A3 which was not seen in SSc lung cells. Furthermore, SSc skin cells expressed much higher levels of all 3 ectoenzymes compared with SSc lung cells, similar to the difference noted between normal fibroblasts derived from the two organs.

Table 7.1: Expression of surface antigens on normal and SSc fibroblasts

Type of antigen	Skin fibroblasts (AFI ± SEM)		Lung fibroblasts (AFI ± SEM)	
	Normal	SSc	Normal	SSc
MHC				
MHC Class I	153 ± 17	156 ± 17	140 ± 25	132 ± 18
MHC Class II	5 ± 4	5 ± 3	4 ± 1	4 ± 1
Adhesion molecules				
ICAM-1	36 ± 2	**57 ± 5***	26 ± 4	**39 ± 5***
LFA-3	7 ± 2	9 ± 2	10 ± 2	9 ± 3
CD29	126 ± 20	130 ± 9	143 ± 38	123 ± 22
CDw49d	22 ± 3	27 ± 2	12 ± 1	9 ± 2
Surface proteases				
CD10	36 ± 11	30 ± 3	8 ± 2	5 ± 1
CD13	526 ± 96	455 ± 68	123 ± 22	113 ± 11
CD26	226 ± 30	182 ± 26	78 ± 28	56 ± 11
New mAbs				
5A3	54 ± 11	**87 ± 8***	32 ± 4	39 ± 5
3D3	651 ± 71	641 ± 62	184 ± 25	261 ± 42
3C4	84 ± 12	**131 ± 21***	28 ± 3	**43 ± 5***
7D3	49 ± 5	53 ± 7	46 ± 6	25 ± 5

* indicates that differences in the expression of antigen between SSc and normal fibroblasts were significant ($p<0.05$).

7.3.5 Expression of ICAM-1, CD26 and 3C4 in SSc skin and FASSc fibroblasts

Analysis of the FCM profiles of ICAM-1, CD26 and 3C4 revealed distinct fibroblast sub-populations in both SSc skin and SSc lung cultures, similar to that described previously for normal dermal and lung cultures. The FCM profiles for both normal and SSc cells derived from the skin and lung are shown in fig 7.4 and fig 7.5, respectively. These sub-populations were classified into high and low antigen expressing cells, as described previously in chapter 6. The numbers of cells in the various sub-populations were analysed for ICAM-1 and 3C4 as

these antigens revealed a significant increase in the mean expression of antigens in both SSc skin and SSc lung fibroblasts.

Analysis of the FCM profiles for ICAM-1 expression in all the SSc dermal cultures revealed that 52 $\pm$ 3% cells expressed high levels of ICAM-1 in contrast to normal dermal cultures where 37 $\pm$ 5% cells expressed high levels of ICAM-1. Similarly, FASSc cultures had 43 $\pm$ 7% ICAM-1 high cells compared with normal cultures which had 35 $\pm$ 10% ICAM-1 high cells. Thus, both SSc skin and lung cells showed an expanded sub-population of ICAM-1 high expressing cells.

The FCM profiles for CD26 expression in all the SSc dermal cultures revealed that 49 $\pm$ 9% cells expressed high levels of CD26 (AFI of 245) and 51 $\pm$ 10% expressed low levels of CD26 (AFI of 82). In contrast, normal dermal cultures had 66 $\pm$ 10% CD26-high cells (AFI of 312) and 32 $\pm$ 7% CD26-low cells (AFI of 51). Similarly, FASSc cultures had lower numbers of CD26 high cells (49 $\pm$ 12%, AFI of 78) compared with normal lung cultures which had 71 $\pm$ 15% CD26 high cells (AFI of 97). Thus, although both SSc skin and lung cells showed a decreased sub-population of CD26 high expressing cells, these changes were not statistically significant.

The results in table 7.2 show a more detailed analysis of the proportion of cells expressing high and low levels of the 3C4 antigen. Again, SSc dermal fibroblast populations were comprised of 57% 3C4-high expressing cells (AFI of 286) compared with 40% of 3C4-high (AFI of 182) normal dermal cells. FASSc fibroblasts had 33% 3C4-high cells (AFI of 81) compared with 23% 3C4-high normal lung cells (AFI of 72). Thus, in both SSc dermal and FASSc cultures, there was an increase in the proportion of 3C4-high expressing cells (significant) compared with normal cultures. However, only SSc dermal cells showed a significant increase in the level of 3C4 expression by the high-antigen expressing sub-population of cells.

Figure 7.3: FCM profiles of ICAM-1, CD26 and 3C4 expression in skin fibroblasts

FCM profiles of (A) ICAM-1, (B) CD26 and (C) 3C4 expression by normal skin fibroblasts (top panel) and SSc skin fibroblasts (lower panel). Note the presence of sub-populations of fibroblasts in both cultures (low antigen expressing cells are shown by small arrows and high antigen expressing cells are shown by large, open arrows). The FCM profiles with ICAM-1, CD26 and 3C4, are shown by dotted lines and the control cells stained with isotype-matched mAbs are shown by continuous lines.

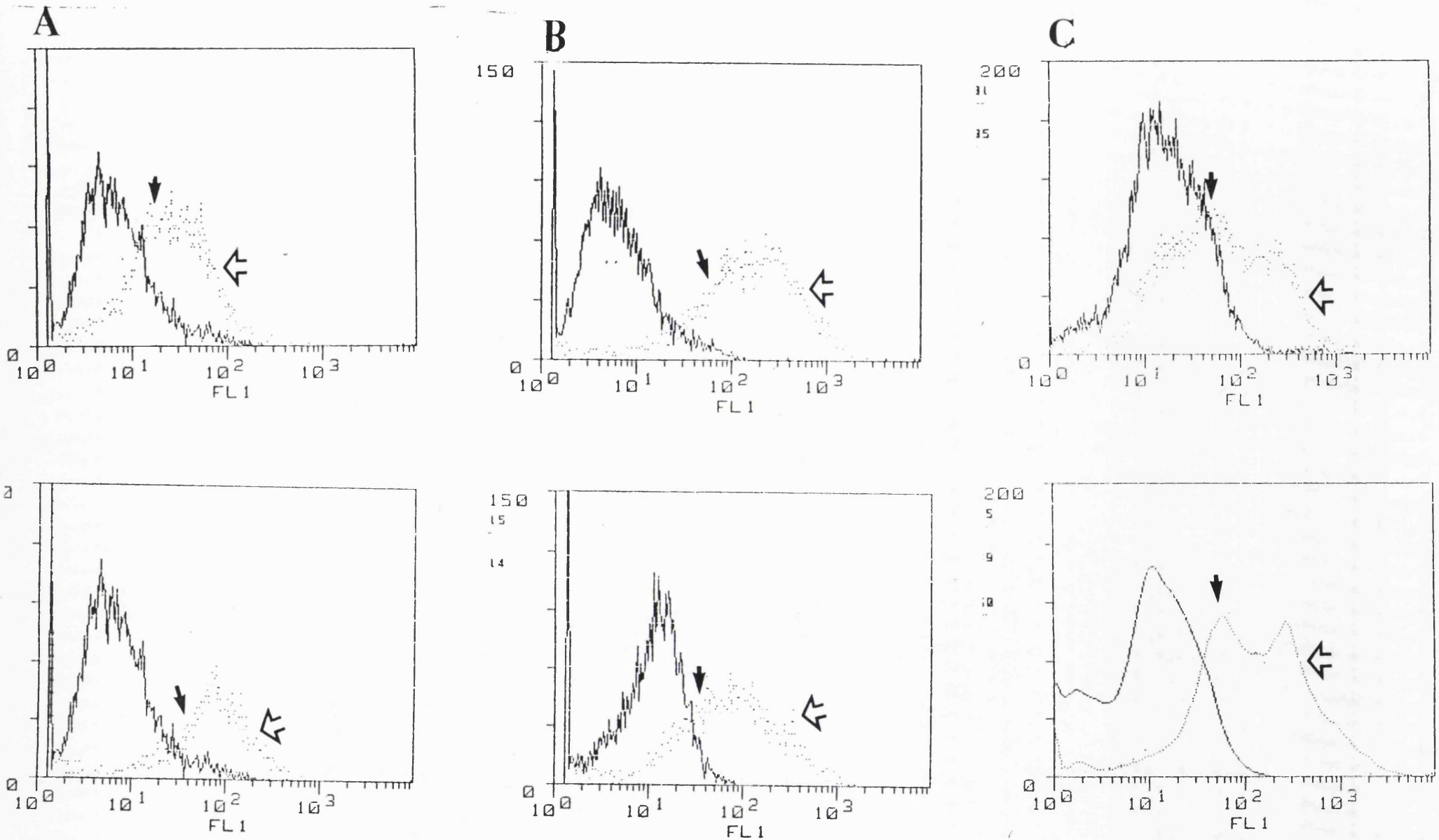

A
B
C
150
200
FL1

Figure 7.4: FCM profiles of ICAM-1, CD26 and 3C4 expression in lung fibroblasts

FCM profiles of (A) ICAM-1, (B) CD26 and (C) 3C4 expression on normal lung fibroblasts (top panel) and SSc lung fibroblasts (lower panel). Note the presence of sub-populations of fibroblasts in both cultures (low antigen expressing cells are shown by small arrows and high antigen expressing cells are shown by large, open arrows). The FCM profiles with ICAM-1, CD26 and 3C4, are shown by dotted lines and the control cells stained with isotype-matched mAbs are shown by continuous lines.

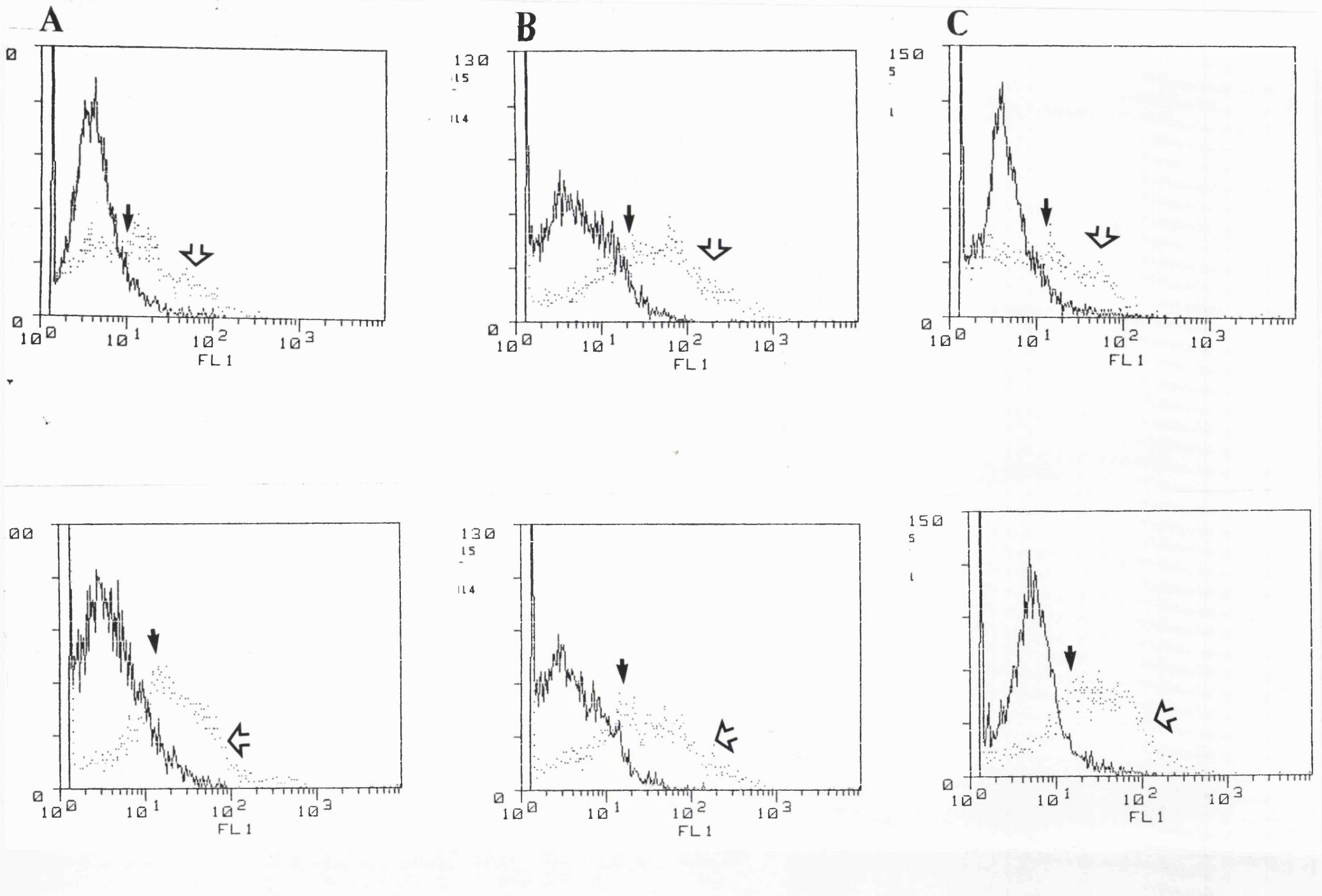

A
B
C
FL1
FL1
FL1
FL1
FL1
FL1

Table 7.2. Distribution and levels of 3C4 antigen in normal and SSc dermal and lung fibroblasts

Fibroblast sub-populations	Normal fibroblasts		SSc fibroblasts	
	AFI + SEM	% cells ±SEM	AFI + SEM	% cells ±SEM
Dermal fibroblasts				
3C4 low *	23.2 ± 2.92	59 ± 1.9	29.9 ± 4.5	43 ± 4.3†
3C4 high **	181.5 ± 29.2	40 ± 1.8	285.9 ± 25.8†	54 ± 4.1†
Pulmonary fibroblasts				
3C4 low‡	18.3 ± 2.6	75 ± 8.2	20.6 ± 3.2	62 ± 5.8
3C4 high‡‡	72.1 ± 5.1	23 ± 4.3	81.2 ± 10.3	33 ± 6.0†

† P value was less that 0.05 on comparison of SSc with normal fibroblasts

* Low 3C4 expressing dermal fibroblasts were taken as those cells with an AFI less than 100 and ** High 3C4 expressing dermal fibroblasts were taken as those cells with an AFI greater than 101.

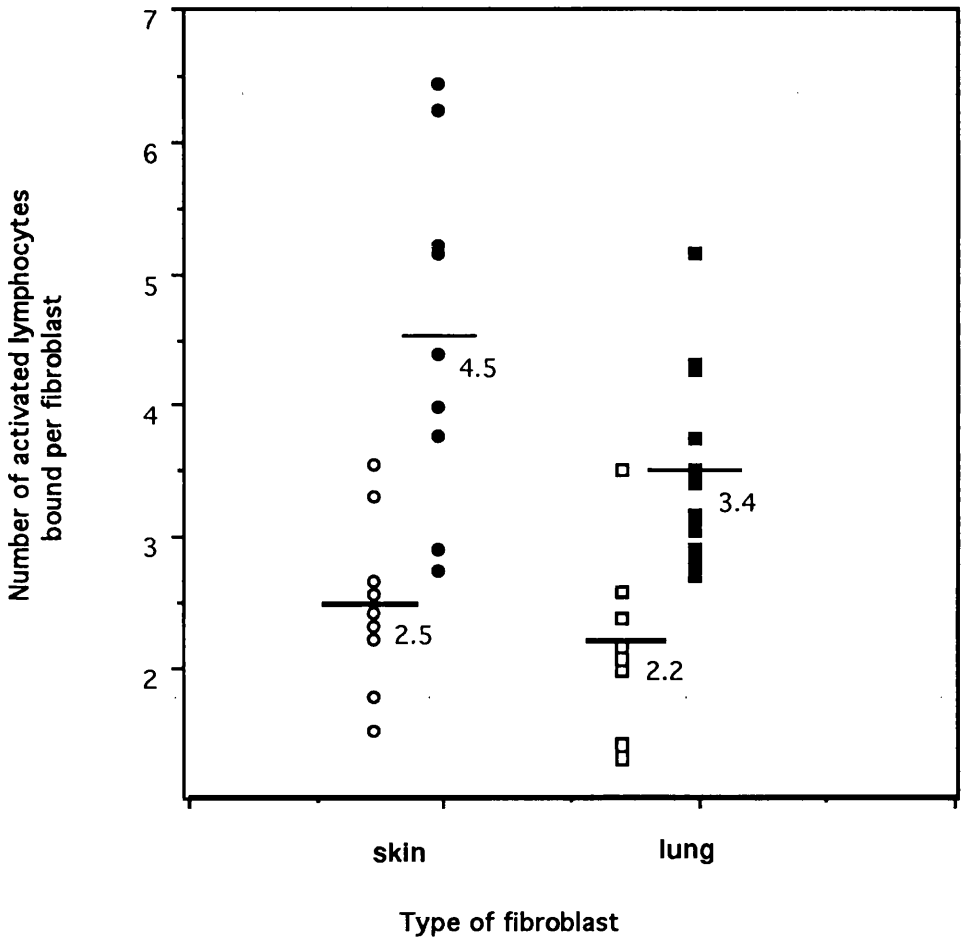
‡ Low 3C4 expressing pulmonary fibroblasts were taken as those cells with an AFI less than 30 and ‡‡ High 3C4 expressing pulmonary fibroblasts were taken as those cells with an AFI greater than 31.

7.3.6 Interactions of T lymphocytes with SSc dermal and FASSc fibroblasts *in vitro*

The adhesion of activated T lymphocytes to SSc dermal and SSc lung fibroblasts was examined and compared with matched normal cells. The results in fig 7.6 show that the binding of T cells to both SSc dermal and pulmonary fibroblasts was significantly higher than that shown by normal cells (4.5 ± 0.4 T cells per fibroblast compared with 2.5 ± 0.2, $p<0.001$, and 3.4 ± 0.2 compared with 2.2 ± 0.2, $p<0.001$, respectively). In addition, the binding of T cells was significantly higher to SSc dermal fibroblasts compared with FASSc fibroblasts

(4.5 T cells per dermal fibroblast compared with 3.4 T cells per lung fibroblast, p=0.007).

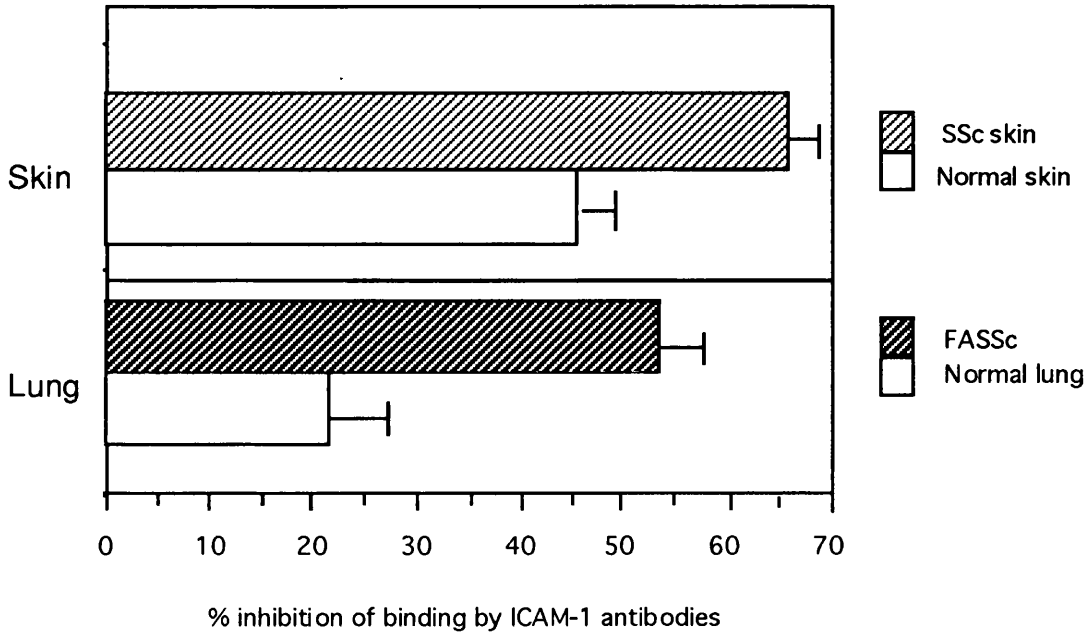
Figure 7.5: Lymphocyte binding to SSc dermal and lung fibroblasts *in vitro*



Activated lymphocytes were radio-labelled with [^{3}H] leucine, and their adhesion to SSc and normal skin and lung fibroblasts measured as described previously. Each data points shown in the graph represents the average binding of lymphocytes to triplicate wells of one fibroblast cell culture. Binding of lymphocytes to SSc fibroblasts (closed symbols) are compared with normal fibroblasts (open symbols). The mean level of binding for each group is shown by a bar and a value.

Antibody blockade experiments were carried out to delineate the functional role of specific adhesion molecules in the binding between the activated T cells and fibroblasts. These experiments showed that neither mAbs against the MHC Class I antigen nor non specific IgG had any inhibitory effect on adhesion (data not shown). In contrast, the mAb to LFA-3 inhibited the binding of activated lymphocytes to both normal dermal and lung fibroblasts equally, by approximately 25%. Identical results were obtained with SSc skin and lung fibroblasts with mAbs to LFA-3. However, the mAb to ICAM-1 caused a significantly higher level of inhibition of binding of activated T cells to both SSc dermal (67%) and FASSc fibroblasts (54%), compared with it's effect on normal dermal (45%) and normal lung (20%) cells (fig 7.7). There was no significant difference in the level of ICAM-1 mediated binding between FASSc and SSc dermal fibroblasts.

Figure 7.6: ICAM-1-dependant lymphocyte interactions with SSc dermal and lung fibroblasts.



The effect of saturating concentrations of mAbs to ICAM-1 on normal skin and lung fibroblasts (open bars) and to SSc skin and lung fibroblasts (hatched bars) are shown. Values are the average of the percentage inhibition of binding of lymphocytes to 5 cell cultures in each group, and are expressed as mean $\pm$ standard error. Prior incubation of fibroblasts with saturating concentrations of the mAbs to MHC class I and non-specific IgG caused less than 5% inhibition of T cell binding to both the SSc and the normal fibroblasts.

7.4 DISCUSSION

In view of the significant fibroblast heterogeneity noted between normal skin and normal lung fibroblasts in the previous chapter, I examined the abnormalities shown by SSc fibroblasts derived from skin and lung lesions. Previous investigations of SSc dermal cells have shown that the main fibroblast abnormalities include increased extracellular matrix synthesis (LeRoy EC., 1974; Jimenez SA., *et al.*, 1986; Scharfetter K., *et al.*, 1988), elevated surface ICAM-1 (Needleman 1990a; Abraham *et al.*, 1991; Shi-Wen *et al.*, 1994) and reduced levels of the ectoenzyme DPPIV (Bou-Gharios *et al.*, 1995). The data described in this chapter show that there is an increased expression of the matrix-related proteins fibronectin, tenascin, prolyl-hydroxylase and an increased secretion of type I collagen in SSc dermal fibroblasts compared with normal dermal cells. However, only tenascin and type I collagen secretion was significantly increased in FASSc cells. Thus, the amount of matrix proteins expressed by SSc skin fibroblasts were markedly higher than that of FASSc cells. This observation is consistent with the more marked end-stage fibrosis noted in SSc dermal lesions compared with FASSc lesions. This data is the first report of significantly altered matrix expression in FASSc fibroblasts *in vitro*.

A number of fibroblast cell surface antigens known to be abnormally expressed in SSc dermal fibroblasts also showed similar changes in SSc pulmonary fibroblasts. Thus, significantly increased levels of ICAM-1 were present in cultures of both cell types which additionally revealed an expanded sub-population of ICAM-1-high cells. Furthermore, enhanced levels of T lymphocyte adhesion *in vitro* was shown by both SSc skin and SSc lung cells compared with matched normal fibroblasts but SSc dermal cells bound more T cells than FASSc fibroblasts. Although this result is consistent with the relative levels of cell surface ICAM-1 expressed by SSc skin and lung cells, *in vitro*, it does not reflect the higher levels of inflammatory infiltrates (lymphocytes and

neutrophils) seen in pulmonary SSc lesions compared with dermal lesions *in vivo* (Harrison *et al.*, 1991; Gruschwitz *et al.*, 1992).

Recent studies which examined fibroblasts grown in three dimensional collagen gels revealed a lack of down regulation of collagen synthesis in SSc dermal fibroblasts compared with normal cells. This impaired regulation of collagen pro-αI (I) mRNA was suggested to be due to an aberrant expression of the collagen binding α1β1 integrins, in particular, reduced levels of the α1 sub-unit (Ivarsson *et al.*, 1993). The results of this chapter show that there was no significant difference in the expression of CD29 (β1 integrin) or CDw49d between SSc dermal and FASSc fibroblasts relative to their normal controls suggesting that these integrins are not abnormal in both SSc cell types.

A recent study showed that the surface protease CD26 (DPPIV) is substantially lower in SSc dermal fibroblasts compared with normal cells (Bou-Gharios *et al.*, 1995). The results of the present study provide evidence that CD10, CD13 as well as CD26 are down regulated in both SSc skin and SSc lung fibroblasts (albeit not significantly). Since these ectoenzyme could have an important role in cell activation, regulation of cellular interactions with matrix components and the metabolism of peptide hormones (Kenny *et al.*, 1987), the reduction of all three membrane antigens noted in SSc dermal and FASSc cells suggests that this abnormality could play a role in the increased expression of peptide hormones observed in SSc lesions and consequently on fibrosis. Murphy *et al.*, 1993, recently showed that CD10 is the major endothelin converting enzyme involved in the processing of ET-1, thus, the reduced levels of CD10 in both SSc dermal and lung fibroblasts may partly explain the raised levels of ET-1 noted previously in both SSc skin and lung biopsies.

The newly developed mAbs raised against fibroblast surface antigens showed an increased staining with the mAbs 5A3 and 3C4 of both SSc skin and lung fibroblasts suggesting that the corresponding fibroblast membrane antigens may be markers for activated fibroblasts. The markedly high level of staining with the mAb 5A3 in SSc dermal fibroblasts compared with SSc lung cells may

reflect the differences in matrix expression shown by these cells since recent data suggests that the mAb 5A3 identifies a residual matrix component on fibroblasts (Korn *et al.*, submitted).

The observation of two distinct sub-populations of fibroblasts detected with the mAb 3C4 in both skin and lung cultures, strongly suggests fibroblast heterogeneity within both organs. Furthermore, analysis of both sub-populations revealed a significant expansion of the 3C4-high expressing subset in both organs. Immunohistochemical data from the last chapter showed that the mAb 3C4 additionally stained smooth muscle suggesting that this antigen may be similar to other myofibroblastic antigens including actin and desmin which have been shown to be increased in fibroblasts derived from hypertrophic scars, fibromatoses, desmoplasia and SSc skin lesions (Sappino *et al.*, 1990).

Sub-populations of fibroblasts may be expanded in disease by a clonal expansion of highly proliferative cells, the clonal deletion either by selective cell death and/or inhibition of proliferation by regulatory or cytotoxic factors and/or by site-specific migration of fibroblast subsets directed by release of chemoattractants. A large number of reports have shown increased levels of inflammatory mediators and growth factors in SSc lesions (Kahari 1993; Mauch *et al.*, 1993), which could result in an expansion of a normal sub-population of fibroblasts which proliferates faster in response to cytokines and growth factors (Korn *et al.*, 1984). Further study is thus warranted to characterise the 3C4 antigen and to study the functional characteristics of the expanded high 3C4 expressing fibroblast subset in both dermal and FASSc cultures, particularly with regard to response to inflammatory mediators and relationship to abnormal collagen synthesis.

In conclusion, the data shown in this chapter show that there are differences in the level of matrix expression and lymphocytes interactions between SSc skin and SSc lung fibroblasts which may contribute to the differences noted in expression of disease in the skin and lung. Furthermore, both organs are composed of normal fibroblast sub-populations which are expanded in SSc.

CHAPTER EIGHT: GENERAL DISCUSSION

SSc is a multi-system disease of unknown aetiology which is characterised by progressive vascular damage, immune alterations and fibroblast activation leading to the excessive deposition of extracellular matrix components in the skin and internal organs (Black 1993). Skin thickening is the major clinical hallmark of SSc, and extensive skin involvement is correlated with decreased survival and a greater risk of developing scleroderma renal crisis, myocardial and interstitial lung disease. Most studies investigating the pathogenesis of SSc have utilised dermal tissue as this is the most accessible lesion for biopsy. However, a number of histopathological findings suggest that there are differences in the processes that lead to disease expression between the skin and visceral organs, particularly the lung.

The earliest ultrastructural abnormality noted in SSc dermal lesions is endothelial cell damage (Prescott *et al.*, 1992), for example, while pulmonary lesions are reported to have both endothelial and epithelial cell changes (Harrison *et al.*, 1991). Early SSc dermal lesions also show perivascular inflammatory infiltrates, which are made up of lymphocytes predominantly with a paucity of granulocytes and plasma cells (Roumm *et al.*, 1984; Gruschwitz *et al.*, 1992; Ishikawa *et al.*, 1992). In contrast, FASSc lesions are characterised by a more mixed inflammatory profile in which the cellular infiltrate consists initially of lymphocytes which are followed by mast cells and eosinophils and then neutophils and plasma cells (Rossi *et al.*, 1985; Haslam 1990; Harrison *et al.*, 1989), suggesting that the type of inflammatory cell recruited to the lungs differs depending on the stage of disease. In the late stages of skin disease, fibrotic tissue replaces the normal dermal architecture (Fleishmajer *et al.*, 1977a), while lesional lung biopsies taken at post-mortem reveal a combination of inflammation and fibrosis (Ashba *et al.*, 1965).

In order to study the development of the disease process in the skin and lung in SSc, I examined three major features of SSc, i.e., vascular activation,

immune cell interactions and fibroblast abnormalities in both organs. This was done by studying the (i) localisation of ET-1 and the expression of ET receptors in skin and lung biopsies, (ii) the localisation and level of expression of adhesion molecules which mediate immune cell interactions: E-selectin, ICAM-1 and VCAM-1 in SSc skin and lung biopsies and (iii) the functional and phenotypic characteristics of fibroblasts derived from SSc skin and lung biopsies. By performing these studies, I was also able to indirectly examine the characteristics of other resident cells present in SSc lesions, such as keratinocytes in skin and alveolar epithelial type II cells in lung. Additionally, I was able to examine the sequence of events in the disease process particularly in the skin. The results of this project provide strong evidence that vascular, immune and fibroblast abnormalities occur in both SSc skin and SSc lung disease which may contribute to the pathogenesis of the disease process in both organs. Furthermore, there are substantial differences between the disease processes in the two organs which needs to be considered when designing therapeutic regimes in SSc.

Examination of the expression of ET-1 and ET-1 receptors and the adhesion molecules E-selectin, ICAM-1 and VCAM-1 expressed by endothelial cells provided some indication of vascular function in SSc *in vivo* .
The first indication that ET-1 was abnormal in SSc was provided by measurement of circulating ET-1 which was markedly higher in both SSc patients with extensive fibrosis and pulmonary vascular disease compared with SSc patients with more limited fibrosis and normal healthy individuals. Examination of the vascular expression of ET-1 in lesional skin and lung biopsies obtained from dcSSc and FASSc patients, respectively, showed an increased expression of ET-1 in microvessels from both organs, implying microvascular abnormalities in both tissues. Furthermore, expression of ET-1 was increased in both clinically "uninvolved" dcSSc skin biopsies and lesional skin biopsies suggesting that vascular activation is an early prefibrotic event in SSc disease progression. Papillary dermal vessels and small interstitial pulmonary vessels also expressed increased levels of ET-1 receptors. These data provide strong evidence for

vascular abnormalities in both the skin and lung and suggest the involvement of ET-1 in the pathogenesis of fibrosis in SSc.

In view of the significantly different immune cell populations seen in SSc skin and SSc lung lesions and of the differences noted in the timing of the inflammatory process in the two organs, the expression of the major adhesion molecules, i.e. E-selectin, ICAM-1 and VCAM-1, was investigated in the circulation and in dermal and pulmonary biopsies obtained from SSc patients. Previous studies have shown increased levels of E-selectin and ICAM-1 in SSc (Carson CW., *et al.*, 1993, Sfikakis PP., *et al.*, 1993) and altered levels of E-selectin, ICAM-1 and VCAM-1 in dcSSc skin biopsies in semi-quantitative studies (Claman HN., *et al.*, 1991, Gruschwitz M., *et al.*, 1992, Koch AE., *et al.*, 1993, Majewski S., *et al.*, 1991, Sollberg S., *et al.*, 1992). No studies to date have examined the expression of adhesion molecules in SSc lung disease. In this project, I was able to measure circulating levels of E-selectin, ICAM-1 and VCAM-1 in SSc and in RP patients who subsequently developed SSc and in primary RP and morphea. I have also quantified the vascular expression of E-selectin, ICAM-1 and VCAM-1 in both SSc skin and SSc lung disease. Circulating E-selectin levels were raised in all the SSc patient groups but not in primary RP and morphea suggesting that this molecule may be used as a marker for selecting patients with RP patients at risk of developing SSc.

Examination of the dermal vascular expression of E-selectin, ICAM-1 and VCAM-1 in SSc skin lesions showed a significant increase in the expression of E-selectin and ICAM-1 all through disease and a transient early expression of VCAM-1. This was in contrast to the diseased pulmonary vasculature which revealed a significantly raised expression of ICAM-1 and VCAM-1 only. Thus, marked differences were present in the vascular expression of adhesion molecules such as E-selectin and VCAM-1 between dermal and pulmonary vessels in SSc. These observations suggest that although vascular activation occurs in both organs, vessels in the skin express substantially different adhesion molecules to those in the lung. These differences in the expression of adhesion molecules between the

skin and lung were also consistent with the significant variation noted in the inflammatory cell population between the two organs in SSc.

The expression of E-selectin in the dermal vasculature in SSc may result in the adherence of a sub-population of T cells which preferentially migrates to the skin and expresses the cutaneous lymphocyte antigen (CLA) (Picker LJ., *et al.*, 1991). VCAM-1 has been reported to be the major adhesion molecule in eosinophil binding to the endothelium (Walsh G., *et al.*, 1991). The increased expression of VCAM-1 in pulmonary vessels but not dermal vessels, is consistent with the increased proportion of eosinophils present in FASSc lesions compared with the skin. Lastly, ICAM-1 has been shown to mediate neutrophil interactions in the lung (Barton *et al.*, 1989; Argenbright *et al.*, 1991) and, as this molecule is significantly increased in the pulmonary vasculature, it may account for the increased presence of neutrophils in SSc lung disease. Thus, the differential expression of adhesion molecules between the skin and lung in SSc may explain the different immune cell populations in the two organs, which may in turn be responsible for the release of substantially different inflammatory mediators and patho-physiological disease processes.

Perhaps the most distinctive cellular difference between the skin and lung in SSc was the presence of marked epithelial cell abnormalities in FASSc, in contrast to the apparent lack of involvement of keratinocytes in dcSSc lesions. All the FASSc biopsies examined in this project showed a total replacement of alveolar epithelial type I cells, which normally line 90% of the alveolar epithelium, with alveolar epithelial type II cells. This epithelial cell abnormality has been shown to occur very early in disease (Harrison NK., *et al.*, 1991). In this project, alveolar epithelial type II cells expressed significantly increased levels of ET-1, ET-1 receptors and ICAM-1 *in vivo*. Previous studies have found that type II epithelial cells also synthesise a number of growth factors including TGFβ, PDGF, IGF and TNFα (Corrin *et al.*, 1994; Martinet *et al.*, 1987), which are known to be potent stimulators of fibroblast proliferation and matrix expression, particularly when acting in synergy with ET-1 (Guidry *et al.*, 1991). In view of

the presence of increased cellular contacts between alveolar epithelial type II cells and lung fibroblasts (Adamson IYR., *et al.*, 1990), and the ability of alveolar epithelial type II cells to increase fibroblast proliferation and collagen synthesis in co-culture experiments (Young L., *et al.*, 1993), it is conceivable that the increased expression of ET-1 and ET-1 receptors by epithelial type II cells in FASSc may play a significant role in fibrosis in the lung. Increased inflammatory infiltrates have also been reported in close proximity to alveolar epithelial type II cells, prior to fibroblast activation in bleomycin-induced lung fibrosis in rats (Vyalov SL., *et al.*, 1993), suggesting that this cell type can mediate immune cell interactions in SSc. Taken together, the above data strongly suggest that the alveolar epithelial repopulation by alveolar epithelial type II cells is an early and persistent abnormality in SSc lung disease which plays a significant part in both the inflammation and fibrosis in the lung.

Although basal keratinocytes also express increased levels of ET-1 receptors and ICAM-1 in the skin in dcSSc, this cell type does not appear to be involved in the disease process to the same extent as the alveolar epithelial type II cell in SSc lung lesions. For example, the fibrotic zone in SSc skin disease is in the deep dermal zone (Lovell CR., *et al.*, 1979, Scharfetter K., *et al.*, 1988) which is not adjacent to keratinocytes, while pulmonary fibrosis occurs in the interstitium which is adjacent to the alveolar epithelium. Furthermore, although basal keratinocytes express increased levels of ICAM-1 in SSc lesions, previous investigators have consistently reported a lack of inflammatory cells in the epidermis (Koch AE., *et al.*, 1993, Sollberg S., *et al.*, 1992).

Fibroblasts may also contribute to the differences in the expression of disease in the skin and lung in SSc. It has been suggested that the selectivity in the fibrotic response may occur due to the presence of different fibroblast populations in different organs (Dalvi B., 1993, Korn JH., *et al.*, 1992). Evidence for such organ-specific fibroblasts has been shown by studies which documented that embryonic skin and lung fibroblasts are different with respect to growth kinetics and hormone metabolism (Schneider *et al.*, 1977; Kondo *et al.*, 1985;

LeRoy 1985). Furthermore, there is evidence to suggest that sub-populations of fibroblasts are present within both the skin and lung. Fibroblasts within a cell culture express different levels of matrix and respond differently to growth factors and cytokines (Feldman *et al.*, 1993; Akamine *et al.*, 1992; Korn *et al.*, 1985b). *In vivo* examination of both the skin and lung biopsies display heterogeneous levels of collagen mRNA and actin within fibroblasts (Kahari VM., *et al.*, 1988, Sappino A., *et al.*, 1990, Scharfetter K., *et al.*, 1988). Consequently, it is possible that the differences in expression of disease in the skin and lung may occur due to differences in their resident fibroblast populations.

Studies of cell size and proliferation revealed that SSc lung cells were smaller and proliferated faster than SSc skin fibroblasts. Examination of the level of matrix expression between the two types of cells demonstrated that although both SSc skin and SSc lung fibroblasts produced higher levels of a number of matrix molecules including fibronectin, tenascin, prolyl-4-hydroxylase and secreted increased levels of type I collagen compared with normal cells, the level of matrix production by SSc dermal fibroblasts was markedly higher than SSc lung fibroblasts. This difference in the levels of matrix expression between SSc skin fibroblasts and SSc lung cells is interesting and is consistent with the more fibrotic aspect of dermal lesions compared with FASSc lesions. It may also provide a rationale for the more effective use of anti-fibrotic agents in skin as opposed to with lung disease (Steen *et al.*, 1985; de Clerk *et al.*, 1987). The level of expression of surface antigens recognised by the mAbs 5A3, 3D3 and 3C4 and the ectoenzymes was also much higher in SSc dermal fibroblasts. T lymphocytes were found to bind more to SSc skin fibroblasts compared with SSc lung cells. All these observations suggest that SSc skin fibroblasts are functionally and phenotypically distinct to SSc lung fibroblasts.

Within both normal and SSc skin and lung fibroblast cultures, there was clear evidence for distinct sub-populations of cells which expressed different levels of ICAM-1, CD26 and 3C4. Examination of the fibroblast sub-populations

which expressed ICAM-1 and 3C4 revealed an expanded sub-population of high-antigen expressing cells in both SSc skin and SSc lung cultures compared with normal cells of either organ. Thus, these results suggested that a normal high-antigen expressing sub-population of cells was expanded in both SSc skin and SSc lung lesions, providing further evidence for the hypothesis that a normally resident sub-population of cells is expanded in SSc. The expression of 3C4 by the sub-population of cells that were expanded in both SSc skin and SSc lung cells was interesting, suggesting that this mAb may be a marker for this expanded sub-population of cells, and deserves further investigation. Thus, the examination of fibroblast abnormalities in both normal and SSc skin and lung cultures provided clear evidence of organ-specific fibroblast heterogeneity and the presence of sub-populations of cells within both organs. Differences in fibroblast abnormalities between the skin and lung may therefore contribute to the variation in the disease processes in these two organs.

Two additionally important clinical observations were made in this project by measuring the circulating levels of E-selectin and ET-1. Measurement of the circulating levels of E-selectin appeared to select patients with SSc, "autoimmune" RP from those with primary RP and morphea which suggests that this molecule may become a useful marker of evolving SSc and indicates that vascular activation is important in the pathogenesis of this disease. Secondly, levels of circulating ET-1 were markedly high in lcSSc patients with isolated pulmonary hypertension. This is an important finding as it may provide an early serological marker for the development of a fatal complication of SSc and the molecule ET-1 and the ETA receptor (vasoconstrictor) may provide a point of therapeutic attack.

In conclusion, the results of this project provide clear evidence for the presence of altered vascular function, increased immune cell interactions and fibroblast abnormalities in both SSc skin and SSc lung disease. The disease process in the skin appeared to consist of early vascular activation, immune cell infiltration followed by fibrosis while the FASSc lesions showed evidence of early

vascular and epithelial abnormalities, inflammation and fibrosis. Perhaps the most interesting observations made in this project were the tissue-specific cellular differences in the vascular expression of adhesion molecules, the presence of epithelial cell involvement and fibroblast abnormalities between the skin and lung in SSc. These differences suggest that distinct cellular abnormalities contribute to the disease processes in the skin and lung in SSc. It was unlikely that these organ-specific differences represented different stages of disease in the two organs as both types of biopsies were taken at early stage disease. Also, the expression of E-selectin was specific to dermal and not pulmonary disease, the profile of immune cells in both biopsies were significantly different, epithelial cell involvement was restricted to pulmonary lesions and the phenotype and function of skin and lung fibroblasts were significantly different. These data provide vital information for understanding the disease process in the skin and lung in SSc and the tissue selectivity of expression of disease. These observations may also provide valuable insight into the development of rational strategies for the early detection and therapeutic management of pulmonary and dermal involvement in SSc.

CHAPTER NINE: REFERENCES

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